RESEARCH ARTICLE

Chaperone-mediated coupling of subunit availability to activation of flagellar Type III secretion

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Abstract

Bacterial flagellar subunits are exported across the cell membrane by the flagellar Type III Secretion System (fT3SS), powered by the proton motive force (pmf) and a specialized ATPase that enables the flagellar export gate to utilize the pmf electric potential ($\Delta\Psi$). Export gate activation is mediated by the ATPase stalk, FliJ, but how this process is regulated to prevent wasteful dissipation of pmf in the absence of subunit cargo is not known. Here, we show that FliJ activation of the export gate is regulated by flagellar export chaperones. FliJ binds unladen chaperones and, by using novel chaperone variants specifically defective for FliJ binding, we show that disruption of this interaction attenuates motility and cognate subunit export. We demonstrate in vitro that chaperones and the FlhA export gate component compete for binding to FliJ, and show in vivo that unladen chaperones, which would be present in the cell when subunit levels are low, sequester FliJ to prevent activation of the export gate and attenuate subunit export. Our data indicate a mechanism whereby chaperones couple availability of subunit cargo to pmf-driven export by the fT3SS.

KEYWORDS

bacterial flagella biogenesis, proton motive force, protein export, Type III secretion system

1 | INTRODUCTION

Bacterial flagella are large macromolecular rotary motors that enable cell motility. In *Salmonella*, the long helical flagellar filament propeller that extends from the cell surface is connected to a flexible hook attached to a basal body spanning the cell envelope. These flagellar substructures are assembled in a strict order, with the basal body being constructed first, followed by the hook and then the filament (Evans et al., 2014a, 2014b). The filament comprises thousands of copies of the major filament subunit, FliC (flagellin), together with three minor filament-class subunits: 11 copies each of FlgK and FlgL form a junction connecting the semi-rigid filament to the hook, and

five FliD subunits assemble at the distal tip of the nascent flagellum to form a cap that facilitates folding and incorporation of flagellin into the growing filament (Yonekura et al., 2000).

Export of the filament subunits is facilitated by the cytoplasmic chaperones FlgN, FliT, and FliS, which bind the C-terminal domains of their cognate structural subunits (FlgN binds FlgK and FlgL, FliT binds FliD, and FliS binds FliC) (Auvray et al., 2001; Bennett et al., 2001; Fraser et al., 1999). The chaperones then pilot their cognate subunits to the specialized flagellar Type III secretion system (fT3SS) machinery at the base of each flagellum (Bange et al., 2010; Evans et al., 2006; Kinoshita et al., 2013; Minamino et al., 2012; Thomas et al., 2004). Chaperone-subunit complexes

Abbreviations: fT3SS, flagellar Type III secretion system; pmf, proton motive force; T3SS, Type III secretion system; vT3SS, virulence Type III secretion system.

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initially dock at the Flil component of the flagellar export ATPase, which is evolutionarily related to the F1-ATPase (Ibuki et al., 2011). Chaperoned subunits are then thought to interact with the integral membrane export gate component FlhA, where chaperones are released and the subunit cargo is unfolded for translocation across the inner membrane, via the FlhAB-FlpQR export gate, into a narrow central channel in the growing flagellum (Bange et al., 2010; Ibuki et al., 2011; Kinoshita et al., 2013; Kuhlen et al., 2018; Minamino et al., 2012). Once released from their cognate subunit cargo, the unladen chaperones FlgN and FliT—but not the flagellin chaperone, FliS—are recruited by the FliJ stalk component of the ATPase, which transfers them to newly synthesized cognate cargo to create a local cycle of chaperone-subunit binding at the membrane fT3SS (Evans et al., 2006). This is thought to promote export of the minor filament subunits that form the hook-filament junction and the filament cap, which are required for initiation of filament assembly (Homma et al., 1984).

Export of flagellar subunit cargo across the membrane is powered by the pmf and the flagellar ATPase complex (Minamino & Namba, 2008; Paul et al., 2008). Specifically, the ATPase is thought to facilitate unfolding of subunit cargo (Akeda & Galán, 2005). In addition, analogous to F- and V-type ATPases, ATP hydrolysis is proposed to drive rotation of the ATPase stalk, FliJ, which interacts with the nonameric export gate component, FlhA, converting the gate into a highly efficient proton-protein antiporter that utilizes the $\Delta\psi$ electric component of the pmf (Minamino et al., 2011). The FliJ-FlhA interaction is essential for export gate activation, and disruption of this interaction renders the export gate inactive and attenuates protein export (Minamino et al., 2011).

What is currently unclear is how FliJ activation of the export gate is regulated in the absence of subunit cargo to prevent the possibility of constitutive proton influx and wasteful dissipation of the pmf. One way in which export gate activation could be regulated is by sequestration of FliJ by other binding partners, specifically the export chaperones FlgN and FliT. A role for chaperones in the regulation of T3SS activity is supported by the finding that the *Pseudomonas* virulence T3SS chaperone PcrG binds the FliJ homolog, PscO, and that loss of this interaction results in upregulation of effector secretion and renders the T3SS more resistant to pmf collapse (Lee et al., 2014). These observations suggest that the chaperone-FliJ interaction is central to the regulation of T3SS activity; however, the mechanistic basis of this regulation remains obscure.

Here, we identify novel point mutant variants of the chaperones FlgN and FliT that are specifically defective for binding to the FliJ ATPase stalk yet retain the ability to bind cognate subunits and the FlhA component of the fT3SS export gate. We show in vitro that the FlgN and FliT chaperones compete with FlhA for binding to FliJ, and that chaperone sequestration of FliJ in vivo blocks the activating interaction between FliJ and the export gate. This suggests a mechanism whereby binding of FliJ by unladen chaperones acts as a signal to the export machinery that subunit cargo is unavailable and, accordingly, export activity is limited to prevent the possibility of wasteful dissipation of the pmf. This model is supported by ribosome

profiling data, which indicate that the cytoplasmic translation ratios of cognate subunits to chaperones are high for FlgN and FliT, relative to the translation ratio of flagellin to its chaperone, FliS, suggesting that levels of unladen FlgN and FliT would be a sensitive proxy measure of intracellular flagellar subunit levels. Our data indicate a mechanism by which chaperones modulate the FliJ-FlhA interaction to regulate energy use by the T3SS in response to subunit availability.

2 | RESULTS

2.1 | Isolation of chaperone variants that are specifically defective for FliJ binding

To investigate the function of chaperone binding to the ATPase stalk, FliJ, we sought to identify variants of the FlgN and FliT chaperones that did not bind FliJ, yet could interact with cognate subunits and the FlhA component of the fT3SS export gate. To do this, we first constructed mutant alleles of *flgN* encoding variants with three-residue deletions in the FlgN $\alpha 3$ helix (residues 70-102), which contains the overlapping binding sites for cognate subunits and FliJ (Figure 1; Supporting Information Appendix Figure S1) (Evans et al., 2006). Screening of the FlgN deletion variants using affinity chromatography pull-down assays with GST-FlgK subunit or GST-FliJ identified a single-deletion variant, FlgN $\Delta 76-78$, that could bind its cognate subunit but displayed severely reduced binding to the ATPase stalk (10% of wild type binding, Figure 1b; Supporting Information Appendix Figure S2b).

Protein sequence comparisons revealed FlgN W₇₈ to be highly conserved and replacement of this residue with alanine (FlgN-W₇₈A) severely reduced FlgN binding to FliJ but did not affect binding to FlgK subunit (Figures S2c and 1c; FlgN $\Delta 90-100$, which binds neither cognate subunit nor FliJ, and wild type FlgN were included as negative and positive controls, respectively) (Evans et al., 2006). Affinity chromatography pull-down assays confirmed that FlgN $\Delta 76-78$ and FlgN-W₇₈A could still bind the cytoplasmic domain of the export gate component FlhA (GST-FlhA_C; Figure 1c; interactions between FlgN variants and their binding partners are summarized in Supporting Information Appendix Table S3).

Having identified residues in the FlgN $\alpha 3$ helix that, when mutated, could decouple chaperone binding of cognate subunits and FliJ, we targeted conserved residues in the equivalent $\alpha 3$ helix of FliT for site directed mutagenesis to alanine (Figure 1d; Supporting Information Appendix, Figure S1). To facilitate screening for FliJ binding, mutations were introduced into a *fliT* allele encoding a truncated variant, FliT₉₄, which lacks the $\alpha 4$ helix and shows enhanced binding to FliJ in vitro (Imada et al., 2010). Affinity chromatography pull-down assays identified five FliT₉₄ variants (I₆₈A, L₇₂A, N₇₄A, E₇₅A and L₇₈A) that showed severely reduced binding to GST-FliJ (Figure 1e). Three of these FliJ non-binders—FliT₉₄-I₆₈A, FliT₉₄-L₇₂A, and FliT₉₄-N₇₄A—had previously been shown to bind cognate subunit FliD (Imada et al., 2010). For this reason, and after assessing the expression and stability of these three FliT₉₄ variants in *Salmonella*

FIGURE 1 Identification of chaperone variants defective for FliJ binding. (a) Atomic resolution structure of the 140-residue *Salmonella* FlgN (PDB: 5B3D) indicating residues 76–78 (cyan) and the region (residues 70–102; red) in which three-residue scanning deletions were introduced. (b) Affinity chromatography of glutathione sepharose-bound GST-FliJ or GST-FlgK incubated with cell lysates containing wild type FlgN or its three-amino acid deletion variants. After washing, proteins were eluted in SDS-loading buffer, separated by SDS(15%)–PAGE and stained with Coomassie Brilliant Blue. Apparent molecular weights are in kilodaltons (kDa). (c) Affinity chromatography of glutathione sepharose-bound GST-FliJ, GST-FlgK, or GST-FliH_AC incubated with cell lysates containing wild type FlgN or its derivative variants FlgNΔ76–78, FlgN-W78A or FlgNΔ90–100. After washing, proteins were eluted in SDS-loading buffer, separated by SDS(15%)–PAGE and stained with Coomassie Brilliant Blue. Apparent molecular weights are in kilodaltons (kDa). (d) Atomic resolution structure of the 122-residue *Salmonella* FliT (PDB: 3A7 M) indicating the position of conserved residues (red) within the region essential for FliJ binding. In vitro binding to FliJ is enhanced by truncating FliT at residue 94 (dashed red line). (e) Affinity chromatography of glutathione sepharose-bound GST-FliJ with cell lysates containing FliT₉₄ or its point mutant variants. After washing, proteins were eluted in SDS-loading buffer, separated by SDS(15%)–PAGE and stained with Coomassie Brilliant Blue. Apparent molecular weights are in kilodaltons (kDa). (f) Affinity chromatography of glutathione sepharose-bound GST-FliH_AC incubated with cell lysates containing FliD and wild type FliT or its point mutant variants FliT-L72A or FliT-Y106A. After washing, proteins were eluted in SDS-loading buffer, separated by SDS(15%)–PAGE and stained with Coomassie Brilliant Blue (top panel) or were analyzed by immunoblotting with anti-FliT or anti-FliD polyclonal antisera (middle and bottom panels). Apparent molecular weights are in kilodaltons (kDa)

(not shown), we chose to further investigate the function of truncated FliT₉₄-L72A and full-length FliT-L72A.

Binding of FliT-L72A and truncated FliT₉₄-L72A to cognate subunit FliD was confirmed using pull-down assays (GST-FliD; Supporting Information Appendix Figure S2). To assess binding to the export gate component FlhA, pull-down assays were carried out using GST-FliH_AC and FliT chaperones that had been incubated with cell lysates containing FliD cognate subunit, which is required for high affinity binding of FliT to FlhA (Figure 1f) (Bange et al., 2010). The FliT-Y₁₀₆A variant, which cannot bind FlhA, was included as a negative control (Figure 1f) (Kinoshita et al., 2013). Data from the pull-down assays indicated that, when bound to cognate subunit FliD, the FliT-L72A variant can bind the FlhA export gate (Figure 1f; interactions between FliT variants and their binding partners are summarized in Supporting Information Appendix, Table S4).

2.2 | Attenuation of cell motility and cognate subunit export by specific disruption of the chaperone–FliJ interaction

Having identified chaperone variants (FlgNΔ76–78, FlgN-W₇₈A, FliT-L72A) that were specifically defective for FliJ binding yet retained the ability to bind cognate subunits and the fT3SS export gate, we went on to investigate the importance of the chaperone–FliJ interaction for cell motility and subunit export. To enable expression of

chaperones at physiological levels, *Salmonella* strains were engineered to carry variant chaperone genes (*flgN*Δ76–78, *flgN*-W₇₈A or *fliT*-L72A) at the natural genetic loci, replacing the wild type chaperone genes. As negative controls, isogenic *Salmonella* strains in which the chaperone genes were either deleted (Δ*flgN* or Δ*fliT*) or replaced with variant genes encoding export defective chaperones unable to bind either cognate subunit (*flgN*Δ90–100) or the FlhA export gate (*fliT*-Y₁₀₆A) were also constructed. These strains were assessed for swimming and swarming motility, and for subunit export efficiency relative to wild type *Salmonella* (Figure 2).

Cell populations producing the FlgNΔ76–78 or FlgN-W₇₈A variants, which cannot bind FliJ, showed slightly decreased swimming (70%–90%) and swarming (75%–90%) motility relative to wild type *Salmonella*, though their motility was significantly better than that of the *flgN* null strain or cells producing FlgNΔ90–100, which binds neither cognate subunits nor FliJ (Figure 2a and Supporting Information Appendix Figure S2). Secretion of cognate subunit FlgK was reduced to c.50% of wild type in cells producing either FlgNΔ76–78 or FlgN-W₇₈A, and this was accompanied by a concomitant increase in soluble extracellular FliC (to 140%–150% of wild type), suggesting that assembly of the FlgKL hook-filament junction was impaired, causing inefficient initiation of filament assembly and increased release of unpolymerized FliC into culture supernatants (Figure 2b) (Bennett et al., 2001). As expected, this secretion defect was more severe in the *flgN* null strain and in cells producing FlgNΔ90–100 (Figure 2b).

FIGURE 2 FliJ binding to FlgN and FliT is required for efficient cell motility and subunit export. (a) Swimming (top panel; 0.25% soft tryptone agar) and swarming (bottom panels; 0.6% agar-tryptone with 0.5% glucose) motility of recombinant *Salmonella* strains producing chromosomally encoded FlgN variants (W78A, $\Delta 76-78$, or $\Delta 90-100$), the SJW1103 wild type, and the *Salmonella flgN* null strain ($\Delta flgN$). (b) Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of *Salmonella* strains producing chromosomally encoded FlgN variants (W78A, $\Delta 76-78$, or $\Delta 90-100$), the SJW1103 wild type, and the *Salmonella flgN* null strain ($\Delta flgN$) were separated by SDS (15%)–PAGE and analyzed by immunoblotting with anti-FliC, anti-FlgK, or anti-FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa). (c) Swimming (top panel; 0.25% soft tryptone agar) and swarming (bottom panels; 0.6% agar-tryptone with 0.5% glucose) motility of recombinant *Salmonella* strains producing chromosomally encoded FliT variants (L72A and Y106A), the SJW1103 wild type, and the *Salmonella fliT* null strain ($\Delta fliT$). (d) Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of *Salmonella* strains producing chromosomally encoded FliT variants (L72A and Y106A), the SJW1103 wild type, and the *Salmonella fliT* null strain ($\Delta fliT$) were separated by SDS (15%)–PAGE and analyzed by immunoblotting with anti-FliC, anti-FliD, or anti-FliT polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa)

Analysis of motility and export phenotypes associated with the FliT chaperone is complicated by the additional function of FliT as a negative regulator of the flagellar master transcriptional activator FlhD₄C₂ (Kutsukake et al., 1999; Sato et al., 2014; Yamamoto & Kutsukake, 2006). Cells lacking FliT show increased cellular levels of FlhD₄C₂, and a concomitant increase in flagellar gene expression that partially alleviates the motility and export defects typically associated with loss of chaperone activity (Bennett et al., 2001). The increased flagellar gene expression in the *fliT* null is reflected by an increase in the cellular level of non-cognate subunit FlgK, which was observed to be 1.4-fold higher than in wild type (Supporting Information Appendix Figure S3). Similarly, the level of FlgK was 1.4-fold higher in the strain producing the export defective FliT-Y₁₀₆A (Supporting Information Appendix Figure S3), indicating increased flagellar gene expression that may be caused by sequestration of FliT-Y₁₀₆A by cytosolic FliD, which would prevent FliT-Y₁₀₆A binding to FlhC and targeting it for degradation (Kutsukake et al., 1999; Sato et al., 2014; Yamamoto & Kutsukake, 2006). However, cellular levels of FlgK in cells producing FliT-L₇₂A were similar to wild type,

suggesting that while FliT-L₇₂A cannot bind FliJ, it retains the ability to bind FlhC and thus regulate flagellar gene expression (Supporting Information Appendix Figure S3).

Cells producing FliT-L₇₂A displayed wild type swimming motility but showed a significant defect in population swarming motility relative to wild type *Salmonella* (c.50% of wild type; Figure 2a). Moreover, export of cognate subunit FliD was reduced to c.50% of wild type in the FliT-L₇₂A strain. These data suggest that the FliT–FliJ interaction may be required for export of sufficient FliD to promote the increased flagellation required for swarming (Fraser & Hughes, 1999; Kearns, 2010). Cells producing FliT-Y₁₀₆A, which cannot bind FlhA, showed only a slight defect in swimming and swarming motility, while the *fliT* null strain displayed a marginal reduction in swimming to c.80% of wild type, yet an increase in swarming motility (to c.110% of wild type; Figure 2b). Both the *fliT* null and FliT-Y₁₀₆A strain showed a significant reduction in FliD export, to 50%–60% of wild type, accompanied by concomitant release of unpolymerized FliC to culture supernatants (to 110% of wild type; Figure 2b). The confounding influence of increased flagellar gene expression in these strains, however, makes it difficult to interpret the motility and export phenotypes of the *fliT* null and FliT-Y₁₀₆A controls, particularly as overexpression of *flhDC* is known to overcome motility defects associated with chaperone loss (Aldridge et al., 2003; Dufour et al., 1998).

Notwithstanding the difficulties in interpreting the motility and flagellar export phenotypes associated with loss or mutation of *fliT*, the data for FlgN $\Delta 76-78$, FlgN-W₇₈A, and FliT-L₇₂A indicate that specific loss of chaperone interactions with FliJ, which would break the local chaperone cycle at the fT3SS, result in attenuation of cognate subunit export and cell motility. That motility and subunit export are only partially attenuated by disruption of the chaperone–FliJ interaction indicates that the variant chaperones are still able to pilot cognate subunits to the fT3SS machinery to promote export. The data suggest that chaperone binding by FliJ increases the efficiency of, but is not essential for, cognate subunit export (Figure 2). This raises a question as to whether the chaperone–FliJ interaction might have an additional function related to the primary role of FliJ in activating the export machinery to couple efficient use of the pmf to protein export (Minamino et al., 2011).

2.3 | Unladen chaperones disrupt the FliJ–FlhA_C interaction

The identification and characterization of FlgN and FliT chaperone variants that retained their subunit piloting function but were specifically unable to bind the FliJ ATPase stalk opened up the possibility of further investigations into whether chaperones might regulate FliJ activation of the fT3SS. To test whether unladen chaperones could bind FliJ and block the FliJ interaction with the FlhA_C binding to cobalt bead-immobilized (His)₆-FliJ was assessed in the presence of chaperone variants that could interact with FliJ but not

FlhA_C (FliT₉₄ or FlgN-Y₁₂₂A) or were defective in binding both FliJ and FlhA_C (FliT₉₄-L72A or FlgN-Y₁₂₂AΔ76-78; Figure 3a). The chaperone variants were engineered to be defective for binding to FlhA_C (by introduction of the FliT₉₄ or FlgN-Y₁₂₂A mutations) to prevent chaperone-FlhA_C interactions and thus allow specific analysis of the effect of chaperone-FliJ binding on the FlhA_C-FliJ interaction. This assay, therefore, specifically investigates the effect that chaperones have on the FliJ-FlhA_C interaction in the absence of interfering chaperone-FlhA_C interactions (Figure 3a; Bange et al., 2010; Evans et al., 2006; Kinoshita et al., 2013; Minamino et al., 2012).

As anticipated, we found that FlhA_C could bind (His)₆-FliJ in the presence of chaperone variants that could not bind FliJ (Figure 3b). However, when assays were performed with chaperones that could bind FliJ, the amount of FlhA_C pulled down by FliJ was severely reduced (Figure 3b). These data show that FlgN and FliT can compete with FlhA_C for binding to FliJ, that is unladen chaperones bound to FliJ prevent the FliJ-FlhA_C interaction—an interaction known to be essential for activation of the export gate to efficiently couple pmf use to protein export (Minamino et al., 2011).

FIGURE 3 FlgN and FliT chaperones compete with FlhA_C for binding to FliJ in vitro. (a) In vitro competition assay to assess FlhA_C binding to cobalt (Co²⁺) bead-immobilized (His)₆-FliJ in the presence of the FlgN or FliT chaperones, or chaperone variants that cannot bind FliJ (FliJ non-binder). (b) Cell lysates of *E. coli* C41 expressing FliT₉₄, which cannot bind FlhA_C (–), or its L72A variant, which cannot bind FlhA_C or FliJ (left-hand panels), or FlgN Y122A which cannot bind FlhA_C (–) or its Δ76-78 variant, which cannot bind FlhA_C or FliJ (right-hand panels) were mixed with *E. coli* C41 lysates containing FlhA_C (load) and incubated with cobalt bead-immobilized (His)₆-FliJ (bound). After washing, proteins were eluted from the cobalt beads in SDS-loading buffer, separated by SDS(15%)–PAGE and analysed by immunoblotting with anti-FlhA, anti-FliT, anti-FlgN or anti-FliJ polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa)

2.4 | Regulation of ft3SS export gate activation in vivo by chaperone sequestration of the FliJ ATPase stalk

The in vitro competition assay established that the FlgN and FliT chaperones could block FliJ binding to FlhA_C, but did not show whether activation of the FlhA_C export gate was prevented. To investigate this, an in vivo competition assay was developed in which the effect of FlgN-FliJ binding on FlhA_C export activity was tested under conditions where the availability of cognate subunits for FlgN was limited. Specifically, FlgN variants that could bind FliJ but not FlhA_C (FlgN-Y₁₂₂A) or were defective in binding both FliJ and FlhA_C (FlgN-Y₁₂₂AΔ76-78) were expressed *in trans* in a Δ*flgE* strain, in which flagellar assembly does not progress beyond completion of the rod, and the FlgN cognate subunits, FlgK and FlgL, are produced at very low levels (Supporting Information Appendix Figure S5) (Hirano et al., 2003; Hughes et al., 1993; Kutsukake, 1994). As cognate subunit levels are low in the Δ*flgE* strain, we hypothesized that *in trans* overexpression of FlgN-Y₁₂₂A would produce an excess of unladen FlgN-Y₁₂₂A that would bind FliJ and disrupt the FliJ-FlhA_C interaction, preventing activation of the flagellar export gate and inhibiting subunit export. Conversely, we hypothesized that similar overproduction of the FlgN-Y₁₂₂AΔ76-78 variant, which cannot bind FliJ, would not disrupt the FliJ-FlhA_C interaction, and would permit FliJ-dependent activation of the export gate and efficient subunit export.

Assays of subunit export in a Δ*flgE* strain producing FlgN-Y₁₂₂A, which binds FliJ, demonstrated a c.75% reduction in export of the FlgD subunit compared with a Δ*flgE* strain producing FlgN-Y₁₂₂AΔ76-78, which cannot bind FliJ (Figure 3c). These phenotypes are still seen in the absence of the FliH and FliI components of the flagellar export ATPase (assessed in a strain containing the *fliH-fliI* deletion and the gain-of function mutation, P₂₈T—a mutation which partially suppresses the motility and export defects associated with the *fliH-fliI* deletion, allowing motility and subunit export assays to be performed; Supporting Information Appendix Figure S6), indicating that (i) the reduction in FlgD export is not caused by FlgN-Y₁₂₂A binding and inhibiting FliH or FliI activity, and (ii) as has been previously demonstrated by others, that FliJ can still activate FlhA to promote subunit export in the absence of FliH and FliI (Minamino et al., 2011; Minamino & Namba, 2008).

The findings from the in vivo competition assays described above, which indicate that unladen chaperones prevent FliJ activation of FlhA, are further strengthened by data showing that export of the early subunit FlgD is attenuated when the cognate subunits of the FlgN or FliT chaperones are not present (in strains deleted for *flgK*, *flgL* or *fliD*), increasing the number of unladen chaperones in the cell (Supporting Information Appendix Figure S7).

Taken together, the data from the in vitro and in vivo competitive binding assays indicate that unladen chaperones block the FliJ-FlhA interaction and prevent FliJ-dependent activation of the flagellar export machinery.

2.5 | Ribosome profiling reveals high cytoplasmic ratios of minor filament subunits to their cognate chaperones, FlgN and FliT

Our accumulating data suggested that, in addition to piloting subunit cargo to the fT3SS, the FlgN and FliT chaperones might be conditional regulators of fT3SS activity, which sense and respond to the availability of subunit cargo. To function effectively in the cell, this mechanism—in which unladen chaperones are a proxy measure for subunit availability—would require cytoplasmic ratios of FlgN/FliT to their cognate subunits to be high. To obtain an indication of the cytoplasmic levels of chaperones and their cognate subunits, we used RNA sequencing (RNA-Seq) and ribosome profiling (Ribo-Seq) to assess the *Salmonella* global transcript abundance and translome, respectively, at late-log growth phase when flagellar gene expression is at its peak (Ingolia et al., 2009; Lister et al., 2008; Mortazavi et al., 2008; Nagalakshmi et al., 2008). Analysis of these data confirmed that flagellar mRNAs are amongst the most abundant and highly translated in *Salmonella* (Figure 5a). The ribosome profiling data indicated that the translation ratio of FlgN to its cognate subunits (FlgK and FlgL) was 1:3, and the translation ratio of FliT to its cognate subunit FliD was 1:4. In comparison to the chaperone for flagellin, FliS, which does not bind FliJ, the chaperone-subunit ratios for FlgN and FliT are high relative to the 1:43 ratio of FliS chaperone to its cognate subunit FliC (Figure 4b).

The high translation ratios of FlgN and FliT to cognate subunits suggest that the presence of these unbound chaperones in the cell would provide a sensitive proxy measure of cytoplasmic flagellar subunit levels. Taken together with our other findings, these data strengthen the view that, when unladen, the FlgN, and FliT chaperones couple subunit availability to pmf-driven export by modulating FliJ-dependent activation of the FlhA_C export gate.

3 | DISCUSSION

The synthesis and operation of bacterial flagella is energetically expensive, using an estimated 2% of the cell's energy, and numerous regulatory mechanisms have evolved to increase efficiency of flagella biogenesis and function (Macnab, 1996). Here, we have uncovered a mechanism by which fT3SS chaperones couple the availability of subunit cargo to the energy efficiency of flagellar export. By identifying point mutant variants of the FlgN and FliT chaperones that were specifically unable to bind the FliJ stalk component of the fT3SS ATPase, we showed that the chaperone–FliJ interaction prevents activation of the FlhA export gate component when subunit availability is low, reducing the possibility of wasteful dissipation of the pmf by the fT3SS machinery.

3.1 | Mutational analysis of the FlgN and FliT chaperones reveals key residues specifically required for binding to the FliJ ATPase stalk

Our mutational analysis identified FlgN chaperone variants, FlgN W78A and FlgNΔ76-78, which were defective in binding to FliJ but

FIGURE 4 FlgN and FliT chaperones compete with FlhA_C for binding to FliJ in vivo. (a) In vivo competition assay to assess the effect of chaperone–FliJ binding on FlhA_C export activity. Export of the unchaperoned FlgD subunit was assessed in a *flgE* null strain. Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of *Salmonella* Δ*flgE* strains producing pTrc99a-encoded FlgN Y122A (–) or FlgN Y122A, Δ76–78 (Δ76–78) were separated by SDS (15%)–PAGE and analysed by immunoblotting with anti-FlgD, anti-FlgN, anti-FliJ and anti-FlhA polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa). Relative amounts of FlgD secreted into culture supernatants from three independent experiments were quantified using Image Studio Lite software and normalized to FlgD secreted by cells expressing the FlgN Δ76–78 Y122A variant (right-hand bar chart). Export of non-cognate subunit FlgD is attenuated in cells producing FlgN Y122A (–), which binds FliJ but not FlhA, but is significantly higher in cells producing the FlgN variant defective in binding FliJ (FlgN Δ76–78,Y122A). Data shown are the mean ± SEM of three biological replicates. *Statistically significant difference, $p < .01$ (unpaired two-tailed *t*-test)

could still pilot their cognate subunits FlgK and FlgL to dock at the export gate component FlhA_C (Figure 1). Motility of strains producing FlgN W78A or FlgNΔ76–78 was marginally reduced compared with wild type, as was export of FlgK and FlgL, indicating that loss of the FlgN–FliJ interaction reduced the efficiency of, but did not abolish, cognate subunit export (Figure 2).

Mutational analysis of the FliT chaperone showed that several highly conserved, surface exposed residues on the helix required for cognate subunit binding were also critical for binding to FliJ (Figure 1c). Replacement of FliT leucine-72 with alanine was found to disrupt binding to FliJ, but did not affect FliT chaperone interactions with FliD cognate subunit or FlhA_C (Figure 1). Cells producing FliT L72A did not display a swimming motility defect compared with wild type *Salmonella* (Figure 2c). However, a marginal export defect was observed for cognate subunit FliD (Figure 2d). Cells producing FliT L72A displayed reduced population swarming, indicating that the FliT–FliJ interaction may be required for the hyperflagellation associated with this form of surface motility.

In the strains producing the chaperone variants, cognate subunits are produced at levels similar to those in the wild type parent strain. In the assay conditions used, chaperones would therefore be in the subunit-bound state and unable to bind FliJ. This means that in these strains, FliJ is still able to activate the export gate, and this explains the observed mild attenuation of flagellar export and cell motility.

FIGURE 5 Ribosome profiling reveals cytoplasmic ratios of flagellar subunits to their cognate chaperones. (a) Smoothed scatter plot of total translation (Ribosome profiling, measurement of ribosome occupation) and total mRNA abundance (RNA-Seq), showing flagellar chaperones FlgN, FliT and FliS (blue), their respective cognate subunits (red), and the regression between total translation and mRNA abundance (dotted line). (b) Ratios of the normalized translation to gene length for the coding regions corresponding to chaperones and their cognate subunits (FlgN:FlgK+FlgL; FliT:FliD; FliS:FliC)

3.2 | Unladen chaperones modulate the interaction of the FliJ ATPase stalk with the cytoplasmic domain of the FlhA export gate

A previous study showed that FliJ appeared to enhance the binding of a FliT/FliD complex to FlhAC (Bange et al., 2010). Using affinity chromatography of resin-bound GST-FlhAC with a complex of FliT-FliD it was observed that when assays contained FliJ, binding of FliT-FliD to GST-FlhAC appeared to increase (Bange et al., 2010). The addition of excess unladen FliT chaperone to the pull-down assay reversed the FliJ-dependent binding enhancement (Bange et al., 2010). As other studies have shown that unladen FliT chaperone does not bind FlhAC, we propose that FliT would block

the FliJ-FlhAC interaction (Figures 4 and 5), preventing the FliJ-dependent enhancement of FliT/FliD binding to FlhAC, as observed by Bange and colleagues (Bange et al., 2010; Jensen et al., 2020; Kinoshita et al., 2013).

We have previously shown that FliJ-bound chaperones are efficiently captured by their cognate subunits to establish a local chaperone cycle at the membrane export machinery, which increases the efficiency of subunit export (Evans et al., 2006). In this mechanism, the absence of cognate subunits would disrupt the chaperone cycle, resulting in unladen chaperones remaining docked at FliJ. The data presented here show that this chaperone-FliJ interaction would block FliJ-dependent activation of the flagellar export gate, preventing its unnecessary activation in the absence of subunit cargo. In the situation where cellular levels of cognate subunit increase, chaperones would be captured from FliJ, restoring FliJ-dependent activation of the export gate and subsequent subunit export (Figure 6). The recent structure of a SctO/SctV complex (homologs of FliJ and FlhA) from *Chlamydia* indicates that FlgN or FliT binding to the chaperone binding sites on FliJ would prevent the FliJ-FlhA interaction by either occluding the FlhA binding site on FliJ (by FliT) or by physically preventing the FliJ-FlhA interaction (by FlgN), supporting the role of chaperones disrupting FliJ-dependent activation of FlhA (Jensen et al., 2020).

It is well established that an interaction between FliJ and the FlhA export gate protein is required for efficient $\Delta\Psi$ -driven protein export (Minamino et al., 2011). The FliH and FliI components of the ATPase support the FliJ-FlhA interaction, and hydrolysis of ATP by

FIGURE 6 Chaperone regulation of the ft3SS machinery in response to subunit cargo availability. Chaperone-subunit complexes initially dock at the FliI component of the ATPase complex (1) before docking at the cytoplasmic domain of FlhA (2). Subunits are subsequently released from their cognate chaperones and translocated into the export channel (3). Unladen chaperones then bind FliJ from which they are captured by newly synthesized cognate subunits (4). Chaperone-subunit complexes subsequently dock at the FliI component of the ATPase, repeating the cycle (5). Under conditions where subunit availability is limited (right-hand panel), unladen FlgN and FliT chaperones remain bound to FliJ, blocking the interaction of FliJ with FlhA and reducing pmf-driven export by the ft3SS machinery

the ATPase complex is proposed to drive rotation of FliJ, allowing it to contact sequentially all nine monomers of the FlhA nonameric ring, converting the export machinery into a highly efficient proton-protein antiporter (Ibuki et al., 2011; Minamino et al., 2011; Minamino & Namba, 2008; Paul et al., 2008). The $\text{F}_1\text{T}3\text{SS}$ ATPase components share many features of the F_1 ATP synthase: FliI and FliJ share considerable structural similarity to the α/β - and γ -subunits, respectively, and FliH shares considerable amino acid sequence similarity to the β and δ subunits (Pallen et al., 2006). Furthermore, analogous to the F_0 ATP synthase component, the flagellar export machinery harnesses the power of the pmf (Minamino & Namba, 2008; Paul et al., 2008). Multiple mechanisms have been identified that regulate both ATPase activity and pmf use by the F_1F_0 -ATP synthase, including proteins that inhibit efficient coupling between the F_1 and F_0 components (Gledhill et al., 2007; Kato-Yamada et al., 1999; Yagi et al., 2009). Our data demonstrate that unladen export chaperones disrupt FliJ-dependent activation of the flagellar export gate, which might prevent the possibility of wasteful pmf use in the absence of cognate subunits, by effectively preventing a FliJ–FlhA interaction until the chaperone is captured from FliJ by a cognate subunit, relieving the inactive state.

Interestingly, the intracellular concentration of FliC in *B. subtilis* is restricted by a partner switching mechanism between a FliW protein which binds either FliC, or binds and inhibits CsrA, an RNA binding protein which represses FliC expression (Altegoer et al., 2008; Oshiro et al., 2019). Like in *Salmonella*, FliS also regulates FliC expression in *B. subtilis*. Increased levels of FliC, increases the proportion of FliW-free CsrA, repressing FliC translation. Whether unladen chaperones in *B. subtilis* also regulate FliJ–FlhA interactions in conditions of reduced flagellar gene expression (e.g., through downregulation of *flhDC*) is unclear. However, the observation that excess unladen FliT modulates FliJ-dependent interactions between cargo and FlhA_C in *B. subtilis* indicates that a similar regulatory mechanism may take place (Bange et al., 2010).

3.3 | A mechanism coupling export gate activation to availability of subunits for export

Our data support a model whereby, when subunits are at low levels in the cell, unladen FlgN and FliT bind to FliJ, preventing the FliJ–FlhA interaction and subsequent activation of the export gate. Ribosome profiling revealed the amounts of chaperone and cognate subunit(s) produced by the cell and while all three flagellar chaperones are produced at similar levels (Figure 6), the intracellular ratio of chaperone to subunit is high for the FlgN and FliT chaperones (1:3 and 1:4, respectively), compared with the ratio of FliS chaperone to flagellin (1:43), which is produced and exported at far higher levels than FlgK and FlgL (Evans et al., 2006). As a result, the presence of unladen FlgN and FliT would serve as a sensitive proxy measure for changes in the availability of newly synthesized flagellar subunits in the cell, as these chaperones are more likely to be free, compared with the FliS chaperone, when subunit levels drop. This may in part explain why FliS has not evolved to bind FliJ.

How, then, are unladen chaperones released from FliJ, enabling export activity to be restored once cognate subunit levels return to normal? Evans and colleagues previously demonstrated that cognate subunits can capture unladen chaperones from FliJ, which would effectively free FliJ to bind the FlhA export gate and restore subunit export (Evans et al., 2006). This provides an elegant mechanism whereby an unladen chaperone remains docked at FliJ, inhibiting pmf-driven export, until a cognate subunit is available to capture the chaperone from FliJ, allowing export activity to resume.

Whether such a mechanism of chaperone-mediated coupling of subunit availability to export gate activation occurs in the related $\text{vT}3\text{SS}$ is not clear. However, a subset of export chaperones, specifically for the virulence needle translocon and tip subunits, bind to $\text{vT}3\text{SS}$ FliJ homologs (Cherradi et al., 2014; Drehtkopf et al., 2020; Evans & Hughes, 2009; Lee et al., 2014). Notably, an export chaperone (PcrG) in *Pseudomonas aeruginosa* binds to the FliJ homolog (PscO) and modulates pmf use by the $\text{vT}3\text{SS}$, although the mechanistic basis for this was unclear (Lee et al., 2014). Independent to PscO (FliJ) binding, and in the absence of cognate subunit, PcrG can regulate effector secretion through interactions with the FlhA homolog, PcrD, preventing effectors accessing the export machinery (Lee et al., 2014). These findings indicate that interactions between a subset of export chaperones and FliJ homologs may be conserved among $\text{vT}3\text{SS}$. Whether these binding events also serve to regulate export gate activation with respect to subunit availability is yet to be determined.

Chaperone modulation of $\text{F}_1\text{T}3\text{SS}$ activity may be important when *Salmonella* encounters environmental conditions in which flagellar gene expression is down-regulated, including during the early stages of host infection (Adams et al., 2005; Ilyas et al., 2018; Miao & Rajan, 2011; Spöring et al., 2018). Our data support a model for a local chaperone cycle at the $\text{T}3\text{SS}$ machinery that (i) facilitates efficient docking and export of cognate subunit cargo when subunit/effector availability is high, and (ii) reduces the likelihood of constitutive proton influx and wasteful dissipation of the pmf when subunit cargo availability is low (Figure 6).

4 | MATERIALS AND METHODS

4.1 | Bacterial strains, plasmids, and growth conditions

Wild type *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) SJW1103 is motile (Yamaguchi et al., 1984). The $\Delta\text{flgN}::\text{K}_m^R$ and the $\Delta\text{fliT}::\text{K}_m^R$ mutant in which the *flgN* or *fliT* genes were replaced by a kanamycin resistance cassette were constructed using the λ Red recombinase system (Hoffmann et al., 2017). Strains containing chromosomally encoded FliT or FlgN variants were constructed by *aph*-I-SceI Kanamycin-resistance cassette replacement using pWRG730 (Hoffmann et al., 2017) and were cultured at 30–37°C in Luria-Bertani (LB) broth containing, where appropriate, ampicillin (100 $\mu\text{g}/\text{ml}$) or chloramphenicol (20 $\mu\text{g}/\text{ml}$) and collected by

centrifugation (6000 $\times$ g for 10 min). Recombinant proteins used in Figures 4a and S6 were expressed in *Salmonella* from the isopropyl β -D-thiogalactoside (IPTG)-inducible plasmid pTrc99a (Amann et al., 1988) (See “FlgN-FliJ in vivo competition assay” section below).

To construct recombinant plasmids encoding wild type or derivative genes, *Salmonella* genes were amplified from chromosomal DNA by PCR or overlap-extension PCR using Q5 High-Fidelity DNA polymerase. PCR products were inserted BamHI/XhoI into pGEX-4T3 or NdeI/BamHI into pACT7 and pTrc99a. Inserts were verified by DNA sequencing (Department of Biochemistry, University of Cambridge). A full list and description of strains and plasmids used in this study can be found in Supporting Information Appendix Tables S1 and S2. Recombinant proteins for purification were expressed in *E. coli* C41 (Miroux & Walker, 1996) from (IPTG)-inducible pACT7 (Thanabalu et al., 1998) or pGEX-4T3 (Kaelin et al., 1992). Briefly, C41 strains carrying pACT7, pGEX-4T3, or their derivatives were grown at 37°C in 2xYT containing appropriate antibiotics to $A_{600} = 0.4$ – 0.6 and induced with 1 mM IPTG before continuing growth for 12–14 hr at 18°C. Cells were collected by centrifugation (6000 $\times$ g, 15 min) and stored at -20°C .

4.2 | Affinity chromatography co-purification assays

Co-purification of proteins expressed in *E. coli* C41 was performed with glutathione-sepharose 4B. Cells were resuspended in buffer A (50 mM sodium phosphate pH7.4, 150 mM NaCl, 1 mM β -mercaptoethanol) and mechanically lysed with a Constant Systems cell disruptor at 30,000 kPa. Lysates were clarified by centrifugation (30,000 $\times$ g, 40 min). Cleared lysates containing GST-tagged proteins were incubated with affinity resin for 1 hr. After extensive washing (30 column volumes) with buffer A, cell lysates containing overexpressed untagged prey proteins were incubated for 1 hr with the resin pre-bound GST-bait. Resins were washed extensively with buffer A (30 column volumes) and proteins eluted by boiling in SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE and detected by immunoblotting.

4.3 | Flagellar subunit export assay

Salmonella strains were cultured at 37°C in LB broth containing ampicillin and 100 μM IPTG to mid-log phase ($A_{600} 1.0$). Cells were centrifuged (6000 $\times$ g, 5 min), resuspended in fresh media, and grown for a further 60 min at 37°C. Cells were pelleted by centrifugation (16,000 $\times$ g, 5 min) and the supernatant passed through a 0.22 μm nitrocellulose filter. Supernatant proteins were precipitated with 10% trichloroacetic acid (TCA) and 1% Triton-X100 on ice for 1 hr, pelleted by centrifugation (16,000 $\times$ g, 10 min), washed with ice-cold acetone and resuspended in SDS-PAGE loading buffer (volumes calibrated according to cell densities). Fractions were separated by SDS-PAGE and analysed by immunoblotting.

4.4 | Motility assays

For swimming motility, *Salmonella* strains were grown in LB broth to $A_{600} 1$. Two microliters of culture were inoculated into soft tryptone agar (0.25% agar, 10 g/L tryptone, 5g/L NaCl). Plates were incubated at 37°C for between 4 and 6 hr. For swarming motility, one microliter of overnight cultures grown in LB broth was inoculated onto tryptone agar plates (0.6% agar, 10g/L tryptone, 5g/L NaCl) supplemented with 0.3% glucose and incubated at 30°C for 16 hr.

4.5 | Chaperone-FliJ in vitro competition assay

Recombinant proteins were expressed in *E. coli* C41 and cells resuspended in buffer A (50 mM sodium phosphate pH7.4, 150 mM NaCl, 1mM β -mercaptoethanol). Resuspended cells were mechanically lysed as described for the co-purification assays. Lysates were clarified by centrifugation (30,000 $\times$ g, 40 min). Cleared lysates containing His-tagged FliJ were incubated with cobalt affinity resin for 1 hr. After extensive washing (30 column volumes), cell lysates containing overexpressed untagged FliH_C was added to each resin fraction and an equal volume of wild type FliT₉₄, FliT₉₄ L₇₂A, wild type FlgN, or FlgN W₇₈A was added. Proteins were incubated for 1 hr, the resin was washed extensively (30 column volumes) with buffer A and proteins were eluted by boiling in SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE and detected by immunoblotting.

4.6 | FLGN-FliJ in vivo competition assay

Salmonella flgE null strains carrying either pTrc99a encoding flgN-Y₁₂₂A Δ 76-78 or flgN-Y₁₂₂A were grown in LB broth containing ampicillin and inducing agent (50 μM IPTG) to $A_{600} 1.0$. Cells were centrifuged (6000 $\times$ g, 5 min) and resuspended in fresh media and grown for a further 60 min at 37°C. Cells were pelleted by centrifugation (16,000 $\times$ g, 5 min) and the supernatant passed through a 0.22 μm nitrocellulose filter (Sartorius). Proteins were precipitated with 10% trichloroacetic acid (TCA) and 1% Triton-X100 on ice for 1 hr, pelleted by centrifugation (16,000g, 10 min, 4°C), washed with ice-cold acetone and resuspended in SDS-PAGE loading buffer (volumes calibrated according to cell densities). Fractions were separated by SDS-PAGE and analyzed by immunoblotting.

4.7 | Ribosome profiling and RNA-SEQ

Libraries were prepared essentially as described previously (Chung et al., 2015, 2017; Hardcastle, 2015) with the following modifications: Cells were grown in LB at 37°C with 180 RPM shaking to $A_{600} 1.0$. Chloramphenicol was added to the culture to a final concentration of 1,500 $\mu\text{g}/\text{ml}$, followed by rapid cooling of the culture and harvesting of cells by centrifugation (6,000 $\times$ g, 1 min, 4°C). The cell pellet was quickly resuspended in 1 ml ice cold bacterial

profiling buffer (20 mM Tris-Cl pH 7.5, 140 mM KCl, 5 mM MgCl₂, 1,500 µg/ml chloramphenicol, 0.5 mM dithiothreitol (DTT), 0.5% NP40, 1% Triton X-100, 2.5% Sucrose) and flash-frozen for cryo-grinding in liquid nitrogen. The frozen powder was thawed and clarified by centrifugation (13,000 ×g, 2 min, 4°C) followed by adjustment of A_{254 nm}¹⁰. Lysates were either snap frozen in liquid nitrogen for storage at -80°C or nuclease treated with RNase 1 (700 U, Ambion) followed by ribosomal RNA depletion using a bacterial ribozero kit (Illumina) prior to library preparation for ribosome profiling. For parallel RNA-Seq, total RNA was extracted from corresponding lysates followed ribosomal RNA depletion using the bacterial ribozero kit (Illumina) prior to library preparation. For Ribosome profiling, rRNA was further depleted with duplex-specific nuclease as described previously (Chung et al., 2015). Ribosome profiling and RNA-Seq libraries were pooled and sequenced using the NextSeq® 500/550 platform and data were trimmed and mapped to the transcriptome assembly of *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain ST4/74 (GenBank accession CP002487.1). Paired ribosome profiling and RNA-Seq analysis was performed with riboSeqR as previously described (Chung et al., 2015; Hardcastle, 2015).

4.8 | Quantification and statistical analysis

Experiments were performed three times unless otherwise stated. Immunoblot data were quantified from three independent measurements unless otherwise stated using Image Studio Lite. The unpaired two-tailed Student's *t*-test was used to determine *p*-values, and significance was defined as **p* < .05. Data are represented as mean ± standard error of the mean (SEM), unless otherwise specified and reported as biological replicates.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Owain J. Bryant carried out Conceptualization, Data curation, Investigation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. Betty Y-W Chung did Data curation, Formal analysis, Funding acquisition, Methodology, Investigation, Visualization, Software, Writing – original draft, Writing – review & editing. Gillian M. Fraser did Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

- Adams, P., Fowler, R., Kinsella, N., Howell, G., Farris, M., Coote, P. et al (2005) Proteomic detection of PhoPQ- and acid-mediated repression of *Salmonella* motility. *Proteomics*, 1, 597–607. [https://doi.org/10.1002/1615-9861\(200104\)1:4<597:AID-PROT597>3.0.CO;2-P](https://doi.org/10.1002/1615-9861(200104)1:4<597:AID-PROT597>3.0.CO;2-P)
- Akeda, Y. & Galán, J.E. (2005) Chaperone release and unfolding of substrates in type III secretion. *Nature*, 437, 911–915. <https://doi.org/10.1038/nature03992>
- Aldridge, P., Karlinsey, J. & Hughes, K.T. (2003) The type III secretion chaperone FlgN regulates flagellar assembly via a negative feedback loop containing its chaperone substrates FlgK and FlgL. *Molecular Microbiology*, 49, 1333–1345. <https://doi.org/10.1046/j.1365-2958.2003.03637.x>
- Altegoer, F., Mukherjee, S., Steinchen, W., Bedrunke, P., Linne, U., Kearns, D.B. et al (2008) FlhS/flagellin/FlhW heterotrimer couples type III secretion and flagellin homeostasis. *Scientific Reports*, 7(8), 11552.
- Amann, E., Ochs, B. & Abel, K.J. (1988) Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene*, 69, 301–315. [https://doi.org/10.1016/0378-1119\(88\)90440-4](https://doi.org/10.1016/0378-1119(88)90440-4)
- Auvray, F., Thomas, J., Fraser, G.M. & Hughes, C. (2001) Flagellin polymerisation control by a cytosolic export chaperone. *Journal of Molecular Biology*, 308, 221–229.
- Bange, G., Kümmerer, N., Engel, C., Bozkurt, G., Wild, K. & Sinning, I. (2010) FlhA provides the adaptor for coordinated delivery of late flagella building blocks to the type III secretion system. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 11295–11300. <https://doi.org/10.1073/pnas.1001383107>
- Bennett, J.C., Thomas, J., Fraser, G.M. & Hughes, C. (2001) Substrate complexes and domain organization of the *Salmonella* flagellar export chaperones FlgN and FlhT. *Molecular Microbiology*, 39, 781–791. <https://doi.org/10.1046/j.1365-2958.2001.02268.x>
- Cherradi, Y., Hachani, A. & Allaoui, A. (2014) Spa13 of *Shigella flexneri* has a dual role: Chaperone escort and export gate-activator switch of the type III secretion system. *Microbiology*, 160, 130–141.
- Chung, B.Y.W., Deery, M.J., Groen, A.J., Howard, J. & Baulcombe, D.C. (2017) Endogenous miRNA in the green alga *Chlamydomonas* regulates gene expression through CDS-targeting. *Nature Plants*, 3, 787–794. <https://doi.org/10.1038/s41477-017-0024-6>
- Chung, B.Y.W., Hardcastle, T.J., Jones, J.D., Irigoyen, N., Firth, A.E., Baulcombe, D.C. et al (2015) The use of duplex-specific nuclease in ribosome profiling and a user-friendly software package for Ribo-seq data analysis. *RNA*, 21, 1731–1745. <https://doi.org/10.1261/rna.052548.115>
- Drehkopf, S., Otten, C., Hausner, J., Seifert, T. & Büttner, D. (2020) HrpB7 from *Xanthomonas campestris* pv. *vesicatoria* is an essential component of the type III secretion system and shares features of HrpO/FlhJ/YscO family members. *Cellular Microbiology*, 22, e13160.
- Dufour, A., Furness, R.B. & Hughes, C. (1998) Novel genes that upregulate the *Proteus mirabilis* flhDC master operon controlling flagellar biogenesis and swarming. *Molecular Microbiology*, 29, 741–751.
- Evans, L.D.B. & Hughes, C. (2009) Selective binding of virulence type III export chaperones by FlhJ escort orthologues InvI and YscO. *FEMS Microbiology Letters*, 29, 292–297. <https://doi.org/10.1111/j.1574-6968.2009.01535.x>

- Evans, L.D.B., Hughes, C. & Fraser, G.M. (2014a) Building a flagellum outside the bacterial cell. *Trends in Microbiology*, 22, 566–572. <https://doi.org/10.1016/j.tim.2014.05.009>
- Evans, L.D.B., Hughes, C. & Fraser, G.M. (2014b) Building a flagellum in biological outer space. *Microbial Cell*, 1, 64–66. <https://doi.org/10.15698/mic2014.01.128>
- Evans, L.D.B., Stafford, G.P., Ahmed, S., Fraser, G.M. & Hughes, C. (2006) An escort mechanism for cycling of export chaperones during flagellum assembly. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 17474–17479. <https://doi.org/10.1073/pnas.0605197103>
- Fraser, G.M., Bennett, J.C. & Hughes, C. (1999) Substrate-specific binding of hook-associated proteins by FlgN and FliT, putative chaperones for flagellum assembly. *Molecular Microbiology*, 32, 569–580. <https://doi.org/10.1046/j.1365-2958.1999.01372.x>
- Fraser, G.M. & Hughes, C. (1999) Swarming motility. *Current Opinion in Microbiology*, 2, 630–635. [https://doi.org/10.1016/S1369-5274\(99\)00033-8](https://doi.org/10.1016/S1369-5274(99)00033-8)
- Gledhill, J.R., Montgomery, M.G., Leslie, A.G.W. & Walker, J.E. (2007) How the regulatory protein, IF1, inhibits F1-ATPase from bovine mitochondria. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 15671–15676. <https://doi.org/10.1073/pnas.0707326104>
- Hardcastle, T.J. (2015) Data from “Analysis of sequencing data from ribosome profiling experiments”. *Bioconductor*. <https://doi.org/10.18129/B9.bioc.riboSeqR>
- Hirano, T., Minamino, T., Namba, K. & Macnab, T.M. (2003) Substrate specificity classes and the recognition signal for *Salmonella* type III flagellar export. *Journal of Bacteriology*, 185, 2485–2492. <https://doi.org/10.1128/JB.185.8.2485-2492.2003>
- Hoffmann, S., Schmidt, C., Walter, S., Bender, J.K. & Gerlach, R.G. (2017) Scarless deletion of up to seven methylaccepting chemotaxis genes with an optimized method highlights key function of CheM in *Salmonella* Typhimurium. *PLoS One*, 12, e0172630. <https://doi.org/10.1371/journal.pone.0172630>
- Homma, M., Kutsukake, K., Iino, T. & Yamaguchi, S. (1984) Hook-associated proteins essential for flagellar filament formation in *Salmonella* typhimurium. *Journal of Bacteriology*, 157, 100–108. <https://doi.org/10.1128/JB.157.1.100-108.1984>
- Hughes, K.T., Gillen, K.L., Semon, M.J. & Karlinsey, J.E. (1993) Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. *Science*, 19, 1277–1280. <https://doi.org/10.1126/science.8235660>
- 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. *Nature Structural & Molecular Biology*, 18, 277–282. <https://doi.org/10.1038/nsmb.1977>
- Ilyas, B., Mulder, D.T., Little, D.J., Elhenawy, W., Banda, M.M., Perez-Morales, D. et al (2018) Regulatory Evolution drives evasion of host inflammasomes by *Salmonella* Typhimurium. *Cell Reports*, 25, 825–832. <https://doi.org/10.1016/j.celrep.2018.09.078>
- Imada, K., Minamino, T., Kinoshita, M., Furukawa, Y. & Namba, K. (2010) Structural insight into the regulatory mechanisms of interactions of the flagellar type III chaperone FliT with its binding partners. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 8812–8817. <https://doi.org/10.1073/pnas.1001866107>
- Ingolia, N.T., Ghaemmhami, S., Newman, J.R. & Weissman, J.S. (2009) Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. *Science*, 324, 218–223. <https://doi.org/10.1126/science.1168978>
- Jensen, J.L., Yamini, S., Rietsch, A. & Spiller, B.W. (2020) The structure of the type III secretion system export gate with CdsO, an ATPase lever arm. *PLoS Pathogens*, 16, e1008923. <https://doi.org/10.1371/journal.ppat.1008923>
- Kaelin, W.G., Krek, W., Sellers, W.R., DeCaprio, J.A., Ajchenbaum, F., Fuchs, C.S. et al (1992) Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. *Cell*, 70, 351–364. [https://doi.org/10.1016/0092-8674\(92\)90108-O](https://doi.org/10.1016/0092-8674(92)90108-O)
- Kato-Yamada, Y., Bald, D., Koike, M., Motohashi, K., Hisabori, T. & Yoshida, M. (1999) ϵ Subunit, an endogenous inhibitor of bacterial F1-ATPase, also inhibits FOF1-ATPase. *Journal of Biological Chemistry*, 274, 33991–33994. <https://doi.org/10.1074/jbc.274.48.33991>
- Kearns, D.B. (2010) A field guide to bacterial swarming motility. *Nature Reviews Microbiology*, 8, 634–644. <https://doi.org/10.1038/nrmicr2405>
- Kinoshita, M., Hara, N., Imada, K., Namba, K. & Minamino, T. (2013) Interactions of bacterial flagellar chaperone-substrate complexes with FlhA contribute to co-ordinating assembly of the flagellar filament. *Molecular Microbiology*, 90, 1249–1261. <https://doi.org/10.1111/mmi.12430>
- Kuhlen, L., Abrusci, P., Johnson, S., Gault, J., Deme, J., Caesar, J. et al (2018) Structure of the core of the type III secretion system export apparatus. *Nature Structural & Molecular Biology*, 25, 583–590. <https://doi.org/10.1038/s41594-018-0086-9>
- Kutsukake, K. (1994) Excretion of the anti-sigma factor through a flagellar substructure couples flagellar gene expression with flagellar assembly in *Salmonella* typhimurium. *Molecular and General Genetics*, 15, 605–612. <https://doi.org/10.1007/BF00279569>
- Kutsukake, K., Ikebe, T. & Yamamoto, S. (1999) Two novel regulatory genes, fliT and fliz, in the flagellar regulon of *Salmonella*. *Genes & Genetic Systems*, 74, 287–292. <https://doi.org/10.1266/ggs.74.287>
- Lee, P.C., Zmina, S.E., Stopford, C.M., Toska, J. & Rietsch, A. (2014) Control of type III secretion activity and substrate specificity by the cytoplasmic regulator PcrG. *Proceedings of the National Academy of Sciences of the United States of America*, 111, E2027–E2036. <https://doi.org/10.1073/pnas.1402658111>
- Lister, R., O'Malley, R.C., Tonti-Filippini, J., Gregory, D.B., Berry, C.C., Millar, A.H. et al (2008) Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. *Cell*, 133, 523–536. <https://doi.org/10.1016/j.cell.2008.03.029>
- Macnab, R.M. (1996) Flagella and motility. Chapter 10. In: Neidhardt, F.C., Curtiss III, R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M. & Umberger, H.E. (Eds.) *Escherichia coli and Salmonella: Cellular and molecular biology* (2nd ed.). Washington, DC: ASM Press, pp. 123–145.
- Miao, E.A. & Rajan, J.V. (2011) *Salmonella* and Caspase-1: A complex interplay of detection and evasion. *Frontiers in Microbiology*, 2, 85. <https://doi.org/10.3389/fmicb.2011.00085>
- Minamino, T., Kinoshita, M., Hara, N., Takeuchi, S., Hida, A., Koya, S. et al (2012) Interaction of a bacterial flagellar chaperone FlgN with FlhA is required for efficient export of its cognate substrates. *Molecular Microbiology*, 83, 775–788. <https://doi.org/10.1111/j.1365-2958.2011.07964.x>
- Minamino, T., Morimoto, Y.V., Hara, N. & Namba, K. (2011) An energy transduction mechanism used in bacterial flagellar type III protein export. *Nature Communications*, 2, 475. <https://doi.org/10.1038/ncomms1488>
- Minamino, T. & Namba, K. (2008) Distinct roles of the Flil ATPase and proton motive force in bacterial flagellar protein export. *Nature*, 451, 485–488. <https://doi.org/10.1038/nature06449>
- Miroux, B. & Walker, J.E. (1996) Over-production of proteins in *Escherichia coli*: Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *Journal of Molecular Biology*, 260, 289–298. <https://doi.org/10.1006/jmbi.1996.0399>
- Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L. & Wold, B. (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods*, 5, 621–628. <https://doi.org/10.1038/nmeth.1226>
- Nagalakshmi, U., Wang, Z., Waern, K., Shou, C., Raha, D., Gerstein, M. et al (2008) The transcriptional landscape of the yeast genome

- defined by RNA sequencing. *Science*, 320, 1344–1349. <https://doi.org/10.1126/science.1158441>
- Oshiro, R.T., Rajendren, S., Hundley, H.A. & Kearns, D.B. (2019) Robust stoichiometry of FliW-CsrA governs flagellin homeostasis and cytoplasmic organization in *Bacillus Subtilis*. *mBio*, 10, e00533-19.
- Pallen, M.J., Bailey, C.M. & Beatson, S.A. (2006) Evolutionary links between FliH/YscL-like proteins from bacterial type III secretion systems and second-stalk components of the F_oF_1 and vacuolar ATPases. *Protein Science*, 15, 935–941. <https://doi.org/10.1110/ps.051958806>
- Paul, K., Erhardt, M., Hirano, T., Blair, D.F. & Hughes, K.T. (2008) Energy source of flagellar type III secretion. *Nature*, 451, 489–492.
- Sato, Y., Takaya, A., Mouslim, C., Hughes, K.T. & Yamamoto, T. (2014) FliT selectively enhances proteolysis of FlhC Subunit in FlhD₄C₂ complex by an ATP-dependent protease. *ClpXP Journal of Biological Chemistry*, 289, 33001–33011.
- Spöring, I., Felgner, S., Preuße, M., Eckweiler, D., Rohde, M., Häussler, M. et al (2018) Regulation of flagellum biosynthesis in response to cell envelope stress in *Salmonella enterica* Serovar Typhimurium. *Mbio*, 9, e00736-17.
- Thanabalu, T., Koronakis, E., Hughes, C. & Koronakis, V. (1998) Substrate-induced assembly of a contiguous channel for protein export from *E.coli*: Reversible bridging of an inner-membrane translocase to an outer membrane exit pore. *EMBO Journal*, 17, 6487–6496. <https://doi.org/10.1093/emboj/17.22.6487>
- Thomas, J., Stafford, G.P. & Hughes, C. (2004) Docking of cytosolic chaperone-substrate complexes at the membrane ATPase during flagellar type III protein export. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 3945–3950. <https://doi.org/10.1073/pnas.0307223101>
- Yagi, H., Konno, H., Murakami-Fuse, T., Isu, A., Oroguchi, T., Akutsu, H. et al (2009) Structural and functional analysis of the intrinsic inhibitor subunit ϵ of F1 -ATPase from photosynthetic organisms. *The Biochemical Journal*, 425, 85–94.
- Yamaguchi, S., Fujita, H., Taira, T., Kutsukake, K., Homma, M. & Iino, T. (1984) Genetic analysis of three additional fla genes in *Salmonella typhimurium*. *Journal of General Microbiology*, 130, 3339–3342.
- Yamamoto, S. & Kutsukake, K. (2006) FliT acts as an anti-FlhD₂C₂ factor in the transcriptional control of the flagellar regulon in *Salmonella enterica* serovar typhimurium. *Journal of Bacteriology*, 188, 6703–6708. <https://doi.org/10.1128/JB.00799-06>
- Yonekura, K., Maki, S., Morgan, D.G., DeRosier, D.J., Vonderviszt, F., Imada, K. et al (2000) The bacterial flagellar cap as the rotary promoter of flagellin self-assembly. *Science*, 290, 2148–2152. <https://doi.org/10.1126/science.290.5499.2148>

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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