Insight Into Distinct Functional Roles of the Flagellar ATPase Complex for Flagellar Assembly in Salmonella

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Most motile bacteria utilize the flagellar type III secretion system (fT3SS) to construct the flagellum, which is a supramolecular motility machine consisting of basal body rings and an axial structure. Each axial protein is translocated via the fT3SS across the cytoplasmic membrane, diffuses down the central channel of the growing flagellar structure and assembles at the distal end. The fT3SS consists of a transmembrane export complex and a cytoplasmic ATPase ring complex with a stoichiometry of 12 FliH, 6 FliI and 1 FliJ. This complex is structurally similar to the cytoplasmic part of the $F_0F_1$ ATP synthase. The export complex requires the FliH$_{12}$-FliI$_6$-FliJ$_1$ ring complex to serve as an active protein transporter. The FliI$_6$ ring has six catalytic sites and hydrolyzes ATP at an interface between FliI subunits. FliJ binds to the center of the FliI$_6$ ring and acts as the central stalk to activate the export complex. The FliH dimer binds to the N-terminal domain of each of the six FliI subunits and anchors the FliI$_6$-FliJ$_1$ ring to the base of the flagellum. In addition, FliJ exists as a hetero-trimer with the FliH dimer in the cytoplasm. The rapid association-dissociation cycle of this hetero-trimer with the docking platform of the export complex promotes sequential transfer of export substrates from the cytoplasm to the export gate for high-speed protein transport. In this article, we review our current understanding of multiple roles played by the flagellar cytoplasmic ATPase complex during efficient flagellar assembly.

Keywords: ATPase, bacterial flagella, $F_0F_1$ ATP synthase, flagellar assembly, proton motive force (pmf), protein translocation, type III secretion system (T3SS)

INTRODUCTION

Pathogenic bacteria use virulence-associated type III secretion systems (vT3SS), also known as the injectisomes, to inject virulence effector proteins directly into eukaryotic host cells as part of their infection process. Motile bacteria employ the flagellar type III secretion system (fT3SS) to construct a supramolecular motility machine, the flagellum, on the cell surface (Wagner and Diepold, 2020). A remarkable feature of both the vT3SS and fT3SS is that the protein export apparatus is capable of translocating export substrates across the cytoplasmic membrane at a rate of tens of thousands of amino acids per second (Iino, 1974; Chen et al., 2017; Renault et al., 2017). The protein export apparatus of the T3SS is composed of a transmembrane export complex powered by the proton...
motive force (PMF) across the cytoplasmic membrane and a cytoplasmic ATPase ring complex (Figure 1). The transmembrane export complex is composed of five conserved membrane proteins: FliA, FlhB, FliP, FliQ, and FliR in the fT3SS; SctV, SctU, SctR, SctS, and SctT in the vT3SS. The cytoplasmic ATPase ring complex is composed of three cytoplasmic proteins, FliH, FliI, and FliJ in the fT3SS and SctL, SctN, and SctO in the vT3SS. The ATPase ring complex is structurally similar to the cytoplasmic part of the FfOvT3SS. The ATPase ring complex is composed of three cytoplasmic proteins, SctV, SctU, SctR, SctS, and SctT in the vT3SS. The cytoplasmic transmembrane export complex is composed of five conserved motor that couples proton (H\(^+\)) flow through FfO with ATP synthesis by FfO (Minamino, 2014; Minamino et al., 2020b).

The flagellum of Salmonella enterica serovar Typhimurium (hereafter referred to as Salmonella) is composed of about 30 different proteins whose copy numbers range from a few to tens of thousands. The Salmonella flagellum is divided into three main structural parts: the basal body, the hook, and the filament (Figure 2). The basal body is located within the cell envelope and serves as a bi-directional rotary motor fueled by the PMF across the cytoplasmic membrane. The hook and filament extend into the cell exterior. The filament functions as a helical propeller to produce the thrust that pushes the cell body forward. The hook between the basal body and filament acts as a universal joint to transmit torque produced by the motor to the filament (Nakamura and Minamino, 2019).

The axial structure of the Salmonella flagellum is composed of the rod (FliE, FlgB, FlgC, FlgE, FlgG), the hook (FliE), the hook-filament junction (FlgK, FlgL), the filament (flagellin, FliC or FliJ) and the filament cap (FliD) (Figure 2). The assembly of the axial structure begins with the rod, followed by the hook with the help of the hook cap (FlgD). Upon completion of hook assembly, the hook cap is replaced by FlgK, and then FlgK and FlgL self-assemble into the hook-filament junction structure at the hook tip. FliD forms the filament cap at the tip of the junction structure and promotes the assembly of newly transported flagellin molecules into the long helical filament (Macnab, 2003).

To construct the axial structure beyond the cellular membranes, fourteen different proteins are translocated across the cytoplasmic membrane via the fT3SS, diffuse down the narrow central channel, and assemble at the tip of the growing structure (Figure 2). They can be classified into two export classes: one is the rod-type (FliE, FlgB, FlgC, FlgE, FlgG, FlgJ) and hook-type (FlgD, FlgE, FliK) class needed for assembly of the rod and hook. The other is the filament-type class (FlgK, FlgL, FlgM, FliC, FliD) responsible for filament assembly. The fT3SS secretes a molecular ruler protein, FliK, to measure the length of the hook during hook assembly and switches its substrate specificity from rod/hook-type proteins to filament-type proteins when the hook reaches its mature length of about 55 nm. At that point hook assembly terminates and filament assembly initiates (Minamino, 2018).

The fT3SS and vT3SS utilize the PMF across the cytoplasmic membrane and ATP hydrolysis to drive protein translocation across the cytoplasmic membrane (Minamino and Namba, 2008; Paul et al., 2008; Lee et al., 2014). The Salmonella fT3SS has a backup engine powered by a sodium (Na\(^+\)) motive force (SMF) across the cytoplasmic membrane to continue flagellar assembly when the cytoplasmic ATPase ring complex does not work properly, as during biofilm development (Minamino et al., 2016b, 2021a).

Once the transmembrane export complex of the Salmonella fT3SS is activated by ATP hydrolysis in the cytoplasmic ATPase ring complex, it becomes an active H\(^+\)/protein antiporter that couples inward-directed H\(^+\) flow with outward-directed protein export (Minamino et al., 2011). Furthermore, the cytoplasmic ATPase complex allows the export complex to coordinate flagellar protein export with assembly in Salmonella (Minamino et al., 2016a; Inoue et al., 2018). Thus, the cytoplasmic ATPase ring complex acts as an activator of the H\(^+\)-driven export engine and also contributes to efficient and robust protein export by the export complex. This review describes our current understanding of the structure and function of the flagellar cytoplasmic ATPase complex in Salmonella.

**Structure and Function of the Transmembrane Export Complex**

The transmembrane export complex of the fT3SS is located inside the MS-ring formed by the transmembrane protein FlfE (Figure 1; Johnson et al., 2021; Kawamoto et al., 2021; Takekawa et al., 2021; Tan et al., 2021). It consists of nine copies of FlhA, a single copy of FlhB, five copies of FliP, four copies of FliQ, and a single copy of FliR (Abruści et al., 2013; Kuhlen et al., 2018, 2020; Johnson et al., 2019).

FliP and FliR assemble into the Flp5-FliR complex with the help of the FlIO scaffolding protein and form the polypeptide channel for the translocation of export substrates across the cytoplasmic membrane (Figure 3, left panel) (Fabiani et al., 2017; Fukumura et al., 2017). Four FliQ subunits bind to the outside of the FliPs-FliR1 complex to form the FliP5-FliQs-FliR1 complex (Figure 3, middle panel). A flexible loop formed by the highly conserved Met-209, Met-210, and Met-211 residues of Flip (the M-loop) on the cytoplasmic side of the polypeptide channel and a plug loop composed of residues 106–122 of Flir (the R-plug) seem to prevent the leakage of small molecules during high-speed protein translocation (Figure 3, right panel) (Ward et al., 2018; Hüsing et al., 2021). The FliP5-FliQs-FliR1 complex has a helical arrangement of subunits similar to the rod (Figure 3), so FliE, which is the first export substrate transported by the fT3SS (Minamino and Macnab, 1999; Minamino et al., 2000), can directly assemble at the distal end of the FliP5-FliQs-FliR1 complex to form the most proximal part of the rod. Interactions between FliE and FliP not only firmly connect the rod with the MS ring but also open the exit gate of the polypeptide channel through conformational changes of Flip and Flir (Hendriksen et al., 2021).

Salmonella FlhB consists of an N-terminal transmembrane domain (FlhBTm) with four transmembrane helices (TMHs) (residues 1–211) and a large C-terminal cytoplasmic domain (FlhBC) (residues 212–383) (Minamino et al., 1994; Kinoshita et al., 2021). FlhBTm associates with the FliP5-FliQs-FliR1 complex to form the FliP5-FliQs-FliR1-FlhBTm complex (Figure 3, middle panel), and the cytoplasmic loop connecting TMH-2 and TMH-3 (FlhBLoop) wraps around
FIGURE 1  | Schematic diagrams of the flagellar type III export apparatus and $F_0F_1$ ATP synthase. The flagellar type III secretion system (tT3SS) is composed of five membrane proteins, FlhA, FlhB, FliP, FliQ, and FliR and three cytoplasmic proteins, FliH, FliI, and FliJ. FlhA, FlhB, FliP, FliQ and FliR assembles into a transmembrane export complex within the MS-ring of the basal body of the flagellum. FliH, FliI, and FliJ form a cytoplasmic ATPase ring. The $F_0F_1$-ATP synthase. The N-terminal and C-terminal domains of FliH structurally are similar in structure to the $\alpha_3\beta_3\gamma_1$ ring complex of the $F_0F_1$ ATP synthase. The stoichiometry of the c-ring varies dramatically from $c_8$ up to at least $c_{15}$. CM, cytoplasmic membrane.

FIGURE 2  | Schematic diagram of the bacterial flagellum. The bacterial flagellum is composed of basal body rings, namely the C-ring, MS-ring, L-ring, and P-ring, and an axial structure consisting of the rod, the hook, the hook-filament junction, the filament, and the filament cap. To construct the axial structure beyond the cytoplasmic membrane, flagellar axial proteins are translocated through the tT3SS, diffuse down a narrow central channel, and assemble at the tip of the growing structure. OM, outer membrane; PG, peptidoglycan layer; CM, cytoplasmic membrane.

the entrance gate of the FliP$_5$-FliQ$_4$-FliR$_1$ complex through interactions of the loop with each FliQ subunit (Figure 3, right panel). It is thus plausible that FlhB may coordinate gate opening for substrate entry into the polypeptide channel. Recent genetic analysis has suggested that the N-terminal cytoplasmic tail of FlhB and FlhB$_C$ are involved, along with the
cytoplasmic ATPase complex, in the gating function of FlhB (Kinoshita et al., 2021).

Salmonella FlhA is divided into two distinct regions: an N-terminal transmembrane region (FlhATM) with eight TMHs (residues 1–327) and a large C-terminal cytoplasmic region (residues 328–692) (Figure 4A; Minamino et al., 1994; Kinoshita et al., 2021). The crystal structure of the C-terminal cytoplasmic region is composed of a compactly folded domain (FlhAC, residues 362–692) and a flexible linker (FlhAC, residues 328–361) connecting FlhAC with FlhATM (Figure 4A; Saijo-Hamano et al., 2010). FlhA assembles into a homo-nonamer through intermolecular interactions between FlhAC subunits, and the interactions of FlhAC with its neighboring FlhAC subunit stabilize the FlhAC-ring structure (Figure 4B; Terahara et al., 2018; Kuhlen et al., 2021). FlhATM associates not only with the FliP3-FliQ4-FliR1 complex but also with the MS-ring (Kihara et al., 2001).

If either MS-ring or the FliP3-FliQ4-FliR1 complex is missing in Salmonella cells, FlhA cannot efficiently form the oligomer at the flagellar base as monitored with FlhA labeled with yellow fluorescent protein (YFP), suggesting that FlhA assembles into the export complex along with other export-gate proteins during MS-ring formation (Morimoto et al., 2014). The highly conserved Arg-94, Lys-203, Asp-208, and Asp-249 residues of FlhATM are critical for H⁺-coupled protein export (Hara et al., 2011; Erhardt et al., 2017). Over-expression of FlhA in Escherichia coli decreases the intracellular pH. Furthermore, over-expression of FlhA increases intracellular Na⁺ concentration in the presence of 100 mM NaCl. These observations suggest that FlhA forms a pathway for the transit of both H⁺ and Na⁺ across the cytoplasmic membrane. The FlhA(D208A) mutation facilitates the H⁺-channel activity of FlhA, suggesting that Asp-208 of FlhA may coordinate H⁺ flow though the FlhA channel with protein export. However, this mutation does not affect the Na⁺-channel activity of FlhA at all, suggesting that the Na⁺ pathway is different from the H⁺ pathway (Minamino et al., 2016b).

FlhAC and FlhBC 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 export substrates (Minamino and Macnab, 2000c; Minamino et al., 2003, 2010, 2012a; Bange et al., 2010). The FlhAC-FlhBC docking platform determines the order of substrate export to facilitate efficient flagellar assembly and also regulates gate opening of the FlhA ion channel and the FliP3-FliQ4-FliR polypeptide channel (Minamino and Macnab, 2000a; Kinoshita et al., 2013; Inoue et al., 2019; Minamino et al., 2020a, 2021b).

A highly conserved hydrophobic dimple including Phe-459, Asp-456, and Thr-490 of FlhA is critical for substrate recognition by the fT3SS during flagellar assembly (Figure 4A; Xing et al., 2018). The C-terminal part of FlhAC binds to its neighboring FlhAC subunit to stabilize the open conformation of FlhAC in the nonameric ring, allowing flagellar export.
chaperones associated with their cognate substrates to bind to the conserved hydrophobic dimple with a nanomolar affinity (Inoue et al., 2021).

**Catalytic Mechanism of the FliIβ-FliJ1 Ring Complex**

The F1 ATPase is composed of three copies of the α subunit, three copies of the β subunit, a single copy of the γ subunit, and a single copy of the ε subunit (Figure 1). The α and β subunits form a hetero-hexameric αβγ ring, and the γ subunit binds within the central pore of the αβγ ring (Abrahams et al., 1994). The ε subunit binds to the γ subunit to control the ATP hydrolysis activity of the F1 ATPase in an ATP-dependent manner (Kato-Yamada et al., 2000). The αβγ1 subcomplex is the minimum unit that can function as an ATP-driven rotary motor to couple ATP hydrolysis with the rotation of the γ subunit within the αβγ2 ring. ATP binds to three catalytic sites in the αβγ ring, each of which is located at an interface between the α and β subunits. Three catalytic β subunits in the αβγ ring undergo highly cooperative and sequential conformational changes in their C-terminal domains during ATP hydrolysis. These conformational changes drive the γ subunit to rotate within the αβγ ring (Watanabe and Noji, 2013). The FliIβ-FliI subcomplex of the fT3SS, which looks similar to the αβγ1 subcomplex, can act as the ATPase at the base of the flagellum (Figure 1; Ibuki et al., 2011).

FliI is the flagellum-specific ATPase. It has highly conserved Walker A and B motifs (Vogler et al., 1991; Fan and Macnab, 1996). *Salmonella* flagellin consists of three domains: N-terminal (residues 2–97, FliIα), ATPase (residues 109–380, FliIβCAT) and C-terminal (residues 381–456, FliIγ) (Figure 5A; Imada et al., 2007). Residues 98–105, most of which are invisible in the electron density map, form a flexible hinge connecting FliIγ and FliIβCAT, and this flexible hinge loop undergoes conformational changes during ATP binding and hydrolysis (Minamino et al., 2001).

The structures of FliI and its fT3SS homolog SctN are remarkably similar to the α and β subunits of the F1 ATPase (Zarivach et al., 2007). However, in contrast to the F1 ATPase, FliI and SctN form homo-hexamers in an ATP-independent manner (Figure 5B, C), and both hexamers themselves can hydrolyze ATP at the interface between FliI/SctN subunits (Clarét et al., 2003; Kazetani et al., 2009). Thus, the ATPase ring complex of the T3SS has six catalytic sites. The FliIγ and SctNγ ring structures have been identified at the base of the flagellum and injectisome, respectively, by electron cryotomography and sub-tomogram averaging (Chen et al., 2011; Kawamoto et al., 2013).

Intermolecular interactions between FliIβ domains are required for FliI ring formation (Figure 5C; Okabe et al., 2009). The core structure of FliIβ can be superimposed onto the N-terminal domains of the α and β subunits of the F1 ATPase within αβγ hetero-hexamer. In the FliIβ-ring model, which was generated by fitting the crystal structure of FliI into the structures of the α and β subunits, FliIβ shows steric hindrance at the subunit interfaces, suggesting that a conformational change in FliIβ is required for FliI ring formation. Deletion of residues 2–7 of FliIβ suppresses FliI hexameterization and decreases the
ATPase activity of FliI (Minamino et al., 2006), suggesting that the extreme N-terminal region of FliI regulates FliI oligomerization. Recently, it has been reported that Arg-33, Asn-73, and Arg-76 are also responsible for well-regulated FliI ring formation (Figure 5C; Kinoshita et al., 2021).

Amino acid residues in the F$_1$ ATPase that are known to be involved in ATP hydrolysis are highly conserved in the FliI/Stn family. FliI$_{CAT}$ contains the highly conserved P-loop (residues 182–188), the catalytic glutamate (Glu-211), and the arginine finger (Arg-374) (Figure 5A; Walker, 2013). ADP binds to the P-loop of FliI, as it does in the F$_1$ ATPase. The carboxyl group of Glu-190 in the $\beta$ subunit of the thermophilic Bacillus F$_1$ ATPase, which corresponds to Glu-211 of FliI, polarizes a water molecule for the nucleophilic attack on the $\gamma$-phosphate of ATP, and the G190Q substitution results in a complete loss of ATPase activity (Shimabukuro et al., 2003).

The fliI(E211Q) mutation completely abolishes ATPase activity but does not affect the binding of ATP to the P-loop, and FliI with the E211Q substitution can form the hexamer ring in the presence of Mg$^{2+}$-ATP. Thus, Glu-211 of FliI$_{CAT}$ acts as the catalytic glutamate.

Arg-373 in the $\alpha$ subunit of the F$_1$ ATPase, which corresponds to Arg-374 of FliI, functions as the arginine finger that protrudes into the nucleotide-binding site of the adjacent $\beta$-subunit. The side chain of this arginine residue forms a positively charged binding pocket for the negative charge of the $\gamma$-phosphate of ATP (Rees et al., 2012). The fliI(R374A) mutation inhibits FliI ring formation significantly and decreases ATPase activity. This effect indicates that Arg-374 of FliI stabilizes the binding of ATP to the P-loop in a way similar to Arg-373 of the $\alpha$ subunit. These observations suggest that FliI and the F$_1$ ATPase share a similar catalytic pathway for ATP hydrolysis.
The binding of ADP to the P-loop induces a conformational change in FliIC relative to FliICAT, suggesting that the FliI hexamer may undergo conformational changes in its C-terminal domains that are coupled with the catalytic reaction cycle in the same way as in the F1 ATPase. This idea is supported by the asymmetric cryoEM structure of the SctN2-SctO1 ring complex with a non-hydrolyzable ATP analog (Majewski et al., 2019).

FliJ and its vT3SS homolog SctO adopt an antiparallel coiled-coil structure that is similar to the two-stranded α-helical coiled-coil part of the γ subunit of the F1 ATPase (Figure 5D; Ibuki et al., 2011). FliJ binds to the C-terminal region of the first α-helix of FliIC (residues 382–406 of Salmonella FliI), which corresponds to the region of the β subunit that is responsible for interaction with the γ subunit. This interaction facilitates FiiL ring formation and increases the ATPase activity of FliI. FliJ penetrates the central cavity of the FliI6 ring like the γ subunit in the F1 ATPase (Figure 5C). These observations have been confirmed by the cryoEM structure of the SctN2-SctO1 ring complex. FliJ has been shown to exert a rotor-like function in both rotary F1 and V1 ATPases (Kishikawa et al., 2013; Baba et al., 2016). Thus, the FliI6-FliJ1 ring complex may function as an ATP-driven rotary motor that couples ATP hydrolysis with the rotation of FliJ within the FliI hexamer.

Peripheral Stalk of Flagellar ATPase Ring Complex

The b and δ subunits of the F0F1 ATP synthase form the peripheral stalk that connects the α3β3γ1ε1 ring complex to the membrane-embedded F0 unit (Figure 1). The extreme N-terminal region of the b subunit binds to F0, whereas the δ subunit interacts with the extreme N-terminal region of the α subunit of F1 (Walker and Dickson, 2006). The N-terminal and C-terminal regions of FliH and its vT3SS homolog SctL are homologous to the b and δ subunits of the ATP synthase (Pallen et al., 2006). This is confirmed by the crystal structure of an N-terminally truncated variant of Salmonella FliH consisting of residues 99–235 in complex with FliI (Figure 6; Imada et al., 2016).

Salmonella FliH consists of 235 amino-acid residues and forms a homo-dimer through residues 101–140, which form a coiled-coil structure (Minamino and Macnab, 2000b; González-Pedrajo et al., 2002). The FliH dimer binds to each FliIγ domain of the FliIγ ring (Figure 6, left panel) and also to the FliN protein in the C-ring (González-Pedrajo et al., 2006; McMurry et al., 2006; Paul et al., 2006). The interactions of FliH with FliN and FliK are required for efficient and robust association of the FliI6-FliJ ring complex with the flagellar basal body (Figure 1; Minamino et al., 2009). The N-terminal domain of FliH (residues 1–140, FliH1) adopts a quite elongated α-helical coiled coil structure similar to that of the b subunit of the ATP synthase, and the extreme N-terminal region of FliH is involved in the interaction with FliN (Hara et al., 2012). Both C-terminal domains (residues 141–235, FliH2) in the FliH dimer are involved in the interaction with FliI (Minamino et al., 2002). These two FliH2 domains have completely different conformations; one binds to the extreme N-terminal α-helix of FliI consisting of residues 2–21, and the other binds to a positively charged cluster consisting of Arg-26, Arg-27, Arg-30, Arg-33, Arg-76, and Arg-93 of FliIγ (Figure 6, middle and right panels). Because FliI cannot localize to the flagellar base in the absence of FliH, FliH seems to act as a peripheral stalk to firmly anchor the FliI6-FliJ1 ring complex to the C-ring.

Mechanism of Gate Activation

The PMF consists of the electric potential difference (Δψ) and the proton concentration difference (ΔpH) across the cytoplasmic membrane. When the cytoplasmic ATPase ring complex works properly for flagellar assembly, the transmembrane export gate complex uses the Δψ component to drive H+ -coupled protein export under a variety of environmental conditions (Paul et al., 2008; Minamino et al., 2011, 2021b). However, when the ATPase ring complex becomes...
non-functional under certain physiological conditions, the export gate complex prefers to use the SMF over a wide range of external pH, indicating that the transmembrane export complex is intrinsically a dual-fuel export engine that can use either H$^+$ or Na$^+$ as the coupling ion (Minamino et al., 2016b, 2021a). This in turn suggests that the cytoplasmic ATPase ring complex switches the export gate complex from the dual-fuel engine mode to a highly efficient $\Delta \psi$-driven one.

FliJ binds to FlhA$_L$ with high affinity to activate the H$^+$ channel of FlhATM and to unlock the entrance gate of the polypeptide channel. As a result, the export gate complex becomes an active H$^+$/protein antiporter that couples inward-directed H$^+$ flow through the FlhA ion channel with outward-directed protein translocation across the polypeptide channel (Minamino et al., 2011). An inactive export gate complex can also be activated by an increase in $\Delta \psi$ above a certain threshold through an interaction between FliJ and FlhA$_L$, suggesting that $\Delta \psi$ is required for efficient and stable interaction between FliJ and FlhA$_L$ (Minamino et al., 2021b).

A helix-loop-helix formed by Gln-38, Leu-42, Tyr-45, Tyr-49, Phe-72, Leu-76, Ala-79, and His-83 of FliJ, which are highly conserved residues in FliJ homologs, extends out of the FliI$_6$-ring.
Mechanistic Role of ATP Hydrolysis for Flagellar Protein Export

ATP hydrolysis by the FliI ATPase and rapid protein translocation by the export complex are both linked to efficient H⁺ translocation through the FlhA ion channel (Morigoto et al., 2016). Recently, it has been reported that ATP hydrolysis by the FliI ATPase also unlocks the entrance gate of the polypeptide channel formed by FliP, FliQ, and FliR for efficient entry of export substrates into the channel (Kinoshita et al., 2021). Furthermore, the Salmonella ΔfliHI \( fliB(P287T) \) \( fliA(T490M) \) mutant has been isolated as a revertant of the ΔfliHI mutant that has increased motility (Minamino et al., 2021a). The protein-export activity of the transmembrane export complex in cells with both \( fliA(T490M) \) and \( fliB(P287T) \) mutations is almost at the wild-type level under a variety of experimental conditions even in the absence of the FliH₁₂-FliI₆-FliJ₁ ring complex (Minamino et al., 2021b). This finding suggests that the export complex normally requires the FliH₁₂-FliI₆-FliJ₁ complex to serve as a H⁺-coupled protein transporter. Because FliJ requires FliI and FliH to bind efficiently to FlhA₁₂ (Minamino et al., 2011), this observation raises the question of how this ATPase ring complex activates the export complex.

The conserved Glu-211 residue of Fli catalyzes ATP hydrolysis. The E211D substitution decreases Fli ATPase activity by about 100-fold (Minamino et al., 2014). Salmonella wild-type cells produce an average of 4.4 ± 1.6 flagellar filaments per cell. In contrast, more than 90% of Salmonella \( fliI(E211D) \) cells have an average of 2.3 ± 1.5 flagellar filaments, and the average length of those filament is only half that of the wild type. Because the TM \( \alpha \) subunit of the \( fliHIJ flhB(P28T) flhA(T490M) \) mutant has been isolated as a revertant of the ΔfliHI mutant that has increased motility, the ATPase activity is still at about 40% of the wild-type level. Because this deletion does not inhibit the interaction between FliI and FlhA, it may affect conformational changes in the FlhA complex that rotate FliI within the FlhA hexamer. Rotation of FliI may induce conformational changes in the FlhATM domain through an interaction between FliI and FlhA₁₂, thereby activating the FlhA ion channel and unlocking the entrance gate of the polypeptide channel (Figure 7).

The elementary step size of \( \gamma \) rotation within the \( \alpha_3 \beta_3 \) ring is 120°, which is composed of 80° and 40° sub-steps driven by ATP binding–ADP release and ATP hydrolysis–Pi release, respectively (Watanabe and Noji, 2013). The cryoEM structure of the SctN₆-SctO₃ complex has suggested a possible rotational mechanism for causality. In this model, the SctO stalk rotates in the SctN₆ ring through an interaction between each SctN₆ domain and SctO. Because the SctN₆-SctO₃ ring complex has six catalytic sites, the elementary step size of SctO rotation within the SctN₆ ring is probably 60° (Majewski et al., 2019). The FliI(E211Q) substitution in Salmonella, which completely eliminates ATPase activity but not ATP binding to the P-loop of FliCAT, results in only 17% of cells having one or two flagellar filaments about 25% the length of those of the wild type (Minamino et al., 2014). Thus, ATP binding to the P-loop is sufficient to activate the H⁺-driven export engine of the fT3SS to some degree. So, the 60° rotation of FliI may be divided into two sub-steps, and ATP binding may induce the first sub-step, which may be sufficient to activate the H⁺-driven export engine weakly.

The Heterotrimeric FliH₂-FliI₁ Complex Acts as Dynamic Carrier

The FliI monomer interacts with the FliH dimer to form a heterotrimeric complex in the cytoplasm (Minamino and Macnab, 2000b; Auvray et al., 2002). High-resolution imaging of fluorescein-labeled FliI in vivo has revealed that FliH₂-FliI₁ complexes are associated with the basal body through interactions of FliH with FlhA and FlhN. FliI-YFP shows a rapid exchange between the basal body and a freely diffusing cytoplasmic pool. The FliI(K188I) substitution, which inhibits ATP binding to the P-loop of FliCAT, does not affect the exchange rate of FliI-YFP, suggesting that ATP hydrolysis does not drive the association-dissociation cycle (Bai et al., 2014). FliH also suppresses the ATPase activity of the FliH₂-FliI₁ complex (Minamino and Macnab, 2000b). Deletion of \( fliA \) decreases the number of FliI-YFP molecules associated with the basal body but does not affect the exchange rate. The highly conserved Trp-7 and Trp-10 residues of FlhN are directly involved in the interactions of FliH with FlhN and FlhATM (Hara et al., 2012; Notti et al., 2015). Because the interaction between FliH and FlhN is required for efficient localization of the FliH₂-FliI₁ complex to the flagellar basal, the FliI-FliN interaction must be highly dynamic to achieve rapid and efficient flagellar protein export by the fT3SS. Flagellar chaperones in complex with their cognate substrates both bind to FliI₁₂, suggesting that FliI₁₂ is also involved in substrate recognition (Thomas et al., 2004;
Pull-down assays have demonstrated that chaperone-associated export substrates bind to FlhAc and FlhBc even in the absence of FlhHi (Evans et al., 2013; Kinoshita et al., 2013; Inoue et al., 2019). However, they require the FlhH2-Flil1 complex to efficiently interact with FlhAc and FlhBc in vivo (Minamino et al., 2016b; Inoue et al., 2018; Kinoshita et al., 2021).

In vitro protein transport assays using inverted membrane vesicles have shown that addition of the purified FlhH2-Flil1 complex considerably increases the transport of flagellar axial protein into the lumen of the membrane vesicles (Terashima et al., 2018, 2020). Thus, the FlhH2-Flil1 complex acts as a dynamic carrier to deliver chaperone-associated export substrates from the cytoplasm to the flagellar base and to facilitate their docking to FlhAc and FlhBc, thereby allowing the activated export complex to unfold and transport export substrates into the central channel of the flagellum.

The FlhH2Flil Complex Is Required for Efficient Flagellar Assembly

Salmonella cells lacking the FlhH and Flil proteins display a very weak motile phenotype. This defect is considerably alleviated by either an increase in the level of export substrates and chaperones or an increase in the PMF (Erhardt et al., 2014). Expression of Vibrio alginolyticus FlhA, which has 73.2% similarity and 52.9% identity in amino acid sequence with Salmonella FlhA, restores motility in the Salmonella ΔflhA mutant but does not increase motility in the Salmonella ΔflhHi flhB(P28T)ΔflhA mutant (Minamino et al., 2016a). Thus, Vibrio FlhA requires FlhHi and Flil to perform protein export in the Salmonella ΔT3SS. Deletion of flgM, which encodes the negative regulator of the flagellar regulon, increases the expression levels of Flil, export substrates and flagellar chaperones and allows Vibrio FlhA to perform protein transport even in the absence of FlhHi and Flil. These results suggest that FlhA needs FlhHi and Flil to buffer protein export against internal perturbations (Minamino et al., 2016a).

The ΔT3SS utilizes the secreted molecular ruler protein Flk to stop growth of the hook at about 55 nm (Minamino et al., 1999; Erhardt et al., 2011; Kinoshita et al., 2017). The ΔflilHi flhB(P28T) bypass mutant cannot properly control the length of the hook, although it secretes the hook capping protein FlgD and the Flk ruler into the culture media almost at the wild-type level (Inoue et al., 2018). However, secretion level of the hook protein FlgE is about 10-fold lower than the wild-type level. The flhA(F459A) mutation, which targets a residue within FlhAc (Figure 4), significantly increases the secretion of FlgE, so the secreted Flk ruler can measure the length of the hook more precisely. Neither the secretion levels nor control of hook length is affected by the FlhB(P28T) and FlhA(F459A) substitutions when FlhHi and Flil are present. Because FlgD, FlgE, and Flk bind to FlhHi and Flil as well as FlhAc and FlhBc (Minamino and Macnab, 2000c), the FlhH2-Flil complex may coordinate targeting of FlgD, FlgE, and Flk to the FlhAc-FlhBc docking platform to make control of the hook length more robust.

FlgN, FlsI, and FltI act as export chaperones for FlgK/FlgL, FlcI, and FlcD, respectively (Fraser et al., 1999; Auvray et al., 2001). The chaperone-substrate complexes bind FlhAc with nanomolar affinity (Figure 4; Kinoshita et al., 2013). This strong interaction of the chaperone with FlhAc facilitates protein unfolding and transport by the H+--driven export complex (Furukawa et al., 2016; Minamino et al., 2021c). In wild-type cells, more than 90% of flagellin molecules transported by the ΔT3SS assemble into the filament. The ΔflhHi flhB(P28T) flhA(F459A) cannot produce the hook-filament junction and filament cap structures at the hook tip, and hence more than 90% of the flagellin molecules are secreted as monomer into the culture supernatant. Because FlgN and FltI bind to the FlhH2-Flil1 complex whereas FlsI does not (Thomas et al., 2004; Minamino et al., 2012b; Sajó et al., 2014), the FlhH2-Flil1 complex may contribute to hierarchical targeting of the flagellar chaperones to FlhAc, thereby allowing the junction and filament cap structures to be efficiently formed at the hook tip prior to filament formation. Thus, the FlhH2-Flil1 complex works with the FlhAc-FlhBc docking platform to ensure the correct order of protein export.

Energy Coupling Mechanism

The information thus far summarized allows us to propose a model for the energetics of protein export by the ΔT3SS (Figure 7). The transmembrane export complex remains inactive until the cytoplasmic ATPase ring complex localizes to the flagellar base through an interaction between FlhHi and FlN (Step 1). ATP hydrolysis by the Flil ATPase induces the rotation of Flil within the Flil6-ring at the FlhAc-FlhBc docking platform. The interactions of Flil and FlhA1 induce conformational changes in the export complex that activate the FlhA ion channel and unlock the entrance gate of the polypeptide channel (Step 2). Then, cytoplasmic FlhH2-Flil1 complexes escort export substrates and chaperone-substrate complexes from the cytoplasm to the FlhAc-FlhBc docking platform through the interactions of FlhH with FlgN and FlhA (Step 3). The binding of the export substrate to the docking platform induces opening of the gate to the polypeptide channel, and the activated export complex acts as an H+/protein antiporter that couples proton flow through the FlhA ion channel with the translocation of export substrates into the polypeptide channel (Step 4). The association-dissociation cycle of the FlhH2-Flil complex with the docking platform allows the transport of flagellar axial proteins in a highly controlled manner.

CONCLUSION AND PERSPECTIVES

The transmembrane export complex of the ΔT3SS is a dual-fuel export engine that uses either H+ or Na+ as the coupling ion to drive export of flagellar proteins. Interestingly, when the cytoplasmic ATPase ring complex works properly, the export gate preferentially utilizes the PMF to drive H+-coupled protein export. FlhATM acts as a dual ion channel to conduct both H+ and Na+. Because there is not yet structural information about the FlhATM, it remains unknown how the cytoplasmic ATPase ring complex switches the ion channel mode of FlhATM from an inefficient dual-ion channel to a highly efficient H+ channel.
The export complex couples inward-directed ion flow through FlhA with outward-directed protein translocation through the polypeptide channel. Recently, the cryoEM structure of the FliP-FliQ-FlhA complex associated with the basal body has been obtained with near atomic level (Johnson et al., 2021; Tan et al., 2021). Unfortunately, both FlhA and FlhB are lacking in the structure. To clarify the energy coupling mechanism, high-resolution structures of the entire export complex in different states of substrate export will be required.

The entire structure of the FliC-FliJ ring complex looks similar to those of rotary F1 and V1 ATPases. The FliC to FliJ ring complex hydrolyzes ATP at the interfaces between FliJ subunits and may induce sequential and cooperative conformational changes in FliC, which is involved in the interaction with FliJ. These observations lead to the hypothesis that ATP hydrolysis by the FliJ ATPase presumably allows FliJ to rotate within the FliC ring. This idea is supported by the cryoEM structure of the SctN-SctO ring complex. To demonstrate the rotational catalytic mechanism of the FliC-FliJ ring complex directly will require a biophysical approach.

The fT3SS transports fourteen different flagellar proteins in their copy numbers ranging from a few to tens of thousands in a sequential manner so that the flagellum can be built efficiently. The fT3SS must ensure the correct order of export of flagellar proteins for this to be an efficient process. The FliC-FliJ complex is required for efficient and robust flagellar assembly. Although an in vitro protein transport assay using inverted membrane vesicles has been established for the fT3SS, a quantitative measurement of ordered flagellar protein export will be needed to understand how the FliC-FliJ complex contributes to hierarchical protein targeting to the export complex.

**AUTHOR CONTRIBUTIONS**

TM, MK, and KN researched and wrote the review article. All authors contributed to the article and approved the submitted version.

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