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Intrinsic Membrane Targeting of the Flagellar Export ATPase FliI: Interaction with Acidic Phospholipids and FliH

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Abstract

The specialised ATPase FliI is central to export of flagellar axial protein subunits during flagellum assembly. We establish the normal cellular location of FliI and its regulatory accessory protein FliH in motile *Salmonella typhimurium*, and ascertain the regions involved in FliH₂/FliI heterotrimerisation. Both FliI and FliH localised to the cytoplasmic membrane in the presence and in the absence of proteins making up the flagellar export machinery and basal body. Membrane association was tight, and FliI and FliH interacted with *Escherichia coli* phospholipids *in vitro*, both separately and as the preformed FliH₂/FliI complex, in the presence or in the absence of ATP. Yeast two-hybrid analysis and pull-down assays revealed that the C-terminal half of FliH (H105-235) directs FliH homodimerisation, and interacts with the N-terminal region of FliI (I1-155), which in turn has an intra-molecular interaction with the remainder of the protein (I156-456) containing the ATPase domain. The FliH105-235 interaction with FliI was sufficient to exert the FliH-mediated down-regulation of ATPase activity. The basal ATPase activity of isolated FliI was stimulated tenfold by bacterial (acidic) phospholipids, such that activity was 100-fold higher than when bound by FliH in the absence of phospholipids. The results indicate similarities between FliI and the well-characterised SecA ATPase that energises general protein secretion. They suggest that FliI and FliH are intrinsically targeted to the inner membrane before contacting the flagellar secretion machinery, with both FliH105-235 and membrane phospholipids interacting with FliI to couple ATP hydrolysis to flagellum assembly.

Keywords

flagellum assembly; FliI and FliH; membrane ATPase; SecA; type III protein export

Introduction

The motility of enterobacteria like *Escherichia coli* and *Salmonella typhimurium* is mediated by rotating flagella on the bacterial cell surface. The flagellum is a macromolecular structure that spans both membranes and the intervening periplasmic space, and extends 15-20 μ m from the cell surface.^{1,2} Its assembly requires sequential export of subunits making up the axial substructures (the flagellar rod, hook, hook-filament junction, filament and filament cap) by a specialised type III export apparatus in the cytoplasmic membrane.^{3,4} The components of this machinery have been identified,⁵ and include six integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ, and FliR) that are suggested to be located within the FliF basal body MS ring.^{6,7} Premature association and oligomerisation of the axial protein subunits is prevented by substrate-specific cytosolic chaperones,⁸⁻¹⁰ facilitating ordered

export through the lumen of the 25-30 Å flagellum central channel and assembly at the distal end of the growing structure.

The flagellar FliI ATPase is assumed to couple ATP hydrolysis to secretion of the axial subunits, and thus be pivotal to transition from the cytosolic to membrane stages of the translocation pathway.^{11,12} FliI activity is regulated negatively by an accessory protein FliH with which it forms a complex.¹³ FliI and FliH have no obvious transmembrane domains, and they can be isolated from recombinant *E. coli* as a soluble FliH₂/FliI complex.¹³ Together with preliminary affinity blotting experiments these observations have prompted the suggestion that the two proteins are cytoplasmic and contact the export membrane machinery *via* the integral membrane proteins FlhA and FlhB.^{5,14} Nevertheless, a clear view of FliI and FliH cellular localisation is still lacking and is essential to establish the mechanistic details of flagellar export. We have therefore sought to determine whether under physiological conditions the two proteins are cytosolic or are targeted to the membrane, either intrinsically or by docking to the flagellar machinery. We have assayed the possible influence of phospholipids on FliI ATPase activity, and assessed the interactions within the FliH₂/FliI complex.

Results

FliI and FliH localise to the inner membrane of flagellated *S. typhimurium*

We first sought to establish the cellular localisation of FliI and FliH in *S. typhimurium* when expressed normally, i.e. under physiological conditions. Cell lysis was achieved by osmotic shock, a technique that has been used to purify intact flagellar hook basal body complexes,¹⁵ and which is gentler than methods such as mechanical breakage by a French press or sonication.¹⁶ Membrane and cytoplasmic fractions were then separated by centrifugation and immunoblotted with polyclonal antisera raised against FliI and FliH.

Both FliI and FliH were associated predominantly with the membrane fraction, in parallel with FliN (Figure 1(a)) and FliM (not shown), the C-ring proteins that associate with the flagellar basal body membrane complex but are not themselves embedded in the membrane.¹⁷ This was observed in cultures harvested throughout the growth curve (not shown), and was not due to inefficient cell lysis, since the control cytosolic β-galactosidase was released entirely into the soluble fraction (Figure 1(a)). When cell lysis was performed using the French press or sonication, variable amounts (ca 50%) of FliI and FliH were detected in the cytoplasmic fraction, as was the case for FliM and FliN (not shown), and it is possible that heat or shearing forces generated by these procedures disrupted the membrane association of these proteins. In agreement with this view, membrane-associated FliI and FliH prepared from osmotically lysed cells were released into the soluble fraction after sonication (see below) or disruption by the French press (not shown). Furthermore, when the membrane fraction was analysed on sucrose density gradients, both FliI and FliH co-fractionated with the inner membrane marker NADH oxidase, and also with FliM and FliN (Figure 1(b)).

Membrane localisation of FliI and FliH is independent of the flagellar export apparatus and basal body

FliI and FliH membrane localisation could be intrinsic or require binding to specific components of the flagellar export apparatus or basal body, as is the case with the co-fractionating FliM and FliN. To distinguish between these possibilities, cell fractionations were performed with well-characterised mutant *S. typhimurium* strains that have been used to determine the pathway of flagella assembly.^{17,18} We chose mutant strains specifically lacking the proposed FliH/FliI partners, the inner membrane FlhA and FlhB proteins,¹⁴ or other proteins that play an important role in the biogenesis, structure, or function of the

export machinery, specifically FliF, FliO, FliP, FliQ and FliR. In every one of the mutants, FliI and FliH localised to the membrane fraction as they did in the wild-type (Figure 2). In contrast, membrane localisation of the C-ring protein FliN was dependent on FliF (Figure 2), the subunit of the inner membrane MS-ring upon which the C-ring is built.

The inner membrane location of FliI and FliH was confirmed in the total absence of other flagellar proteins by expressing them from plasmids pBADFliI and pBADFliH in the *S. typhimurium* SJW1368 *flhDC* mutant strain lacking the flagellar master regulator FlhD₂C₂. The two proteins, which were expressed at levels comparable to wild-type (not shown), were again found in the membrane fraction (Figure 3), and the same results were obtained when FliI and FliH were expressed in *E. coli* BL21 lacking any flagellar or virulence-related type III secretion system (not shown). These results indicate that FliI and FliH are targeted to the inner membrane independently of any other component of the flagellar export apparatus and basal body.

Characterisation of FliI and FliH association with the inner membrane

To establish the nature of FliI and FliH membrane association, membranes were isolated from *S. typhimurium flhDC* (pBADFliI) or *flhDC* (pBADFliH), which express FliI or FliH at levels comparable to wild-type (not shown), and exposed individually to extraction treatments that distinguish between peripheral and integral membrane proteins. FliI and FliH behaved identically. High concentrations of salt released neither protein from the membrane (Figure 4), but both were extracted partially by sodium carbonate at pH 11, non-ionic detergent (Triton X-100), and with protein denaturant e.g. urea (it is not clear why pelleted FliH migrates slightly slower than the soluble form, but this may reflect the presence of membranes). The same pattern of solubilisation was observed in experiments using membranes from wild-type *S. typhimurium* SJW1103 (data not shown), indicating that other flagellar proteins do not influence FliI and FliH extractability. These extraction characteristics are atypical of both peripheral and integral membrane proteins, and suggest FliI and FliH topologies that are intermediate. Similar conclusions have been drawn for the GSP ATPase subunit SecA,¹⁹ the type IV secretion ATPase VirB11,²⁰ and the histidine permease ATPase subunit HisP.²¹

FliI and FliH both display strong affinity for phospholipid vesicles

Since FliI and FliH associate with the membrane in the absence of other flagellar proteins, we investigated whether they have an intrinsic ability to associate with membrane phospholipids. *S. typhimurium* FliI, FliH, and the FliH₂/FliI complex were each purified from an *E. coli* laboratory strain carrying plasmids pETFliI, pETFliH or pETFliH/I, and mixed with liposomes made from *E. coli* phospholipids (PL). The mixtures were placed at the bottom of a sucrose gradient and centrifuged at 75,000*g* for 16 hours, allowing the separation (flotation) of liposome-associated proteins (top fraction) from liposome-free proteins (bottom fraction). Assayed independently, FliI and FliH each floated to the top fractions with the liposomes (Figure 5(a)) whereas when liposomes were omitted, both proteins remained at the bottom of the gradient. Furthermore, the liposome association of FliI and FliH was not disrupted by the presence of 1 M NaCl, which is consistent with the results of the membrane extraction treatments described in the previous section. Nor, apparently, was liposome association by the two proteins prevented by complex formation (Figure 5(b)). The presence of ATP did not prevent flotation of the FliI ATPase (Figure 5(a)) or the FliH₂/FliI complex (Figure 5(b)).

The C-terminal region of FliH effects homodimerisation and interacts with the N-terminal region of FliI

To identify the regions involved in FliH-FliI interaction and FliH homodimerisation, we first used the yeast two-hybrid system (Clontech). The activation domain (AD) and binding domain (BD) of the GAL4 transcriptional activator were fused independently to residues 1-155 (I1-155) and 156-456 (I156-456) of the 456 residue FliI, and in parallel with residues 1-130 (H1-130) and residues 105-235 (H105-235) of the 235 residue FliH. Plasmids encoding these FliH and FliI fusion proteins, and unfused GAL4 AD or BD, were introduced into the yeast reporter strains Y190 and Y187 (Clontech) to establish pairwise matings. The combinations were tested for interaction by assaying the expression of the GAL4-controlled *lacZ* reporter gene (Table 1). No β -galactosidase activity was detected when any of the FliH and FliI fusion proteins were combined in strains containing the activation or binding domain alone. No interaction was found in any matings involving H1-130, nor was self-interaction seen with I1-155 or I156-456. In contrast, there was a clear self-interaction effected by H105-235, and direct interaction between I1-155 and H105-235, and between I1-155 and I156-456, regardless of which partners were fused to GAL4 AD and BD.

To confirm these findings, we constructed bacteriophage T7 plasmids expressing FliI, FliH and their derivatives I1-155, I156-456, H1-130 and H105-235 (Figure 6(a)), and tested protein interactions by a pull down assay. Mixtures of a His-tagged and an untagged component were applied to a Ni-nitrilotriacetic acid (Ni-NTA) column, and co-elution assayed (Figure 6(b)). FliH/I complex formation has been demonstrated using comparable pull-down assays.¹³ Our assays confirmed that His-tagged FliH co-purified with FliI, and revealed that FliH co-purified with I1-155, but not with I156-456. Furthermore, His-tagged H105-235, but not His-tagged H1-130, was able to pull-down FliI. The results confirm a stable FliH-FliI interaction, and indicate that it is established by the N-terminal region of FliI and the C-terminal region of FliH. Moreover, they indicate that FliH homodimerisation is mediated by its C-terminal region, and reveal intra-molecular interaction between the N and C-terminal regions of FliI.

Activation of the FliI ATPase by acidic phospholipids

Since FliI and FliH are located in the cytoplasmic membrane and have intrinsic affinity for liposomes, we investigated whether phospholipids stimulate FliI activity, as is the case with the SecA ATPase.²² Measured *in vitro* by the malachite green ATP hydrolysis assay, FliI activity was seen to be sevenfold higher in the presence of *E. coli* PL (Figure 7(a)). To ascertain whether PL overcame the inhibitory effect of FliH on FliI ATPase that has been reported,¹³ FliI activity was assayed in the purified FliH₂/FliI complex. The result confirmed the inhibitory effect of FliH (ca tenfold) and revealed that it was mediated by the C-terminal domain of FliH (H105-235). *E. coli* PL did not relieve this FliH inhibitory effect (Figure 7(a)). To investigate whether activation of FliI by PL is dependent on the charge of PL, FliI ATPase activity was assayed in the presence of acidic or zwitterionic phospholipids. As shown for SecA,²² the charged acidic phospholipids phosphatidylglycerol (PG) and cardiolipin (CL), both of which are present in the *E. coli* membrane, stimulated FliI ATPase activity ca tenfold, while the zwitterionic lipid phosphatidylcholine (PC), which is not present in *E. coli* PL, had no effect (Figure 7(a)). Neither protein floated to the top fraction of the density gradients when liposomes were prepared from PC only, but did do so when PG or CL was present in the liposomes (Figure 7(b)). This confirmed that liposome interaction of FliI and FliH was mediated by charged phospholipids.

Discussion

Export of the flagellar axial components involves the co-ordinated participation of more than 20 proteins, acting in the cytoplasm and the subsequent membrane localized steps of translocation. Critical to the export pathway is the flagellar ATPase FliI, which acts at the transition between the cytoplasmic and membrane events, coupling energy from ATP hydrolysis to translocation of the axial subunits into the channel of the growing structure. In this study, we have addressed questions central to understanding FliI action. In particular, does FliI act in the cytosol and/or dock onto specific components of the membrane export machinery, as has been proposed for FliI^{5,14} and its homologues in type III virulence effector secretion systems?²³ Or is FliI targeted intrinsically to the membrane, as is the case with the well-characterised SecA ATPase that underpins the general secretory pathway of proteins across the inner membrane?²² Does membrane interaction modulate FliI ATPase activity and, if so, how does this relate to the interaction of FliI with its accessory regulatory protein FliH?

Despite the expectation arising from ready purification of soluble FliH₂/FliI heterotrimers following overproduction in *E. coli*,¹³ we have established here that in wild-type motile *S. typhimurium*, FliI and FliH are localised to the cytoplasmic membrane. Even though they lack obvious membrane-spanning domains, membrane association is intrinsic for both FliI and FliH. This has been shown to be the case for SecA¹⁹ and other polar membrane-bound ATPases of transport systems, i.e. the histidine permease ATPase subunit HisP21 and the type IV secretion ATPase VirB^{11,20} which similarly lack such hydrophobic sequence signatures. This localisation is not dependent on the presence of other flagellar components that could act as membrane “docking” proteins, as is the case for the C-ring proteins FliM and FliN. The pattern of FliI and FliH extraction from the cytoplasmic membrane indicated a tight association, and this view was supported by the interaction of FliI, FliH or FliH₂/FliI complexes *in vitro* with phospholipids. We found that in further similarity to SecA,²² the ATPase activity of FliI is activated by bacterial phospholipids, particularly acidic phospholipids, indicating that the intrinsic membrane association is critical to the function of FliI in export. It is possible that FliI interacts with the polar head group region of acidic lipids, although our finding that FliI lipid association occurs in high concentrations of salt suggests that, as reported for SecA,²⁴ electrostatic interactions are not the sole determinants.

Data from limited proteolysis and co-purification assays have suggested that FliH interacts with the N-terminal region of FliI.^{13,25} We assessed direct interaction of N and C-terminal regions of FliH and FliI by yeast two-hybrid and pull-down assays. These established that FliH₂/FliI complex formation is mediated by the interaction of the C-terminal half of FliH (H105-235) with the N-terminal third of FliI (I1-155), and that the interaction by H105-235 is sufficient to inhibit the ATPase activity of FliI *in vitro*. In addition, the N and C-terminal regions of FliI are able to interact, an intra-molecular interaction that might modulate the efficiency of ATP hydrolysis, especially since the N-terminal region of FliI has been shown to exert a regulatory effect on the catalytic mechanism.²⁶

How do our data contribute to an improved view of the early steps of export and flagellar assembly? Previous to this work, models suggested that FliH₂/FliI associates with chaperone/substrate complexes in the cytosol, with subsequent recruitment to the export machinery *via* interactions with the cytoplasmic domains of FlhA and FlhB.¹⁴ Our data establish that FliI and FliH associate intrinsically with the inner membrane, and that acidic phospholipids activate ATP hydrolysis at this location. Phospholipids do not relieve the FliH inhibitory effect on FliI. This may indicate that coupling of ATP hydrolysis to protein translocation requires a conformational change in FliI and/or participation of additional export or substrate components to relieve FliH restraint. FliH interaction could restrain ATP

hydrolysis until the export apparatus is in a secretion-competent state and/or substrates are delivered by the cytosolic chaperones. After FliI and FliH associate with the lipid phase of the membrane, they could undergo lateral diffusion towards the flagellar integral membrane proteins, and become incorporated within the assembling MS-ring. Such early events involving FliI and FliH would therefore be central to formation of the functional export apparatus, and secretion of the axial subunits arriving from the cytosol.

Materials and Methods

Bacteria and recombinant plasmids

S. typhimurium SJW1103 has wild-type motility,²⁷ its derivatives are mutated in the flagellar genes *flhDC* (SJW1368),²⁸ *fliI* (SJW2702), *flhA* (SJW1616), *flhB* (SJW1467), *fliOPQR* (SJW192), and *fliF* (SJW1684).¹⁸ *E. coli* BL21(DE3) was used for protein expression.²⁹ Routine DNA manipulation and electroporation were carried out as described,³⁰ using *E. coli* *recA1* XL1 Blue (Stratagene). Plasmids are listed in Table 2. To construct pETFliH, pETFliI, pETFliHI, pETFliH105-235/I and pETFliH/I1-155, the *fliH*, *fliI*, *fliHI*, *fliH105-235/I*, and *fliH/I1-155* DNA sequences were isolated by PCR amplification from *S. typhimurium* SJW1103 chromosomal DNA (1-10 ng) in native *Pfu* buffer (Stratagene), 0.25 mM each dNTP and 50 pmol of the corresponding oligonucleotide pairs, with 2.5 units of native *Pfu* DNA polymerase (Stratagene) in a Perkin-Elmer thermal cycler. Amplified DNA was purified using a Qiaquick PCR purification kit (Qiagen), digested with *Nde* I and *Bam* HI and ligated to *Nde* I/*Bam* HI-digested T7 expression vector pET15b (Novagen). Plasmids pETFliH1-130/I and pETFliH/I156-456, were constructed using the overlap extension SOE-PCR method developed by Horton *et al.*³¹ (Table 2). The PCR products containing the deletions were digested with *Nde* I and *Bam* HI, and ligated to *Nde* I/*Bam* HI-digested vector pET15b. Automated DNA sequencing was used to confirm that there were no mutations and that the reading frame after the deletion junction was maintained. To construct pBADFliH, pBADFliI, and pBADFliHI, the *fliH*, *fliI* and *fliHI* containing fragments were excised from pETFliH, pETFliI, and pETFliHI, respectively, by *Xba* I/*Hind*III digestion, and ligated into the arabinose-inducible expression vector pBAD18.32 To construct vectors expressing FliH and FliI N and C-terminal regions for the yeast-two-hybrid assay, regions of FliH (residues 1-130 and 105-235) and FliI (residues 1-155 and 156-456) were PCR-amplified from *S. typhimurium* SJW1103 chromosomal DNA and the PCR products digested with *Nde* I and *Bam* HI, and ligated to *Nde* I/*Bam* HI-digested vector pET15b. The resulting plasmids, pETFliH1-130, pETFliH105-235, pETFliI1-155, and pETFliI156-456, (Table 2) were each used as templates in a subsequent PCR reaction using primers that contain *Nco* I and *Bam* HI restriction sites. The PCR products were digested with *Nco* I/*Bam* HI and inserted into the GAL4 activation domain (pACT2-1) and binding domain (pAS2-1) yeast two-hybrid vectors. The sequences of the cloned FliH and FliI regions were confirmed by automated DNA sequencing. Plasmid pOZ172 encoding β -galactosidase was described by Ozin.³³

Pull-down assay

Complex formation was assayed as co-purification, analysed by Ni-NTA affinity chromatography as described.¹³

Cell fractionation

Cells were grown at 37 °C in Luria-Bertani (LB) broth, harvested at mid/late-exponential growth phase (A_{600} 1.0), and fractionated using a modification of a previous protocol.³⁴ To create spheroplasts, cell pellets were resuspended in one volume of 0.5 M sucrose, 40 mM Tris-HCl (pH 7.4), 5 mM EDTA before the addition of lysozyme (80 μ g ml⁻¹) and one volume of sterile, distilled water. To stabilise the cytoplasmic membranes, MgCl₂ (20 mM

final concentration) was added to the suspension. Spheroplasts were pelleted by centrifugation (16,000g, 15 minutes) and lysed by resuspension in a hypotonic solution of 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM Pefabloc. DNase I (50 $\mu\text{g ml}^{-1}$) was added and the suspension agitated until viscosity decreased. Membranes were pelleted by centrifugation (16,000g, 15 minutes), the supernatant being the cytosolic fraction.

Preparation of membrane fractions from *S. typhimurium*

S. typhimurium strains were grown in LB broth in the absence of arabinose until late-exponential growth phase (A_{600} 1.0). Cells were pelleted and resuspended in 20 mM Tris-HCl (pH 7.4), 1 mM Pefabloc, and passed through a French pressure cell (Aminco) three times at 82,800 kPa. Following removal of intact cells by centrifugation (2000g, ten minutes), 20 mM MgCl_2 was added and total membranes were separated from soluble proteins by high-speed centrifugation (100,000g, two hours). Membrane pellets were resuspended in 20 mM Tris-HCl (pH 7.5). Separation of bacterial inner and outer membranes was performed according to the method of Osborn & Munson.³⁵ Isolated membranes (with additional 5 mM EDTA) were layered onto sucrose gradients (16 ml) prepared in a stepwise fashion with layers of 1.0 M, 1.2 M, 1.4 M, 1.6 M, 1.8 M and 2.0 M sucrose containing 5 mM EDTA. Gradients were centrifuged for 16 hours at 75,000g (Beckman L8-M Ultracentrifuge) after which 1 ml fractions were removed and precipitated with 10% (w/v) trichloroacetic acid.

Membrane extraction

Isolated membranes were treated with the following reagents: 2 M NaCl, 0.5% (v/v) Triton X-100, 5 M urea, 200 mM Na_2CO_3 (pH 11.0), at a final protein concentration of 1 mg ml^{-1} . Samples were incubated at room temperature for 30 minutes and centrifuged in a TLA-100.2 rotor at 100,000g for 30 minutes (Beckman Optima TLX Ultracentrifuge). Pellets were resuspended in volumes equal to those of the corresponding supernatants.

Liposome flotation

FliI, FliH and FliH/FliI complex were purified from *E. coli* BL21(DE3) carrying plasmid pETFliI, pETFliH or pETFliH/I, respectively, as described.^{13,26} Purified proteins were dialysed against 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT. *E. coli* phospholipids were obtained from Avanti Polar Lipids (USA). DOPC, PG and CL were obtained from Fluka (UK). A lipid stock solution in chloroform-methanol (9:1, v/v) was dried under nitrogen and resuspended to 10 mg ml^{-1} in buffer A (20 mM Tris-HCl (pH 7.4), 150 mM NaCl), sonicated and stored at 4 °C. Purified protein (1 μg) was added to buffer A-liposome solution (40 μl) and the mixture incubated at room temperature for 15 minutes, in the presence or in the absence of 20 mM ATP, 10 mM MgCl_2 . A sucrose-buffer A solution was added to this mixture to a final concentration of 50% (w/v) sucrose in a total volume of 1 ml. The samples were overlaid with 3.5 ml of 40% (w/v) sucrose and 0.5 ml of buffer A. Centrifugation was carried out overnight at 75,000g for 16 hours (Beckman L8-M Ultracentrifuge) at 16 °C. Ten fractions (0.5 ml) were collected from the gradient and precipitated with 10% trichloroacetic acid, fractions 1-4, 5-7 and 8-10, corresponding to top (T), middle (M) and bottom (B) fractions, respectively.

Protein analysis and immunodetection

Proteins were resolved by SDS-PAGE using 10%, 12.5% or 15% (w/v) polyacrylamide gels, stained with Coomassie blue or transferred onto PVDF (ProBlot, Applied Biosystems) or Nitrocellulose (Amersham) membranes blocked with 3% (w/v) bovine serum albumin and probed with rabbit polyclonal anti-sera raised against FliH, FliI1-155 (Scottish Antibody production Unit, Carlisle, UK), FliN and FliM (Covance, USA). Antibodies against FliH

and FliI1-155 were purified as described.³⁰ Goat anti-rabbit IgG-HRP conjugate (Pierce) was used to probe the blot before visualisation with SuperSignal substrate (Pierce). β -Galactosidase was detected using a mouse monoclonal antibody (Promega) probed with goat anti-mouse antibody-HRP conjugate (Amersham).

ATPase activity measurements

FliI ATPase activity was determined essentially as described by Fan & Macnab.²⁶ DOPC, PG, CL and *E. coli* PL, were prepared as described above and used at 10-20 mg ml⁻¹ in a 1 ml reaction.

Yeast two-hybrid system

The Matchmaker Two-hybrid system (Clontech) was used as described to test pairwise interactions between FliH1-130, FliH105-235, FliI1-155 and FliI156-456.³³ The protocols recommended by the manufacturer (Clontech) were followed closely. Each of the FliH and FliI regions cloned into the GAL4 binding domain (pAS2-1) or activation domain (pACT2-1) vectors was transformed independently into *Saccharomyces cerevisiae* strains Y187 (MAT α , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3,112*, *gal4* Δ , *met-*, *gal80* Δ , URA3::GAL1_{UAS}-GAL1_{TATA}-HIS3) and Y190 (MAT α , *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3,112*, *gal4* Δ , *gal80* Δ , *cyh*^r2, LYS2::URA::GAL1_{UAS}-HIS3_{TATA}-HIS3, URA3::GAL1_{UAS}-GAL1_{TATA}-HIS3), respectively. Resulting transformants were used in pairwise matings, selecting for both leucine and tryptophan nutritional markers encoded by the pACT2-1 and pAS2 vectors. Positive interactions were detected as activation of the GAL4-controlled β -galactosidase reporter gene by colony lift and liquid assays.

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Abbreviations used

AD	activation domain
BD	binding domain
CL	cardiolipin
DOPC	dioleoyl phosphatidylcholine
PC	phosphatidylcholine
PG	phosphatidylglycerol
PL	phospholipid(s)

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Figure 1.

FliI and FliH localisation in wild-type *S. typhimurium*. (a) Cell lysates of the flagellated *S. typhimurium* SJW1103 strain expressing β -galactosidase from plasmid pOZ172 were fractionated. Proteins from cytoplasmic (c), and membrane (m) fractions were analysed by SDS-12.5% PAGE and immunoblotting with anti- β -galactosidase, anti-FliI, anti-FliH, and anti-FliN antisera. (b) Membrane fractions were separated on a 0.8 M-2.0 M sucrose gradient. Proteins were resolved by SDS-12.5% PAGE and either stained with Coomassie blue (top panel, molecular mass markers are in kDa), or immunoblotted with anti-FliI, anti-FliH, anti-FliN and anti-FliM antisera (bottom panels). Positions of co-separated inner membrane NADH oxidase and outer membrane proteins (OMPs) are indicated.

Figure 2.

FliI and FliH localisation in *S. typhimurium* flagellar mutant strains. Cell lysates from SJW2702 (*fliI* mutant), SJW1616 (*flhA*), SJW1467 (*flhB*), SJW192 (*fliOPQR*), and SJW1684 (*fliF*) were fractionated. Proteins from cytoplasmic (c), and membrane (m) fractions were analysed by SDS-12.5% PAGE and immunoblotting with anti-FliI, anti-FliH and anti-FliN antisera.

Figure 3.

Localisation of FliI and FliH artificially expressed in a *flhDC* mutant. (a) Cell lysates from the non-flagellated *S. typhimurium* SJW1368 *flhDC* mutant expressing either FliI or FliH from plasmids pBADFliI or pBADFliH were fractionated. Proteins from cytoplasmic (c), and membrane (m) fractions were analysed by SDS-12.5% PAGE and immunoblotting with anti-FliI or anti-FliH antisera. (b) Membrane fractions were separated on a 0.8 M-2.0 M sucrose gradient and analysed as above, using inner membrane NADH oxidase and outer membrane proteins (OMPs) as markers.

Figure 4.

Extraction of membrane-associated FliI and FliH. Membrane pellets from the *S. typhimurium* SJW1368 *flhDC* mutant expressing FliI or FliH were either sonicated for 30 seconds, or resuspended in one of the solutions indicated. Soluble (s) and pellet (p) material was separated by centrifugation, analysed by SDS-12.5% PAGE and immunoblotting with anti-FliI or anti-FliH antisera.

Figure 5.

Flotation of purified FliI and FliH with liposomes. (a) Purified proteins FliI, FliH and (negative control) FlgN were added to *E. coli* phospholipid (PL) vesicles and mixed with 55% (w/v) sucrose, overlaid with 40% (w/v) sucrose and allowed to float through the density-gradient during centrifugation (75,000g, 16 hours). Flotations were also performed with PL in the presence of 1 M NaCl or 20 mM ATP, or in the absence of PL, as indicated. Top (T), middle (M) and bottom (B) fractions were analysed by SDS-12.5% PAGE and immunoblotting with anti-FliI, -FliH or -FlgN antisera. (b) Purified FliH₂/FliI complex was mixed with *E. coli* PL in the absence or presence of 20 mM ATP and analysed as above.

Figure 6.

Complex formation by FliH and FliI truncated derivatives. (a) Plasmid constructs co-expressing His-tagged FliH and FliI, or their deletion derivatives. The *fliH* and *fliI* genes, represented by grey and black bars, respectively, are co-transcribed in an operon. The phage T7 promoter (broken arrow) and histidine-tag (hatched bar) are represented 5' of the FliH coding sequence. (b) Pull-down assay (Ni-NTA affinity chromatography) using the protein pairs shown in (a). Lysates of IPTG uninduced (-) and induced (+) cell cultures, and proteins eluted from the nickel column (eluate) were analysed by SDS-12.5% (1, 3 and 5) or 15% (2 and 4) PAGE and Coomassie blue staining. Molecular mass markers were the same in each gel, as indicated.

(a)

lipid	ATPase activity		
	FliI	FliI/H	FliI/H105-235
none	0.30	0.04	0.03
<i>E. coli</i> PL	2.20	0.04	0.05
DOPC	0.39	ND	ND
DOPC/PG	2.80	ND	ND
DOPC/CL	3.20	ND	ND

(b) **Figure 7.**

Role of acidic phospholipids in liposome binding and activation of FliI. (a) ATP hydrolysis ($\pm 10\%$ nmol phosphate $\text{min}^{-1} \mu\text{g}^{-1}$ FliI) was assayed at 37 °C with FliI ($10 \mu\text{g ml}^{-1}$), FliH/FliI or FliH105-235/FliI complex ($30 \mu\text{g ml}^{-1}$), in the presence or absence of phospholipids (PL) ($10 \mu\text{g ml}^{-1}$). We used *E. coli* PL, dioleoyl phosphatidylcholine (DOPC) alone, or mixtures (5:1 ratio) of DOPC/phosphatidylglycerol (PG) and DOPC/cardiophilin (CL).

Released inorganic phosphate was measured using the malachite green assay.²⁶ ND, not determined. (b) Flotation of purified FliI or FliH to DOPC, DOPC/PG, or DOPC/CL, as measured by density-gradient centrifugation as in Figure 5.

Table 1

Interactions detected by the yeast two-hybrid system

BD fusion	AD fusion			
	Control (-)	I1-155	I1-156-456	H1-130 H105-235
Control (-)	-	-	-	-
I1-155	-	-	+	++
I156-456	-	+	-	-
H1-130	-	-	-	-
H105-235	-	++	-	+

FluH/FluH interaction was detected as Lac+ colony phenotype in colony lift assays and as Miller units (MU) in liquid β -galactosidase assays (Clontech). The development of blue colour in one hour (++) c 6.5 ± 0.1 MU, two hours (+); c 2.5 ± 0.1 MU, and >eight hours (-; 0.1 MU) was determined by testing three colonies for each mating. Cloning vectors PACT2-1 (activation domain, AD) and pAS2-1 (binding domain, BD) were used as negative controls.

Table 2

Plasmids	
pET15b	Amp ^r , T7 expression vector, 6×His translational fusion ^a
pBAD18	Amp ^r , P _{ara} expression vector ^b
pETFliH	pET15b P _{T7} <i>fliH</i> (<i>fliH</i> amplified by PCR)
pETFliH1-130	pET15b P _{T7} <i>fliH1-130</i> (<i>fliH1-130</i> amplified by PCR)
pETFliH105-235	pET15b P _{T7} <i>fliH105-235</i> (<i>fliH105-235</i> amplified by PCR)
pETFliI	pET15b P _{T7} <i>fliI</i> (<i>fliI</i> amplified by PCR)
pETFliI1-155	pET15b P _{T7} <i>fliI1-155</i> (<i>fliI1-155</i> amplified by PCR)
pETFliI56-456	pET15b P _{T7} <i>fliI56-456</i> (<i>fliI56-456</i> amplified by PCR)
pETFliHI	pET15b P _{T7} <i>fliH/fliI</i> (<i>fliH/fliI</i> amplified by PCR)
pETFliHI1-155	pET15b P _{T7} <i>fliH/fliI1-155</i> (<i>fliH/fliI1-155</i> amplified by PCR)
pETFliHI156-456	pET15b P _{T7} <i>fliH/fliI56-456</i> (<i>fliH/fliI56-456</i> amplified by PCR)
pETFliH1-130/I	pET15b P _{T7} <i>fliH1-130/fliI</i> (<i>fliH1-130/fliI</i> amplified by PCR)
pETFliH105-235/I	pET15b P _{T7} <i>fliH105-235/fliI</i> (<i>fliH105-235/fliI</i> amplified by PCR)
pBADFliH	pBAD18 P _{ara} <i>fliH</i> (<i>Xba</i> I- <i>Hind</i> III <i>fliH</i> -containing fragment from pET-FliH, subcloned into pBAD18)
pBADFliI	pBAD18 P _{ara} <i>fliI</i> (<i>Xba</i> I- <i>Hind</i> III <i>fliI</i> -containing fragment from pET-FliI, subcloned into pBAD18)

All the other material except the first two were from this study.

^aNovagen.

^bGuzman *et al.*³²