The FliI ATPase couples ATP hydrolysis to substrate

switching in bacterial flagellar type-III secretion

Rosa Einenkel[a], Mario Delgadillo-Guevara[a,b], Lasse Hallenga[a], Christian Goosmann[c],

and Marc Erhardt[a,d]#

[a]Humboldt-Universität zu Berlin, Institute of Biology, 10115 Berlin, Germany

[b]Department of Biochemistry and Pharmacology, Bio21 Molecular Science and

Biotechnology Institute, The University of Melbourne, Melbourne, VIC, Australia

[c]Max Planck Institute for Infection Biology, 10117 Berlin, Germany

[d]Max Planck Unit for the Science of Pathogens, 10117 Berlin, Germany

Running Head: FliI ATPase Controls Flagellar Secretion Specificity

#Correspondence to: marc.erhardt@hu-berlin.de

Word count abstract: 159

Word count significance: 119

Word count text: 6655

Abstract:

Bacterial flagella are assembled by a specialized type III secretion system that exports structural subunits in a defined order. While the ATPase FliI is known to couple ATP hydrolysis to substrate translocation, its role in the transition between early and late secretion stages has remained unclear. Here, we systematically analyzed *Salmonella enterica* strains with defined FliI point mutations and found that FliI activity is dispensable for early substrate export and hook-basal body formation but is important for triggering the substrate specificity switch and promoting late substrate export. Mutant strains showed delayed gene expression from class 3 promoters, prolonged early secretion, and impaired flagellar filament assembly, despite normal FliI localization and oligomerization. These findings support the involvement of FliI in controlling the temporal dynamics of flagellar assembly. We propose that FliI contributes to substrate switching, ensuring robust and orderly fT3SS function. This study highlights the multifaceted role of the fT3SS ATPase in optimizing the efficiency and robustness of flagellum assembly.

Significance:

The ordered export of substrates by bacterial type III secretion systems is essential for the assembly of complex surface structures such as the flagellum, yet the mechanisms that control the timing of substrate switching remain poorly understood. The bacterial flagellar ATPase FliI is best known for being involved in energizing the flagellar type III secretion system. Here, we show that FliI contributes to the transition from early to late substrate export during flagellar biogenesis. Using targeted FliI mutants in *Salmonella*, we show that ATPase activity is dispensable for early export but important for proper substrate switching and late-stage flagellar assembly. These findings highlight the multifaceted roles of FliI during flagellar assembly beyond activating the flagellar type III secretion system.

Introduction:

Bacterial flagella are complex nanomachines that play a crucial role in motility, enabling bacteria to navigate through their environment (1). This motility is essential for many bacterial processes, including colonization and adhesion to host cells (2). The flagellum consists of three major structural components: the basal body, the hook and the filament (**Fig. 1A**). The basal body (BB) anchors the flagellum to the bacterial cell envelope and serves as the motor apparatus, generating the rotational force that drives motility (3, 4). It is composed of several ring-like structures, including the peptidoglycan-spanning P-ring the outer membrane L-ring and the inner membrane MS-ring, which connects to the rotor (3, 4). The rotor, or C-ring links the flagella mediated motility to the process of chemotaxis and acts as the rotary switch complex (5-7). The stator units generate the rotational force that drives the rotation of the C-ring and hence the flagellar filament (8). The proximal rod and the distal rod form the secretion channel through the cell envelope. The hook acts as a flexible joint between the BB and the filament (9), with its length precisely regulated (10, 11). The hook-filament junction connects the hook to the filament, which consists of tens of thousands of subunits of a single protein, the flagellin (either FliC or FljB in *S. enterica*) and acts as a mechanical buffer zone to smoothly connect the flexible hook and the rigid filament (12). The filament's distal end is capped by a pentamer of FliD, a specialized structure, that facilitates the assembly of flagellin subunits into a functional filament (12, 13).

The secretion of axial proteins is tightly regulated and mediated by the flagellar type III secretion system (fT3SS), which is responsible for the translocation of most extra-cytoplasmic flagellar components from the bacterial cytoplasm to the site of assembly, a

process that is mainly powered by the proton motive force (14). The fT3SS as part of the

BB consists of the membrane embedded export gate (FlhA, FlhB) and secretion pore

(FliP, FliQ and FliR), as well as a cytoplasmic ATPase complex (FliH, FliI and FliJ) (15).

FliI, a Walker-type AAA$^+$-ATPase with two highly conserved motifs for nucleotide binding

(Walker A) and ATP hydrolysis (Walker B), forms a hexameric ring structure at the base

of the flagellum with FliJ as the central stalk, anchored to the basal body by 12 FliH

molecules through interactions with the motor protein FliN (16-21). The ATPase complex

is thought to couple ATP hydrolysis to the activation of the export gate through

interactions of FliJ with the export gate protein FlhA to ensure efficient and robust protein

export (22). While a previous study had proposed a potential interaction between the

second messenger c-di-GMP and the fT3SS ATPase FliI (23), the exact role of this

interaction remains unclear. Furthermore, heterotrimers of 2 FliH and 1 FliI are thought to

play an important role in the dynamic delivery of substrates to the export gate, ensuring

the timely assembly of the flagellar components (24). Based on the known functions of

the evolutionarily related ATPase InvC from the virulence-associated T3SS, as well as

insights from mathematical modeling, FliI has been proposed to play roles in substrate

unfolding and in dissociating substrates from their cognate chaperones (25, 26).

However, although extensively studied, the exact molecular mechanisms of the fT3SS

ATPase complex remain incompletely understood (27).

Flagellum biogenesis and flagellar gene expression are tightly regulated through

a hierarchical system to ensure proper assembly and efficient energy utilization. This

process involves three distinct classes of promoters, starting from the class 1 promoter

that drives the expression of the master regulator $FlhD_4C_2$. $FlhD_4C_2$ activates expression

from class 2 promoters, that encode structural components of the hook-basal body (HBB)

as well as regulatory proteins like the flagellar-specific transcription factor σ^{28} and its anti-

σ factor FlgM. FlgM coordinates the temporal progression of flagellar gene expression by

sequestering σ^{28}, thereby inhibiting transcription from class 3 promoters until hook

assembly is completed. Hook completion acts as a checkpoint that signals the export

apparatus to switch substrate specificity from early substrates (rod and hook components)

to late substrates (filament components). This transition, mediated by FlhB and FliK,

ensures proper hook length, which is essential for optimal motility and flagellar bundle

formation (10, 11, 26). Late substrates require chaperones for their efficient export and to

prevent premature interactions and degradation. Upon the substrate specificity switch,

FlgM is recognized as a late substrate and secreted via the fT3SS, freeing σ^{28} to finally

initiate transcription from class 3 promoters, thereby elegantly connecting proper

flagellum assembly with gene regulation (25, 28, 29). Genes expressed from class 3

promoters encode, among others, the flagellins, the stator units and key components of

the chemotaxis system (30).

In this study, we dissected the role of the flagellar ATPase FliI by generating

targeted point mutations in its ATPase domain and adjacent regions implicated in

secretion control. Through a combination of mutagenesis and functional assays, we

identified mutants that exhibited selective defects in the secretion of late substrates and

in the substrate specificity switch, while early substrate export and hook assembly

remained unaffected. These results demonstrate that FliI is dispensable for early stages

of flagellar assembly but important for triggering the transition to late-stage export. Our

findings support a model in which FliI not only powers secretion via ATP hydrolysis but

116 also actively supports the substrate switching mechanism, thereby ensuring the temporal

117 fidelity and efficiency of flagellar biogenesis.

Results:

The Walker-type AAA+ ATPase FliI from *S. enterica* plays a crucial role in flagellar assembly by ensuring a robust export of substrates through the fT3SS. To investigate the molecular function of FliI, we generated point mutations at residues implicated in its catalytic activity and putative regulatory sites (23, 31). All mutations were introduced into the *S. enterica* genome to maintain native expression conditions. Specifically, we created mutations at residues located at the interface between FliI monomers (R177H and E215Q), and in the nucleotide-binding Walker A motif within the central cavity of the FliI hexamer (G183A). Additionally, E215 was previously shown to be involved in the catalytic activity of FliI (19). We included two previously characterized Walker B mutants (E211D and E211Q), known to drastically reduce ATPase activity - by 100-fold and completely, respectively - resulting in impaired motility and substrate secretion (31).

The effect of *fliI* point mutations on swimming motility was assessed using a soft-agar motility assay (**Fig. 1B-C**). As anticipated, the *fliI* deletion mutant (Δ*fliI*) was non-motile, consistent with previous reports (14). The R177H mutant displayed 48% motility, while the E215Q mutant was non-motile. The Walker A motif mutant G183A exhibited a slight reduction in motility to 93% of the WT level. The Walker B motif mutants E211D and E211Q showed 25% motility or loss of motility, respectively. These phenotypes suggest defects in flagellar assembly, prompting further investigation into how these specific mutations impair motility.

Fig. 1 FliI is required for flagellar motility and efficient assembly of the flagellar export apparatus. (A) Schematic of a gram-negative bacterial flagellum and the flagellar type III secretion system (fT3SS). The flagellar motor spans the inner membrane (IM), periplasmic space including the peptidoglycan (PG), and outer membrane (OM), and assembles an external filament via a central secretion channel. The cytoplasmic ATPase complex (FliHIJ) delivers substrates and activates the export gate through ATP hydrolysis. (B) Relative motility of wildtype (WT) and *fliI* mutants analyzed using soft-agar motility plates, quantified after 4-5 h incubation at 37 °C. Diameters of the motility halos were measured using Fiji and normalized to the WT. Bar graphs represent the mean of at least 3 biological replicates with standard deviation error bars. Replicates shown as individual data points. ***$p < 0.001$ (ordinary one-way ANOVA with Dunnett's multiple comparison test vs. WT). (C) Representative swimming halos of the WT and *fliI* point mutants. (D) Representative microscopy images of WT and *fliI* mutants. FliI-HaloTag labeled with TMR-ligand (magenta), basal bodies visualized with mNeonGreen-FliG (cyan); PC (PhaseContrast). Percentage of FliI-FliG foci pairs below the colocalization threshold (2 px) is indicated for each strain. White arrows show examples of colocalized foci. Scale-bar: 2 µm.

To investigate whether the phenotypes observed in the FliI mutants were caused by

subcellular mislocalization of the ATPase, we performed *in vivo* fluorescence microscopy.

In order to observe FliI localization, we constructed strains expressing FliI with a C-

terminal HaloTag fusion. To assess colocalization of the ATPase with the basal body, the

strains also expressed the C-ring component FliG with an N-terminal mNeonGreen

fusion. Flagellar biosynthesis was synchronized by replacing the native promoter of the

flhDC master regulator operon with an anhydrotetracycline (AnTc) inducible P_{tetA}

promoter (32). FliI-HaloTag was labeled with the cell-permeant ligand

tetramethylrhodamine (TMR), and fluorescence microscopy was performed on live cells

(**Fig. 1D**). We then quantified the spatial colocalization between foci detected in the

respective fluorescence channels by calculating pairwise distances between detected

spots across individual cells. Spots were considered colocalized if their distance was

below a predefined threshold (2 px or ~144 nm). The fraction of colocalized foci pairs was

calculated as the number of colocalized pairs divided by the total number of detected

pairs within a maximal distance (30 px or ~2μm).

In WT cells, FliI strongly colocalized with FliG at the basal body, forming distinct

overlapping foci with 76% of foci pairs below the colocalization threshold (**Fig. 1D**). As a

negative control, the FliI Δaa2-7 mutant, which has been previously shown to be deficient

in hexamer formation (33) and interaction with FliH (34), displayed a diffuse cytoplasmic

localization, with no distinct FliI foci visible by microscopy. Due to the nature of the

analysis pipeline, which detects local intensity maxima, foci pairs were still found,

however only 16% were considered colocalized. This confirms that FliI Δaa2-7 is unable

to localize to the basal body, likely due to its inability to properly form hexamers and

disrupted interaction with FliH. The FliI point mutants (R177H, G183A, E211D, E211Q,

E215Q) localized to the basal body similarly to the WT, as indicated by the presence of

discrete FliI foci (Figure 4D). The percentage of colocalized foci pairs was 72% for R177H,

71% for G183A, 73% for E211D, 72% for E211Q, and 70% for E215Q. These results

suggest that the FliI point mutations do not substantially impair the subcellular targeting

of FliI. Additionally, we confirmed that proper localization of FliI depends on FliH and FliN

but not on FliJ or FlhA, as previously reported (**Fig. S1A**) (21).

To assess whether the FliI point mutants affect hexamer formation, we performed *in vivo*

site-specific photo-crosslinking of FliI variants containing a photoactivatable pBpa residue

(FliI*) at the FliI-FliI interface (**Fig. S1B**). UV-dependent formation of higher molecular

weight FliI adducts was observed for WT FliI* and all its point mutants studied here,

indicating that these variants retain the ability to oligomerize (**Fig. S1C**). In contrast, FliI*

Δaa2-7 exhibited only a faint high molecular weight band, suggesting premature

oligomerization in the cytoplasm, likely due to the high-level plasmid expression of FliI*.

However, the reduced level of cross-links compared to the WT confirms that hexamer

formation in this mutant is defective but may still occur at low levels under the

experimental conditions. Taken together, these results demonstrate that while the

mutations in FliI impair motility to varying degrees, they do not substantially alter its

subcellular localization or ability to form oligomers. Thus, the observed motility defects in

the FliI point mutants may instead reflect disruptions in the secretion activity of the fT3SS.

FliI facilitates secretion of late flagellar substrates

The fT3SS mediates the assembly of the axial structures of the flagellum through two distinct secretion modes: early and late. Upon hook completion, the fT3SS undergoes conformational changes, allowing recognition and export of late substrates (11, 35). To determine whether the motility defects of the FliI point mutants result from impaired secretion, we systematically analyzed early and late substrate export in these strains. First, we confirmed FliI localization in strains deleted for components that lock the fT3SS in either the early ($\Delta flgBC$ (proximal rod), $\Delta flgE$ (hook)) or late ($\Delta flgKL$ (hook-filament junction)) stages of flagellar assembly (**Fig. S1A**). Fluorescence microscopy revealed that FliI colocalized with the basal body in both secretion states, indicating its presence at the basal body throughout the flagellar assembly process. To analyze the secretion efficiency of early substrates in FliI point mutants, we employed a hook protein / β-lactamase (FlgE-Bla) reporter system (**Fig. 2A**) (36, 37). Deletion of the rod components FlgBC leads to secretion of flagellar substrates into the periplasm (38), resulting in the ability to resist ampicillin in correlation to the quantity of secreted FlgE-Bla. Therefore, we analyzed the ability of the FliI mutants to grow in the presence of increasing ampicillin concentrations and determined the concentration of ampicillin leading to inhibition of bacterial growth to half of its maximum (IC_{50} [Amp], **Fig. S2A**) and then normalized it to WT levels (**Fig. 2B**). A mutant lacking the MS-ring and therefore the entire fT3SS ($\Delta fliF$), was used as a negative control, representing the basal level of resistance against ampicillin (**Fig. S2A**). Deletion of *fliI* resulted is a severe reduction of early substrate secretion to 2% of WT levels, similar to the $\Delta fliF$ control strain (1%), consistent with previous observations (14). Among the point mutants, G183A exhibited the highest secretion efficiency, with an IC_{50}

of 356 µg/mL corresponding to 138% of WT levels. The R177H mutant displayed

secretion comparable to WT (IC_{50} of 257 µg/mL compared to 256 µg/mL for WT), despite

reduced significantly motility. Thus, both R177H and G183A were proficient in early

substrate secretion, despite motility defects. In contrast, the ATPase activity mutants

showed impaired secretion. E215Q exhibited the most severe defect, with only 13% of

WT levels (32 µg/mL), though still 11-fold higher than the Δ*fliF* control. The Walker B motif

mutants E211D and E211Q retained partial secretion activity (62% and 37% of WT,

respectively). Importantly, this still represents a significant 52- and 31-fold increase of the

IC_{50} of the ATPase activity mutants E211D (IC_{50} = 157 µg/mL) and E211Q

(IC_{50} = 95 µg/mL) compared to the Δ*fliF* control strain, respectively (**Fig. S2A**).

For quantification of late substrate secretion, we next employed the Nano-Glo

extracellular detection system (Promega) and the split luciferase NanoBit (**Fig. 2A**) (39,

40). Strains were engineered to express late flagellar substrates translationally fused to

the small luciferase subunit HiBiT (41). Secreted HiBiT-tagged proteins accumulated in

the supernatant and were detected via luminescence (**Fig. 2C-F**).

All mutants with impaired early secretion (Δ*fliI*, E211D, E211Q, and E215Q) also exhibited

strong defects in the secretion of FlgM-HiBiT (**Fig. 2C**). Secretion levels were reduced to

0.1% (Δ*fliI*) , 0.4% (E215Q) , 0.7% (E211Q) and 7% (E211D) of WT levels. The R177H

mutant also showed a significant reduction (12.3%), while G183A retained 69.9% of WT

levels. FlgK-HiBiT secretion (**Fig. 2D**) was also reduced across all mutants. The strongest

defects were observed in Δ*fliI*, E215Q, and E211Q, each exhibiting less than 0.3% of WT

levels. E211D retained 8.2%, while R177H and G183A secreted 27.5% and 85.2% of WT

levels, respectively.

A similar pattern was observed for FliD-HiBiT (**Fig. 2E**), with secretion entirely abolished

in *ΔfliI*, E215Q, and E211Q (0.0%). E211D retained minimal export (1.8%), and secretion

was strongly to moderately reduced in R177H (19.6%) and G183A (60.5%).

E211D retained partial FliC-HiBiT (**Fig. 2F**) secretion (13.5% of WT), while the *ΔfliI* and

E215Q mutants showed only 0.1%, and E211Q 0.3% of WT levels. Secretion of FliC-

HiBiT was moderately reduced to 75.4% in G183A and more strongly in R177H (41.7%),

consistent with their motility phenotypes. Western blot analysis of cellular and secreted

fractions (**Fig. S2B–E**) confirmed the luminescence data. Additionally, reduced cellular

levels of FliC-HiBiT in most mutants suggested a delayed substrate specificity switch,

likely contributing to the impaired secretion phenotypes (**Fig. S2E**).

Secretion of all tested late substrates was consistently reduced across the *fliI*

mutants, suggesting a general requirement for FliI in late substrate export. Together,

these results are consistent with previous reports that early substrates can be secreted

with minimal ATP hydrolysis (31). The increased early substrate secretion in the G183A

mutant, and the unaffected secretion in the R177H mutant, indicate that FliI activity is

likely more critical for late substrate export than for early stages of assembly. This is

further supported by the observation that all ATPase activity mutants (E211D, E211Q and

E215Q) retained partial early secretion, despite strongly impaired ATPase activity.

Together, these findings suggest that FliI may promote late substrate export secretion

either by enhancing transport efficiency or by contributing to the substrate specificity

switch.

Fig. 2 FliI facilitates secretion of late substrates. (A) Schematic of experimental set-ups to quantify secretion of early (FlgE-Bla) and late substrates (example for FliC-HiBiT). Left: early substrate secretion was measured in strains lacking proximal rod components, allowing secretion of FlgE-Bla into the periplasm, where it confers ampicillin-resistance (Amp^R), proportional to the amount of secreted FlgE-Bla. Right: late substrate secretion was quantified using the split Luciferase NanoBit. Strains were locked in FliC production (*fliC*^{ON}) and lacked the hook-filament junction to prevent filament assembly ($\Delta flgKL$). Cells expressed a translational fusion of FliC to HiBiT. Addition of LargeBit and substrate to the culture supernatant enabled luminescence detection proportional to secreted FliC-HiBiT levels. (B) Quantification of early substrate (FlgE-Bla) secretion. Relative ampicillin resistance normalized to WT. Bars represent means ± SD from at least 3 independent experiments; **$p < 0.01$, ***$p < 0.001$; ns, not significant (ordinary one-way ANOVA with Dunnett's multiple comparison test vs. WT). (C-F) Quantification of late substrate secretion, normalized to the WT. Bars represent means ± SD from at least 3 independent experiments; ***$p < 0.001$ (ordinary one-way ANOVA with Dunnett's multiple comparison test vs. WT).

FliI contributes to the substrate specificity switch

The observed decrease of late substrate secretion in the *fliI* mutant strains could have resulted from either a defect in secretion efficiency of late substrates or from a defect or delay in the substrate specificity switch. Before hook completion, the late substrate and anti–σ^{28} factor FlgM inhibits the transcription of genes from class 3 promoters by complexing the flagellar specific σ^{28} (42). The fT3SS undergoes a substrate specificity switch upon complete formation of the HBB, which results in the secretion of FlgM, thereby allowing formation of the σ^{28}-RNA-Polymerase holoenzyme and transcription from class 3 promoters (43). Inefficient late substrate secretion would therefore not only result in lower FliC secretion levels but simultaneously result in a lower abundance of late substrates due to prolonged sequestration of σ^{28} by FlgM.

To address possible delays in the switch mechanism, gene expression from class 3 promoters was monitored using strains with an inducible *flhDC* master operon for synchronized flagella formation and a plasmid harboring the reporter genes *luxCDABE* under control of the class 3 promoter P*motA* (44). We found for the WT a half-maximal induction time of 93 ± 3 min of the P*motA* promoter after *flhDC* induction (**Fig. 3A**, dotted line). Further, the relative class 3 gene expression at T_{90} and T_{360} was determined (**Fig. S2A**). The previously described slow hook polymerization mutant *flgE*$_{T149N}$ was utilized as a control (45, 46). Since the hook polymerization in this mutant takes much longer, the substrate specificity switch and therefore activation of transcription from class 3 promoters is delayed.

The G183A mutant displayed the mildest defect in the capability to perform the substrate

specificity switch with a relative class 3 gene expression of 78% and 85% at T_{90} and T_{360},

respectively. While P_{motA} showed only 18% of the WT activity at T_{90} in the R177H mutant,

the relative promoter activity reached 63% at T_{360}. The ATPase activity mutant E211D

showed a steady increase of relative class 3 gene expression over time, starting from 3%

at T_{90} to 71% at T_{360}. The E211Q mutant displayed a relative class 3 gene expression of

3% at T_{90} and T_{360}. In both $\Delta fliI$ and E215Q mutants the relative class 3 gene expression

stayed approximately the same over the course of the experiment, with 2% (T_{90}) and 1%

(T_{360}) relative expression observed. The $flgE_{T149N}$ mutant showed a delayed substrate

specificity switch, as expected, resulting in relative class 3 gene expression of 2% at T_{90}

and 11% at T_{360}.

A possible reason for the observed reduction of class 3 gene expression in the mutant

strains could be a reduced number of hooks. A slower hook polymerization, similar to the

$flgE_{T149N}$ control strain, and hence a reduced number of completed hooks would delay the

substrate specificity switch. Only upon completion of the HBB, the substrate specificity

switch occurs and the anti-σ28 factor FlgM will be recognized as a substrate of the fT3SS.

Accordingly, a delayed and/or decreased HBB formation frequency, resulting in FlgM

remaining inside the cell, would also explain the observed delay in class 3 gene

expression.

Fig. 3 FliI contributes to the substrate specificity switch. (A) Time course of P$_{motA}$ activity (class III gene expression). Luciferase reporter activity was monitored in strains with synchronized flagella synthesis. Shown are the mean RLU (lines and symbols) ± SD (transparent lines) measured in 6 biological replicates. Dashed line indicates half-maximal induction time for P$_{motA}$ in the wildtype (WT). (B) Quantification of hook number per cell determined by fluorescence microscopy. Violin plots represent distribution of the data from 3 biological replicates; symbols represent the mean of the biological replicates. ***p < 0.001; ns, not significant (ordinary one-way ANOVA on the means with Dunnett's multiple comparison test vs. WT). (C) Representative fluorescence microscopy images of WT and *fliI* point mutants. Filaments (FliC T237C) labeled with Star Green Maleimide (green), hooks (FlgE$_{3\times HA}$) immunostained with α-HA-Alexa555 (red) and DNA counterstained with DAPI. Percentage of hooks with an attached filament indicated in white. Scale-bar: 2 µm. (D) Hook length measurements from purified hook basal bodies. Symbols represent individual measurements, horizontal black line indicates mean ± SD error bars. (E) Representative negative-stain Transmission Electron Microscopy images of purified hook basal bodies. Scale bar = 50 nm.

To address this possible reason for the observed delayed substrate specificity

switch in the *fliI* mutants, we determined the number of hooks per cell (**Fig. 3B**). This was

especially interesting for the R177H and G183A mutant, since both mutants did not

display a reduction of early substrate secretion but showed reduced class 3 gene

expression levels (**Fig. 2C**, **Fig. 3A**). Furthermore, we determined the number of HBBs

with an attached filament (**Fig. 3C**, **Fig. S4**). We hypothesized, if the substrate specificity

switch was intact, that the ratio of attached filaments to the HBBs would be similar to the

WT. On the other hand, an increased number of HBBs without attached filaments would

suggest an impairment in the substrate specificity switch. To determine the number of

hooks and attached filaments per cell, we simultaneously visualized the hook and filament

using strains expressing *flgE*$_{3\times HA}$ for immunostaining of the hook and *fliC*$_{T237C}$ for

maleimide staining of the filament (**Fig. 3C**). In agreement to the early substrate secretion

levels, both R177H and G183A mutants did not show a difference in the amount of

assembled HBBs compared to the WT. The ATPase activity mutant E211D displayed a

slight, but non-significant reduction of hooks per cell. In contrast, E211Q displayed a

significantly reduced number of hooks with a maximum of five hooks per cell, compared

to nine in the WT. The number of cells without hooks just increased slightly from 8% (WT)

to 10% in E211D, whereas 55% of the E211Q mutant did not assemble any hooks. The

number of hooks per cell in the E215Q mutant only ranged from zero to four. However,

84% of the E215Q mutant cells did not assemble any hooks. Importantly, these findings

indicate that the reduced class 3 gene expression in the R177H and G183A mutants did

not result from a reduced number of hooks or slower hook polymerization. The reduced

number of hooks in the mutants E211Q and E215Q likely influenced the prolonged

suppression of class 3 gene expression.

The simultaneous hook and filament labelling also served for determining the percentage

of attached filaments per cell (**Fig. 3C**, **Fig. S4**). Therefore, filaments were counted on

previously analyzed cells with determined hook numbers. Since a filament can only be

assembled if the HBB formation is completed and the fT3SS has undergone the substrate

specificity switch, all cells without HBBs were excluded from this analysis. We therefore

reasoned that the frequency of filament formation, meaning the ratio of hooks with an

attached filament, would be reduced in mutants with a defective substrate specificity

switch. The distribution of attached filaments in the G183A mutant was similar to the WT

distribution (**Fig. S4**), however overall percentage of hooks with attached filaments has

decreased from 59% in the WT to 50% in G183A (**Fig. 3C**). Even though the R177H

mutant did not have a defect in HBB assembly, the ratio of hooks with attached filaments

was reduced to 38% in the mutant strain. In the ATPase activity mutants E211D and

E211Q, the maximum number of filaments per cell was reduced to four and two,

respectively. Consequently, the percentage of hooks with attached filaments decreased

to 25% and 11%, respectively. Nonetheless, both mutants (E211D, E211Q) showed

filament formation to some extent, whereas no filaments were detected in E215Q,

independent from the number of assembled HBBs. In summary, the reduction of hooks

with attached filaments in the FliI mutant strains support a defective substrate specificity

switch.

To further confirm the involvement of the FliI ATPase in the substrate specificity switch,

we measured the hook lengths of the WT and R177H and G183A mutants (**Fig. 3D-E**).

We hypothesized that the delayed substrate specificity switch in the mutants would

prolong the operating time of the T3SS in its early substrate secretion mode, resulting in

elongated hooks. Therefore, we purified HBB complexes from the WT, and R177H and

G183A mutants and measured the lengths of the hooks. Importantly, the lengths of the

hooks of both mutants were slightly increased. The WT displayed hooks with an average

length of 54.4 nm, whereas the hooks of the R177H mutant were on average 57.1 nm. In

line with the increased secretion of early substrates (**Fig. 2C**) in the G183A mutant, the

hooks from this mutant were even longer, on average 58.5 nm. The elongated hooks

assembled by the FliI mutants further confirm an involvement of the fT3SS ATPase FliI in

the substrate specificity switch.

Collectively, our results demonstrate that FliI couples ATPase activity to substrate

specificity switching of the fT3SS. The introduced point mutations impaired the transition

to late substrate secretion, as reflected in reduced class 3 gene expression, fewer hooks

with attached filaments, and elongated hooks. These effects are not due to defects in FliI

oligomerization or basal body localization, as confirmed by *in vivo* photo-crosslinking and

fluorescence microscopy. Thus, while FliI ATPase activity is dispensable for early

substrate secretion, it is important for efficient progression to the late assembly mode.

Discussion:

ATP hydrolysis by the FliI ATPase is essential for efficient operation of the fT3SS, but its precise contribution to the temporal regulation of flagellar assembly has remained unclear. In this study, we show that FliI activity is dispensable for early substrate secretion but critically required for the substrate specificity switch and efficient export of late substrates. The observed defect in the secretion of late substrates was not limited to FliC, as secretion of other late substrates (FlgM, FlgK, FliD) was similarly impaired (**Fig. S2C**), supporting a general role of FliI in facilitating late substrate export. Our analysis of FliI point mutants demonstrates that they exhibit impaired late-stage assembly, with active early substrate export and HBB formation, revealing a role for FliI in supporting the transition to late secretion.

Table 1. Characteristics of the analyzed FliI mutants

	Relative motility	Relative early substrate secretion	Relative FliC-HiBiT secretion	Relative class 3 gene expression T_{90}	Hooks per cell	Hooks with attached filament [%]	Hook length [nm]
WT	1.00	1.00	1.000	1.000	3.1	58.7	54.4
Δ*fliI*	0.04	0.02	0.001	0.017	NA	NA	NA
R177H	0.48	1.00	0.417	0.183	3.1	38.2	57.1
G183A	0.93	1.38	0.754	0.778	3.0	50.5	58.5
E211D	0.25	0.62	0.135	0.034	2.6	24.9	NA
E211Q	0.09	0.37	0.003	0.027	0.5	11.4	NA
E215Q	0.06	0.13	0.001	0.022	0.1	0.0	NA

The key findings are summarized in **Table 1**. Notably, mutants such as R177H and G183A, despite retaining early secretion and WT numbers of HBBs, exhibited strong

defects in late substrate secretion and reduced gene expression from class 3 promoters.

Mutants with impaired ATPase activity (E211D, E211Q, E215Q) showed progressively

more severe defects in all late assembly metrics. Importantly, these defects were not due

to loss of FliI oligomerization or localization, as demonstrated by *in vivo* photo-crosslinking

and fluorescence microscopy. Together, our data suggest that the function of the ATPase

activity of FliI is not merely to contribute to protein translocation but also facilitates the

transition of the fT3SS to late substrate export.

Recent structural and functional studies have proposed that FliHI modulate FlhA

conformation to stabilize the late export state (47). Our findings are fully consistent with

this model. It is likely that reduced ATPase activity or altered protein-protein interfaces in

our mutants impair this remodeling function, thereby disrupting efficient switching to late

substrate export. Additionally, we cannot exclude that FliI activity may contribute to

dissociation of late chaperone-substrate complexes, as shown for the ATPase InvC of the

evolutionary related virulence associated T3SS - processes that may be more ATP-

dependent than early substrate export (26).

Taken together, our findings support a model in which FliI performs multiple, coordinated

functions during flagellar assembly. First, FliI acts as a dynamic substrate carrier,

delivering substrates to the export gate (24). Second, it serves as an energy-coupling

ATPase that facilitates the efficient translocation of substrates through the fT3SS (27).

Third, FliI plays an active role in controlling the substrate specificity switch, likely by

modulating the conformational state of the export gate and promoting the transition from

early to late substrate export (47). Hence, rather than serving solely as an energy-

coupling translocase, FliI operates as a multi-functional ATPase that integrates substrate

delivery, energy coupling, and specificity switching, underscoring its central role in
flagellar biogenesis.

Fig. 4 Schematic model of FliI function in wildtype and mutant strains. In wildtype cells (top), FliI facilitates
the transition from early to late secretion upon hook completion. This enables efficient FlgM secretion, timely
induction of class 3 gene expression, and robust export of late substrates, resulting in many hooks with
attached filaments. In FliI mutants (bottom), prolonged early secretion, delayed FlgM export, impaired class
3 gene expression, and inefficient late substrate export are observed. Consequently, hooks are elongated,
and fewer filaments are assembled.

Materials and Methods:

Bacterial strains, plasmids and media

All bacterial strains and plasmids used in this study are listed in Table S1 and were derived from *S. enterica* subsp. *enterica* serovar Typhimurium LT2. Cells were cultivated at 37 °C under aerobic conditions in lysogeny broth (LB) at 180 rpm. If not stated otherwise, main cultures were diluted 1:100 and grown to mid-exponential phase. If required, cultures were supplemented with ampicillin (cf = 100 µg/mL), chloramphenicol (cf = 12.5 or 10 µg/mL) and/or AnTc (cf = 100 ng/mL). The FliI point mutations were introduced chromosomally by site-specific mutagenesis using λ-Red homologous recombination (48). Oligonucleotides used in this study are listed in Table S2. Furthermore, the generalized transducing phage of *Salmonella enterica* serovar Typhimurium P22 HT105/1 int-201 was used (49). Plasmids were constructed using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, catalogue number E2621L) ore one-step mutagenesis (50).

Swimming motility assay

Swimming motility was studied using tryptone broth-based soft agar swim plates containing 0.3% Bacto agar. Motility plates were inoculated with 2 µL of overnight culture and incubated at 37 °C for 4-5 h. Images were acquired by scanning the plates and the diameters of the swimming halos were measured using Fiji (51). The swimming diameters of the mutant strains were normalized to those of the WT.

Subcellular localization of FliI

The strains carried translational fusions of *fliG* to mNeonGreen and of HaloTag to *fliI*. Additionally, the strains carried an AnTc inducible P_{tetA} promoter for the flagellar master regulator operon *flhDC* (32). Overnight cultures were grown in 2 mL LB. Cultures were diluted 1:100 in 10 mL of fresh LB and incubated at 37 °C for 1.5 h. The expression of *flhDC* was induced by the addition of AnTc (cf = 100 ng/μL), followed by 1 h incubation at 37 °C. A 500 μL sample of each culture was collected and centrifuged at 2,550 × g for 3 min. Pellets were resuspended in 1 × PBS and adjusted to an OD_{600} of 0.3. A 150 μL sample of each strain was transferred to a fresh 1.5 mL reaction tube and HaloTag labeling was performed by adding the HaloTag ligand tetramethylrhodamine (cf = 0.5 μM, Promega, catalogue number G8251) and incubating the samples for 30 min at 37 °C. Cells were washed twice with 500 μL of 1 × PBS and resuspended in 300 μL of 1 × PBS for microscopy. For imaging, 1 μL of labeled cells was spotted onto a 1% agarose pad in 1 × PBS. The cell suspension spot was air-dried for approximately 1 min and plasma-cleaned glass coverslips were applied. Fluorescence microscopy was performed using a Ti-2 Nikon inverted microscope equipped with a CFI Plan Apochromat DM 60× Lambda oil Ph3/1.40 (Nikon) oil objective, an Orca Fusion BT camera (Hamamatsu), and a SPECTRA III LED light source (Lumencor). Z-stacks were acquired in a 1.6 μm range with 5 stacks at 0.4 μm intervals. Microscopy data was analyzed using a custom Jupyter Notebook (See data and code availability statement) and Ilastik v1.4.0 (52).

In vivo photo-crosslinking of the FliI hexamer

The protocol for *in vivo* photo-crosslinking to study the ability of FliI to form hexamers was adapted from previously published protocols (53, 54). Strains were deleted for *fliI*, carried pSUP and expressed FliI$_{3\times FLAG}$ (with amber mutation and respective point mutations) constitutively from pTrc99A-FF4. Overnight cultures were grown in LB, supplemented with chloramphenicol (cf = 10 µg/mL) and ampicillin (cf = 100 µg/mL) at 37 °C, 180 rpm. Main cultures were diluted 1:100 in 10 mL LB with the respective supplements in the presence of 1 mM *p*Bpa at 37 °C until they reached mid-exponential phase. 9 mL of each culture was harvested at 4,000 × g, 4 °C for 5 min. Cells were washed once with 2 mL cold 1 × PBS and pelleted again with centrifugation. Cells were resuspended in 2 mL cold 1 × PBS. 1 mL of the cell culture was transferred into a fresh 1.5 mL reaction tube; another 1 mL was transferred into a 6-well cell culture dish and irradiated for 30 min with UV light (λ = 366 nm) on ice. Afterwards, the cells were transferred into a fresh 1.5 mL reaction tube harvested at 10,000 × g at 4 °C for 3 min. Proteins were precipitated using 10% (v/v) TCA, followed by 30 min incubation on ice. Proteins were pelleted by centrifugation at 20,000 × g for 30 min at 4 °C. Protein pellets were washed with ice-cold acetone and air-dried. Samples were adjusted to 50 OD units/µL. 500 OD units were analyzed under denaturing conditions using SDS-PAGE. Immunoblotting was performed using primary α-FLAG antibody (Sigma-Aldrich, catalogue number F1804-200UG, 1:10,000 in 1 × TBS-T). Proteins were detected using secondary α-mouse antibodies conjugated to horseradish peroxidase (Bio-Rad Immun-Star Goat Anti-Mouse (GAM)-HRP Conjugate, catalog number 170-5047, 1:20,000 in 1 × TBS-T).

Minimal inhibitory concentration assay (FlgE-Bla secretion)

The minimal inhibitory concentration (MIC) of ampicillin correlating with the amount of secreted hook-protein-β-lactamase fusion protein (FlgE-Bla) was determined as described by Lee et al. (36) and adapted by Singer et al. (37) to fit a 96-well plate format. Briefly, cultures were grown overnight in 200 µL LB in a 96-well plate at 37 °C, 180 rpm in biological triplicates. The cultures were diluted 1:100 in fresh LB and grown to mid-exponential phase at 37 °C at 180 rpm. The cultures were then diluted 1:10 in 300 µL LB and finally diluted 1:10 in 200 µL LB media supplemented with increasing concentrations of fresh ampicillin (Amp) in technical triplicates. Cells were grown for 4.5 h at 37 °C at 180 rpm and OD_{600} was measured with a microplate reader. The IC_{50} was calculated using a custom Jupyter Notebook.

Secretion Assay using the split NanoBit® enzyme

Nano-Glo® HiBiT Extracellular Detection System was purchased from Promega (N2420). Quantification of the secreted HiBiT-tagged proteins was determined as described previously (41). Briefly, overnight cultures were diluted 1:100 and grown to late exponential phase at 37 °C at 180 rpm. OD_{600} was measured and 1 mL sample of each culture was transferred into a 1.5 mL Eppendorf tube on ice. Samples were centrifuged at 13,000 x g at 4 °C for 3 min. 500 µL of the supernatant (SN) was transferred into a fresh Eppendorf tube and stored on ice. 25 µL of the SN was transferred in technical triplicates into a white 384-well plate with LB-blanks included. The working solution was prepared and SN samples and working solution were mixed as described by the manufacturer. Luminescence was measured at T_0 = 0 min and T_{10} = 10 min using a

529   microplate reader. The relative light units (RLU) of each strain were calculated using

530   following equation:

531
$$RLU = (t_{10} - t_0) - (mean\ of\ blank)$$

The calculated values were normalized to the measured OD_{600} and to the WT strain.

Secretion Assay analyzed by SDS-PAGE and Western Blot

For analysis with SDS-PAGE and Western Blotting, the same cultures were used as for

the secretion assay using the split luciferase. A 1.9 mL aliquot was taken, and cells were

harvested by centrifugation at 13,000 × g for 5 min. 1 mL of the supernatant was

transferred into a fresh tube, the remaining supernatant was discarded, and the cell

pellets were resuspended in 1 mL of double-distilled water. Proteins were precipitated

using 10% (v/v) TCA, followed by 30 min incubation on ice. Proteins were pelleted by

centrifugation at 20,000 × g for 30 min at 4 °C. Protein pellets were washed with ice-cold

acetone and air-dried. Samples were adjusted to 20 OD units/µL. 200 OD units were

analyzed under denaturing conditions using SDS-PAGE. The samples of all three

biological replicates were pooled to obtain the mean protein amount of all replicates.

Immunoblotting was performed using primary α-FlgM (gift from K. Hughes, 1:10,000 in

1 × TBS-T), α-FlgK (gift from T. Minamino, 1:10,000 in 1 × TBS-T), α-FliD (gift from T.

Minamino, 1:10,000 in 1 × TBS-T) or α-FliC (Difco, catalog number 228241 Salmonella H

Antiserum I, 1:5,000 in 1 × TBS-T) antibodies. Proteins were detected using secondary

α-rabbit antibodies conjugated to horseradish peroxidase (Bio-Rad Immun-Star Goat

Anti-Rabbit (GAR)-HRP Conjugate, catalog number 170-5046, 1:20,000 in 1 × TBS-T).

The relative amounts of secreted and cellular proteins were determined by normalization

to the housekeeping protein DnaK using the Image Lab software (Bio-Rad). DnaK was

detected using a primary α-DnaK (abcam, catalog number ab69617, 1:10,000 in 1 × TBS-

T) antibody and secondary α-mouse antibodies conjugated to horseradish peroxidase

(Bio-Rad Immun-Star Goat Anti-Mouse (GAM)-HRP Conjugate, catalog number 170-

5047, 1:20,000 in 1 × TBS-T).

Luciferase assay – P$_{motA}$-*luxCDABE*

Strains harboring the plasmid pRG19 (P$_{motA}$-*luxCDABE*) were used to monitor flagellar

class 3 gene expression (44). ON-cultures of 6 biological replicates were grown in 200 µL

LB supplemented with Chloramphenicol (Cm) with cf = 12.5 µg/ml in a 96-well plate at 37

°C, 200 rpm. To prevent evaporation, the plate was covered with a Breath-Easy sealing

membrane. The cultures were diluted 1:10 in 200 µL LB supplemented with Cm and again

1:10 in LB supplemented with Cm and AnTc to a final concentration of 100 ng/ml to induce

expression of the flagellar master regulator from P$_{tetA}$ (32) in a white 96-well plate for final

dilution to 1:100. The OD$_{600}$ was measured, as well as luminescence with a microplate

reader for 6 h every 15 min. The relative light units (RLU) were normalized to the

measured OD$_{600}$ of each sample and analyzed as described previously (45, 55). The

relative class 3 gene expression in respect to the WT was calculated for three timepoints

T$_{360}$ (360 min).

Hook and Filament Staining

For determination of the number of hooks per cell and the number of hooks with an

attached filament, cells were grown to mid-log phase. 500 µL samples were taken and

centrifuged at 2500 × g for 5 min. The cells were carefully resuspended in 500 µL fresh 1

× PBS. Star Green maleimide dye (Abberior, catalogue number STGREEN-0003-1MG)

with cf = 10 µM and 0.5 µL α-HA-Alexa555 (Thermo Fisher Scientific, catalogue number

26183-A555) were added to stain the filaments ($fliC_{T237C}$) (56) with maleimide and the

hook ($flgE_{3\times HA}$) with immunostaining. The cells were incubated for 1 h at 37 °C at 300

rpm. The cells were washed twice with 1 × PBS before loading the samples on a poly-L-

lysine coated coverslip as described previously (57, 58). The cells were fixed using 2%

paraformaldehyde-0.2% glutaraldehyde solution and rinsed with 1 × PBS before staining

the DNA fluoroshield containing DAPI (Sigma Aldrich, catalogue number F6057-20ML).

Cells were observed using a Zeiss Inverted Microscope Axio Observer Z1 at 100 ×

magnification and z-stack experiments were performed with 9 slices, 4 µm range with 0.5

µm slice interval. Analysis was done using Fiji for image processing equipped with the

MicrobeJ Plugin to determine the number of hooks per cell (51, 59). Filaments were

counted manually on cells with determined hook numbers.

Hook Length Measurements

The purification of hook basal body complexes was adapted from a previously published

protocol (6, 12, 60). Briefly, an overnight culture was inoculated with a single colony in LB

medium. The next day, 500 mL LB medium was inoculated 1:100 with the overnight

culture and grown for 2.5 h. We utilized strains with an AnTc inducible P$_{tetA}$ promoter for

the flagellar master regulator operon *flhDC* (32). To induce flagella biosynthesis, AnTc

was added, and the cells were further inoculated for 1 h. Cells were harvested at

4,000 × g at 4 °C for 15 min. The cell pellet was carefully resuspended in 20 mL ice-cold

sucrose solution (0.5 M sucrose, 0.15 M Trizma base, unaltered pH) on ice. Lysozyme

and EDTA pH 4.7 were added slowly to final concentrations of 0.1 mg/mL and 2 mM,

respectively, while stirring the cell suspension on ice. After 5 min of stirring on ice, the

suspension was transferred to RT and slowly stirred for 1 h to allow spheroplast formation.

For cell lysis, Triton X-100 was added to a final concentration of 1%. After rapidly stirring

the suspension for 10 min until it became translucent, it was slowly stirred further for 30-

45 min on ice. To degrade DNA, 2 mg DNAseI and $MgSO_4$ were added to a final

concentration of 5 mM while stirring on ice. After 5 min, EDTA pH 4.7 was added at a final

concentration of 5 mM. To pellet cell debris and unlysed cells, the suspension was

centrifuged at 15,000 × g at 4 °C for 10 min. The supernatant was collected and

centrifuged at 37,000 rpm (T-647.5 Fixed Angle Rotor, Thermo Fisher Scientific) for 1 h

at 4 °C to pellet the flagella. The flagella were washed carefully with 50 mL buffer A (0.1

M KCl, 0.3 M sucrose, 0.1% Triton X-100) and collected again at 37,000 rpm (T-647.5

Fixed Angle Rotor, Thermo Fisher Scientific) for 1 h at 4 °C. To depolymerize the flagellin

filament, the pellet was carefully resuspended in 50 mL buffer B (50 mM glycine, 0.1%

Triton X-100, pH 2.5), followed by 30 min incubation at room temperature. Finally, the

hook basal bodies were collected again by centrifugation at 37,000 rpm (T-647.5 Fixed

Angle Rotor, Thermo Fisher Scientific) for 1 h at 4 °C. Hook basal bodies were

resuspended carefully in 50 µL buffer C (10 mM Tris pH 8, 5 mM EDTA pH 8, 0.1% Triton

X-100) and incubated overnight on a rolling platform at 4 °C. Grids were prepared as

described previously (61). Briefly, aliquots of HBB samples were applied to freshly glow

discharged carbon-film-coated copper grids and allowed to adsorb for 10 minutes. After

three washes with distilled water, the grids were contrasted with 4% phospho-tungstic-

acid/1% trehalose, touched on filter paper and air-dried. The grids were analyzed in a Leo

906 transmission electron microscope (Zeiss) at 100 kV acceleration voltage.

Micrographs were scanned using a Morada side-mount camera (SIS) with ImageSP

software from TRS (Tröndle). Hook lengths were measured using Fiji (51).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 10 (GraphPad Software), and

values of $P < 0.05$ were considered statistically significant.

Data and code availability

The Jupyter notebook used for colocalization analyses of the microscopy data is available

at **https://github.com/SalmoLab/FliI_ATPase**.

Acknowledgements:

M.E. acknowledges funding from the Deutsche Forschungsgemeinschaft (DFG) research

grant no. 322866343 and from the Max Planck Society as Max Planck Fellow. M.D.G.

was supported by a Grand Challenge Initiative Global Health grant of the Berlin University

Alliance (no.113_MC_GH_MEL-BER_Erhardt_HU). We thank K. Hughes (The University

of Utah) and T. Minamino (Osaka University) for providing antibodies. We thank members

of the Erhardt lab for helpful discussions and critical comments on the manuscript, and

Heidi Landmesser and Raúl Trepel-Washington for technical assistance.

Author Contributions:

M.E. conceptualized and supervised the research project and ensured funding. R.E. and M.D.G. generated *S. enterica* mutants. R.E. performed the experiments, wrote the first draft of the manuscript and prepared figures. L.H. wrote the code for colocalization analyses for the microscopy data. C.G. prepared TEM grids and observed the purified HBB samples. M.E. reviewed and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

References:

1. Berg, HC, Anderson, RA. 1973. Bacteria Swim by Rotating their Flagellar Filaments. Nature 245:380–382.

2. Horstmann, JA, Lunelli, M, Cazzola, H, Heidemann, J, Kühne, C, Steffen, P, Szefs, S, Rossi, C, Lokareddy, RK, Wang, C, Lemaire, L, Hughes, KT, Uetrecht, C, Schlüter, H, Grassl, GA, Stradal, TEB, Rossez, Y, Kolbe, M, Erhardt, M. 2020. Methylation of *Salmonella* Typhimurium flagella promotes bacterial adhesion and host cell invasion. Nat. Commun. 11:2013.

3. Tan, J, Zhang, X, Wang, X, Xu, C, Chang, S, Wu, H, Wang, T, Liang, H, Gao, H, Zhou, Y, Zhu, Y. 2021. Structural basis of assembly and torque transmission of the bacterial flagellar motor. Cell 184:2665–2679.e19.

4. Johnson, S, Furlong, EJ, Deme, JC, Nord, AL, Caesar, JJE, Chevance, FFV, Berry, RM, Hughes, KT, Lea, SM. 2021. Molecular structure of the intact bacterial flagellar basal body. Nat. Microbiol. 6:712–721.

5. Singh, PK, Sharma, P, Afanzar, O, Goldfarb, MH, Maklashina, E, Eisenbach, M, Cecchini, G, Iverson, TM. 2024. CryoEM structures reveal how the bacterial flagellum rotates and switches direction. Nat. Microbiol. 9:1271–1281.

6. Johnson, S, Deme, JC, Furlong, EJ, Caesar, JJE, Chevance, FFV, Hughes, KT, Lea, SM. 2024. Structural basis of directional switching by the bacterial flagellum. Nat. Microbiol.

7. Tan, J, Zhang, L, Zhou, X, Han, S, Zhou, Y, Zhu, Y. 2024. Structural basis of the bacterial flagellar motor rotational switching. Cell Res. 34:788–801.

8. Santiveri, M, Roa-Eguiara, A, Kühne, C, Wadhwa, N, Hu, H, Berg, HC, Erhardt, M, Taylor, NMI. 2020. Structure and Function of Stator Units of the Bacterial Flagellar Motor. Cell 183:244–257.e16.

9. Kato, T, Makino, F, Miyata, T, Horváth, P, Namba, K. 2019. Structure of the native supercoiled flagellar hook as a universal joint. Nat. Commun. 10:5295.

10. Spöring, I, Martinez, VA, Hotz, C, Schwarz-Linek, J, Grady, KL, Nava-Sedeño, JM,
Vissers, T, Singer, HM, Rohde, M, Bourquin, C, Hatzikirou, H, Poon, WCK, Dufour,
YS, Erhardt, M. 2018. Hook length of the bacterial flagellum is optimized for maximal
stability of the flagellar bundle. PLoS Biol. 16:e2006989.

11. Erhardt, M, Singer, HM, Wee, DH, Keener, JP, Hughes, KT. 2011. An infrequent
molecular ruler controls flagellar hook length in *Salmonella enterica*. EMBO J.
30:2948–2961.

12. Einenkel, R, Qin, K, Schmidt, J, Al-Otaibi, NS, Mann, D, Drobnič, T, Cohen, EJ,
Gonzalez-Rodriguez, N, Harrowell, J, Shmakova, E, Beeby, M, Erhardt, M,
Bergeron, JRC. 2025. The structure of the complete extracellular bacterial flagellum
reveals the mechanism of flagellin incorporation. Nat. Microbiol.

13. Al-Otaibi, NS, Taylor, AJ, Farrell, DP, Tzokov, SB, DiMaio, F, Kelly, DJ, Bergeron,
JRC. 2020. The cryo-EM structure of the bacterial flagellum cap complex suggests
a molecular mechanism for filament elongation. Nat. Commun. 11:3210.

14. Erhardt, M, Mertens, ME, Fabiani, FD, Hughes, KT. 2014. ATPase-Independent
Type-III Protein Secretion in *Salmonella enterica*. PLoS Genet. 10:e1004800.

15. Halte, M, Erhardt, M. 2021. Protein Export via the Type III Secretion System of the
Bacterial Flagellum. Biomolecules 11

16. Chen, S, Beeby, M, Murphy, GE, Leadbetter, JR, Hendrixson, DR, Briegel, A, Li, Z,
Shi, J, Tocheva, EI, Müller, A, Dobro, MJ, Jensen, GJ. 2011. Structural diversity of
bacterial flagellar motors. EMBO J. 30:2972–2981.

17. Claret, L, Calder, SR, Higgins, M, Hughes, C. 2003. Oligomerization and activation
of the FliI ATPase central to bacterial flagellum assembly. Mol. Microbiol. 48:1349–
1355.

18. Ibuki, T, Imada, K, Minamino, T, Kato, T, Miyata, T, Namba, K. 2011. Common
architecture of the flagellar type III protein export apparatus and F- and V-type
ATPases. Nat. Struct. Mol. Biol. 18:277–282.

19.   Imada, K, Minamino, T, Tahara, A, Namba, K. 2007. Structural similarity between
the flagellar type III ATPase FliI and F1-ATPase subunits. Proc. Natl. Acad. Sci.
U.S.A. 104:485–490.

20.   Imada, K, Minamino, T, Uchida, Y, Kinoshita, M, Namba, K. 2016. Insight into the
flagella type III export revealed by the complex structure of the type III ATPase and
its regulator. Proc. Natl. Acad. Sci. U.S.A. 113:3633–3638.

21.   Minamino, T, Yoshimura, SDJ, Morimoto, YV, González-Pedrajo, B, Kami-ike, N,
Namba, K. 2009. Roles of the extreme N-terminal region of FliH for efficient
localization of the FliH–FliI complex to the bacterial flagellar type III export
apparatus. Mol. Microbiol. 74:1471–1483.

22.   Minamino, T, Morimoto, YV, Hara, N, Namba, K. 2011. An energy transduction
mechanism used in bacterial flagellar type III protein export. Nat. Commun. 2:475.

23.   Trampari, E, Stevenson, CE, Little, RH, Wilhelm, T, Lawson, DM, Malone, JG. 2015.
Bacterial rotary export ATPases are allosterically regulated by the nucleotide second
messenger cyclic-di-GMP. J. Biol. Chem. 290:24470–24483.

24.   Bai, F, Morimoto, YV, Yoshimura, SDJ, Hara, N, Kami-ike, N, Namba, K, Minamino,
T. 2014. Assembly dynamics and the roles of FliI ATPase of the bacterial flagellar
export apparatus. Sci. Rep. 4:6528.

25.   Kucera, J, Terentjev, EM. 2020. FliI$_6$-FliJ molecular motor assists with unfolding in
the type III secretion export apparatus. Sci. Rep. 10:7127.

26.   Akeda, Y, Galán, JE. 2005. Chaperone release and unfolding of substrates in type
III secretion. Nature 437:911–915.

27.   Minamino, T, Kinoshita, M, Namba, K. 2022. Insight Into Distinct Functional Roles
of the Flagellar ATPase Complex for Flagellar Assembly in *Salmonella*. Front.
Microbiol. 13

28.   Gillen, KL, Hughes, KT. 1991. Molecular characterization of *flgM*, a gene encoding
a negative regulator of flagellin synthesis in *Salmonella typhimurium*. J. Bacteriol.
173:6453–6459.

29. Hughes, KT, Gillen, KL, Semon, MJ, Karlinsey, JE. 1993. Sensing Structural
Intermediates in Bacterial Flagellar Assembly by Export of a Negative Regulator.
Science 262:1277–1280.

30. Chevance, FF, Hughes, KT. 2008. Coordinating assembly of a bacterial
macromolecular machine. Nat. Rev. Microbiol. 6:455–465.

31. Minamino, T, Morimoto, YV, Kinoshita, M, Aldridge, PD, Namba, K. 2014. The
bacterial flagellar protein export apparatus processively transports flagellar proteins
even with extremely infrequent ATP hydrolysis. Sci. Rep. 4:7579.

32. Karlinsey, JE, Tanaka, S, Bettenworth, V, Yamaguchi, S, Boos, W, Aizawa, S-I,
Hughes, KT. 2000. Completion of the hook–basal body complex of the *Salmonella*
typhimurium flagellum is coupled to FlgM secretion and *fliC* transcription. Mol.
Microbiol. 37:1220–1231.

33. Minamino, T, Kazetani, K-i, Tahara, A, Suzuki, H, Furukawa, Y, Kihara, M, Namba,
K. 2006. Oligomerization of the Bacterial Flagellar ATPase FliI is Controlled by its
Extreme N-terminal Region. J. Mol. Biol. 360:510–519.

34. Okabe, M, Minamino, T, Imada, K, Namba, K, Kihara, M. 2009. Role of the N-
terminal domain of FliI ATPase in bacterial flagellar protein export. FEBS Lett.
583:743–748.

35. Minamino, T, Inoue, Y, Kinoshita, M, Namba, K. 2020. FliK-Driven Conformational
Rearrangements of FlhA and FlhB Are Required for Export Switching of the Flagellar
Protein Export Apparatus. J. Bacteriol. 202

36. Lee, HJ, Hughes, KT. 2006. Posttranscriptional control of the *Salmonella enterica*
flagellar hook protein FlgE. J. Bacteriol. 188:3308–3316.

37. Singer, HM, Erhardt, M, Hughes, KT. 2014. Comparative analysis of the secretion
capability of early and late flagellar type III secretion substrates. Mol. Microbiol.
93:505–520.

38. Minamino, T, Macnab, R. 1999. Components of the *Salmonella* Flagellar Export
Apparatus and Classification of Export Substrates. J. Bacteriol. 181:1388–1394.

39. Dixon, AS, Schwinn, MK, Hall, MP, Zimmerman, K, Otto, P, Lubben, TH, Butler, BL,
Binkowski, BF, Machleidt, T, Kirkland, TA, Wood, MG, Eggers, CT, Encell, LP,
Wood, KV. 2016. NanoLuc Complementation Reporter Optimized for Accurate
Measurement of Protein Interactions in Cells. ACS Chem. Biol. 11:400–408.

40. Hall, MP, Unch, J, Binkowski, BF, Valley, MP, Butler, BL, Wood, MG, Otto, P,
Zimmerman, K, Vidugiris, G, Machleidt, T, Robers, MB, Benink, HA, Eggers, CT,
Slater, MR, Meisenheimer, PL, Klaubert, DH, Fan, F, Encell, LP, Wood, KV. 2012.
Engineered Luciferase Reporter from a Deep Sea Shrimp Utilizing a Novel
Imidazopyrazinone Substrate. ACS Chem. Biol. 7:1848–1857.

41. Einenkel, R, Halte, M, Erhardt, M. *In* Journet, L, Cascales, E (ed), Quantifying
Substrate Protein Secretion via the Type III Secretion System of the Bacterial
Flagellum Bacterial Secretion Systems, p. 577–592. Bacterial Secretion Systems:
Methods and Protocols, Humana, New York, NY.

42. Ohnishi, K, Kutsukake, K, Suzuki, H, Lino, T. 1992. A novel transcriptional regulation
mechanism in the flagellar regulon of *Salmonella typhimurium*: an anti-sigma factor
inhibits the activity of the flagellum-specific Sigma factor, σF. Mol. Microbiol. 6:3149–
3157.

43. Chadsey, MS, Karlinsey, JE, Hughes, KT. 1998. The flagellar anti-σ factor FlgM
actively dissociates *Salmonella typhimurium* σ28 RNA polymerase holoenzyme.
Genes Dev. 12:3123–3136.

44. Goodier, RI, Ahmer, BM. 2001. SirA orthologs affect both motility and virulence. J.
Bacteriol. 183:2249–2258.

45. Erhardt, M, Hirano, T, Su, Y, Paul, K, Wee, DH, Mizuno, S, Aizawa, S, Hughes, KT.
2010. The role of the FliK molecular ruler in hook-length control in *Salmonella*
enterica. Mol. Microbiol. 75:1272–1284.

46. Moriya, N, Minamino, T, Hughes, KT, Macnab, RM, Namba, K. 2006. The Type III
Flagellar Export Specificity Switch is Dependent on FliK Ruler and a Molecular
Clock. J. Mol. Biol. 359:466–477.

47. Kinoshita, M, Minamino, T, Uchihashi, T, Namba, K. 2024. FliH and FliI help FlhA
bring strict order to flagellar protein export in *Salmonella*. Commun. Biol. 7:366.

48. Blank, K, Hensel, M, Gerlach, RG. 2011. Rapid and Highly Efficient Method for
Scarless Mutagenesis within the *Salmonella enterica* Chromosome. PLoS One
6:e15763.

49. Sanderson, KE, Roth, JR. 1983. Linkage map of *Salmonella typhimurium*, Edition
VI. Microbiol. Rev. 47:410–453.

50. Liu, H, Naismith, JH. 2008. An efficient one-step site-directed deletion, insertion,
single and multiple-site plasmid mutagenesis protocol. An efficient one-step site-
directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol
8:91.

51. Schindelin, J, Arganda-Carreras, I, Frise, E, Kaynig, V, Longair, M, Pietzsch, T,
Preibisch, S, Rueden, C, Saalfeld, S, Schmid, B, Tinevez, J-Y, White, DJ,
Hartenstein, V, Eliceiri, K, Tomancak, P, Cardona, A. 2012. Fiji: an open-source
platform for biological-image analysis. Nat. Methods 9:676–682.

52. Berg, S, Kutra, D, Kroeger, T, Straehle, CN, Kausler, BX, Haubold, C, Schiegg, M,
Ales, J, Beier, T, Rudy, M, Eren, K, Cervantes, JI, Xu, B, Beuttenmueller, F, Wolny,
A, Zhang, C, Koethe, U, Hamprecht, FA, Kreshuk, A. 2019. ilastik: interactive
machine learning for (bio)image analysis. Nat. Methods 16:1226–1232.

53. Dietsche, T, Tesfazgi Mebrhatu, M, Brunner, MJ, Abrusci, P, Yan, J, Franz-Wachtel,
M, Schärfe, C, Zilkenat, S, Grin, I, Galán, JE, Kohlbacher, O, Lea, S, Macek, B,
Marlovits, TC, Robinson, CV, Wagner, S. 2016. Structural and Functional
Characterization of the Bacterial Type III Secretion Export Apparatus. PLoS Pathog.
12:e1006071.

54. Miyazaki, R, Akiyama, Y, Mori, H. 2020. A photo-cross-linking approach to monitor
protein dynamics in living cells. BBA - Gen. Subj. 1864:129317.

55.   Brown, JD, Saini, S, Aldridge, C, Herbert, J, Rao, CV, Aldridge, PD. 2008. The rate
of protein secretion dictates the temporal dynamics of flagellar gene expression.
Mol. Microbiol. 70:924–937.

56.   Renault, TT, Abraham, AO, Bergmiller, T, Paradis, G, Rainville, S, Charpentier, E,
Guet, CC, Tu, Y, Namba, K, Keener, JP, Minamino, T, Erhardt, M. 2017. Bacterial
flagella grow through an injection-diffusion mechanism. eLife 6

57.   Erhardt, M. 2017. Fluorescent Microscopy Techniques to Study Hook Length Control
and Flagella Formation. Methods Mol. Biol. 1593:37–46.

58.   Wozniak, CE, Chevance, FF, Hughes, KT. 2010. Multiple promoters contribute to
swarming and the coordination of transcription with flagellar assembly in *Salmonella*.
J. Bacteriol. 192:4752–4762.

59.   Ducret, A, Quardokus, EM, Brun, YV. 2016. MicrobeJ, a tool for high throughput
bacterial cell detection and quantitative analysis. Nat. Microbiol. 1:16077.

60.   Francis, NR, Sosinsky, GE, Thomas, D, DeRosier, DJ. 1994. Isolation,
characterization and structure of bacterial flagellar motors containing the switch
complex. J. Mol. Biol. 235:1261–1270.

61.   Halte, M, Andrianova, EP, Goosmann, C, Chevance, FFV, Hughes, KT, Zhulin, IB,
Erhardt, M. 2024. FlhE functions as a chaperone to prevent formation of periplasmic
flagella in Gram-negative bacteria. Nat. Commun. 15:5921.