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# Soluble components of the flagellar export apparatus, FliI, FliJ, and FliH, do not deliver flagellin, the major filament protein, from the cytosol to the export gate ☆

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ABSTRACT

Flagella, the locomotion organelles of bacteria, extend from the cytoplasm to the cell exterior. External flagellar proteins are synthesized in the cytoplasm and exported by the flagellar type III secretion system. Soluble components of the flagellar export apparatus, FliI, FliH, and FliJ, have been implicated to carry late export substrates in complex with their cognate chaperones from the cytoplasm to the export gate. The importance of the soluble components in the delivery of the three minor late substrates FlgK, FlgL (hook–filament junction) and FliD (filament-cap) has been convincingly demonstrated, but their role in the transport of the major filament component flagellin (FliC) is still unclear.

We have used continuous ATPase activity measurements and quartz crystal microbalance (QCM) studies to characterize interactions between the soluble export components and flagellin or the FliC:FliS substrate–chaperone complex. As controls, interactions between soluble export component pairs were characterized providing $K_d$ values. FliC or FliC:FliS did not influence the ATPase activity of FliI alone or in complex with FliH and/or FliJ suggesting lack of interaction in solution. Immobilized FliI, FliH, or FliJ did not interact with FliC or FliC:FliS detected by QCM. The lack of interaction in the fluid phase between FliC or FliC:FliS and the soluble export components, in particular with the ATPase FliI, suggests that cells use different mechanisms for the export of late minor substrates, and the major substrate, FliC. It seems that the abundantly produced flagellin does not require the assistance of the soluble export components to efficiently reach the export gate.



## 1. Introduction

Flagella are the locomotion organelles of bacteria. The flagellum consists of three major parts: the basal body (including the rod), the hook, and the filament. Assembly of the flagellum requires the coordinated expression and transport of about 20 structural components, and numerous other proteins play a role in the regulation of the assembly process [1–3]. Outer components of the flagellum starting with the rod proteins are transported to the assembly site by a specialized type III export apparatus [4], which is related to the type III secretion system (T3SS) for virulence factors of certain pathogenic bacteria [5].

The flagellar T3SS of *Salmonella enterica* serotype *typhimurium* (*S. typhimurium*) is composed of six membrane proteins (FlhA, FlhB, FliO, FliP, FliQ, FliR) forming the export gate, which is found within the MS ring of the basal body [4,6]. Three additional proteins FliI, FliH, and FliJ constitute the soluble components of the export apparatus. Export substrates are thought to be delivered from the cytosol to the export gate by the soluble components [3,7], which also play a role in recycling of export chaperones [8].

FliI is an ATPase that was shown to be related to the α and β subunits of the $F_0F_1$ ATP synthase by sequence similarity [9]. The structure of FliI confirmed the homology [10], and based on biochemical and electron microscopic data the functional form of FliI seems to be a homohexamer [11–13] analogous to the $\alpha_3\beta_3$ hexamer ring of the $F_1$ ATPase. For a long time it was widely believed that FliI provides the energy for the flagellar export system [9,14,15]. This assumption was further supported by mutations at the nucleotide-binding site that markedly reduce the ATPase activity of FliI and motility of the cells [14,16]. Later it turned out that the proton motive force (PMF) is the driving force of the

*Abbreviations:* FliI, a soluble ATPase component of the flagellar export apparatus; FliH and FliJ, soluble regulatory components of the export apparatus; FliC, flagellin, the major filament protein; FliS, chaperone for FliC; T3SS, type III secretion system; FlhA FlhB, FliO, FliP, FliQ and FliR, membrane components of the flagellar export apparatus; FlgK and FlgL, hook–filament junction proteins; FlgN, chaperone for FlgK and FlgL; FliD, filament-cap protein; FliT, chaperone for FliD; FliN, a C-ring protein

☆ Complexes with defined stoichiometry (e.g. FliC:FliS) are indicated with a colon (:), while complexes with varying or unknown stoichiometry (e.g. FliI–FliJ–FliH) are indicated with a hyphen (-).

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flagellar export apparatus [17,18] and FliI along with FliH is not absolutely essential for export although their absence results in a highly paralyzed filament formation [19].

FliH also shows sequence similarity to $F_0F_1$ ATP synthase subunits, namely to the b and δ subunits [20]. Originally FliH was thought to be the regulator of FliI, because it reduces its ATPase activity potentially preventing futile ATPase hydrolysis in the cytosol [15]. However, null mutation of FliH can be substantially bypassed by overexpressing FliI or certain FlhA or FlhB mutations [21], and now it seems likely that FliH is primarily required for anchoring the FliI hexamer to the export gate [1]. Interestingly overexpression of FliH in otherwise wild-type cells also reduces motility [22], which can be explained by the excessive formation of a FliI:$FliH_2$ heterotrimer [15] preventing FliI hexamerization. In all, FliI and FliH are both required for efficient export, and they also must be expressed in a proper ratio.

Originally FliJ was thought to function as a general chaperone [23]. A recent structural work established that it is homologous to the γ subunit of the $F_0F_1$ ATP synthase [13] and probably functions as an integral component of the FliI–FliJ–FliH ATPase complex. FliJ is essential for efficient flagellar export, lack of FliJ results in a leaky motile phenotype [23]. Similarly to FliH, overexpression of FliJ in otherwise wild-type cells also reduces motility [22] possibly because it may form 1:1 complexes with FliI when too much FliJ is present [13] preventing the formation of the functional FliI hexamer ring.

The soluble components (FliI, FliH, FliJ) were identified to preferentially associate with membranes [8,11,24]. FliI interacts in vitro strongly with acidic phospholipids, which in turn promote hexamerization and increase the ATPase activity of FliI [11,24]. However, on electronmicroscopic images the FliI hexameric ring structure seems to be located under a (likely nonameric) ring formed by the cytoplasmic domains ($FlhA_C$) of FlhA molecules [25,26] further from the inner membrane. FliI, FliJ, and FliH were all shown to interact with $FlhA_C$ [1] and FliH also binds to the C-ring protein FliN [27] that is thought to play an important role in the localization of the ATPase complex.

FliJ was also shown to have a moonlighting role to cycle export chaperones FlgN (chaperone for hook–filament junction proteins FlgK and FlgL) and FliT (chaperone for filament cap protein FliD), but not FliS, the chaperone of the major filament protein, FliC (flagellin) [8]. FliS binds to the disordered C-terminal part of FliC [28], while the N-terminal disordered segment of FliC carries the export signal of flagellin [29,30].

FliI (alone or in complex with FliH) was shown to interact with FlgN–FlgK and FlgN–FlgL chaperone–substrate complexes in solution [7], and FliT or the FliT:FliD complex was shown to bind FliI [31]. These observations led to the idea that export substrates are escorted from the cytoplasm to the export gate by the soluble export components. This mechanism seems to be justified for the minor late substrates FlgK, FlgL (hook–filament junction) and FliD (filament-cap), but in the case of the major filament component flagellin (FliC) data are contradictory [15,22,32]. An earlier report showed that FliC interacts with FliI and increases its ATPase activity [32], suggesting a role for the FliI–FliC interaction in the export process. Others could not reproduce this ATPase activity enhancement by FliC in the presence or absence of FliH [15] and suggested that other components (e.g. FliJ) might be required. Affinity blots showed that FliC interacts with the soluble export components, FliI, FliH, and FliJ [22], but these interactions were not confirmed by other, more reliable methods. In all, it is assumed that the soluble export components deliver substrate–chaperone complexes of late substrates from the cytoplasm to the export gate, however this assumption was convincingly demonstrated only for the three minor late substrates FlgK, FlgL (hook–filament junction) and FliD (filament-cap). Fig. 1 summarizes the current view of substrate delivery.

In this study our aim was to clarify the role of the soluble components of the flagellar export apparatus in the recognition and delivery of the major export substrate, flagellin. We used a continuous ATPase activity assay to detect changes in FliI activity in the presence or absence of the other two components, FliH and FliJ, upon the addition of FliC or the FliC:FliS complex. To detect physical interaction, regardless of the activity change, quartz crystal microbalance (QCM) measurements were carried out. No interaction was detected between FliC or the FliC:FliS complex and the soluble components in solution by any of the applied methods. We came to the conclusion that, in contrast to minor late export substrates where such mechanism was convincingly demonstrated, the soluble components of the flagellar export system do not deliver flagellin from the cytoplasm to the export gate.

## 2. Materials and methods

### 2.1. Genes and strains

The genes encoding N-terminally $His_6$-tagged FliJ, FliH and FliS were produced by PCR amplification using genomic DNA from the wild-type *S. typhimurium* strain SJW1103 [33]. The genomic DNA was purified using the NucleoSpin Tissue DNA isolation kit (Macherey-Nagel GmbH). The amplified DNA fragments were cloned into the pET17b vector (Novagen-Merck) between the NdeI and HindIII sites for FliJ and FliH, and the NdeI and XhoI sites for FliS. The gene encoding FliI was also amplified from genomic DNA and cloned into the pET19b vector (Novagen-Merck) between the NdeI and BamHI sites. The final FliI construct encodes a vector derived tag including a $His_{10}$ sequence. All PCR primers are listed in Table 1. Finally the plasmids were transformed into *Escherichia coli* BL21(DE3)pLysS (Novagen-Merck) cells for expression.

### 2.2. Protein expression and purification

FliC was purified as previously described [34] with some modifications as follows. 3 × 100 mL of *S. typhimurium* SJW1103 culture was grown for 8 h at 37 °C and 250 rpm in 3% YE (yeast extract solution) medium. 3 × 1 L of 5% YE medium in 3-liter Erlenmeyer flasks was inoculated with the 100 mL cultures and 0.015% (final concentration) antifoam A (Sigma) was added. The cultures were grown for 16 h at 37 °C and 80 rpm with aeration using a sparger. 2% PEG-6000 and 1% NaCl (final concentrations) were added to the cultures in order to aggregate detached flagella, then they were shaken for an additional hour at 37 °C without aeration. The cells were collected by centrifugation at 6 °C, 30 min, 4400 *g*, then the pellet was resuspended in 30 mL 20 mM Tris, 150 mM NaCl, pH = 7.8. Flagella were detached by shearing the cells using a blender with continuous cooling on ice, then the cells were removed by centrifugation at 6 °C, 30 min, 10,000 *g*. Flagella were collected by centrifuging the supernatant at 10 °C, 60 min, 178,000 *g* (40,000 rpm, T-647.5 rotor, Thermo Scientific). The pellet was washed with 5 mL 20 mM Tris, 150 mM NaCl, pH = 7.8, then resuspended in 5 mL 20 mM Tris, pH = 7.8 buffer containing a Complete ULTRA mini EDTA-free protease inhibitor cocktail tablet (Roche). Flagella were monomerized by heat treatment at 65 °C for 10 min. The aggregates were removed by centrifugation at 4 °C, 30 min, 340,000 *g* (70,000 rpm, MLA-80 rotor, Beckman). Flagellin was purified by polymerization by adding $(NH_4)_2SO_4$ to a final concentration of 0.8 M, then washed twice with 3 mL buffer containing 20 mM Tris, 150 mM NaCl, pH = 7.8. The flagellin filaments were resuspended in 3 mL buffer containing 20 mM Tris, 1 mM EDTA, pH = 7.8, monomerized again, then purified by anion-exchange chromatography in the same buffer using a linear gradient of 50–150 mM NaCl. Fractions containing FliC were combined, then dialyzed overnight against a buffer containing 20 mM Tris, 50 mM NaCl, 1 mM EDTA, pH = 7.8.

FliI, FliJ, FliH, FliS were grown in LB medium until they reached $OD_{600}$ = 0.6, and subsequently induced with 0.4 mM (final) IPTG. After induction the cells were grown overnight at 25 °C, 180 rpm. The cells were harvested by centrifugation and disrupted by ultrasonic treatment. In the case of FliI, FliJ, and FliH the soluble fraction contained the protein of interest, while FliS formed inclusion bodies.