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Flagellin Polymerisation Control by a Cytosolic Export Chaperone

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Abstract

Assembly of the long helical filament of the bacterial flagellum requires polymerisation of ca 20,000 flagellin (FliC) monomeric subunits into the growing structure extending from the cell surface. Here, we show that export of *Salmonella* flagellin is facilitated specifically by a cytosolic protein, FliS, and that FliS binds to the FliC C-terminal helical domain, which contributes to stabilisation of flagellin subunit interactions during polymerisation. Stable complexes of FliS with flagellin were assembled efficiently *in vitro*, apparently by FliS homodimers binding to FliC monomers. The data suggest that FliS acts as a substrate-specific chaperone, preventing premature interaction of newly synthesised flagellin subunits in the cytosol. Compatible with this view, FliS was able to prevent *in vitro* polymerisation of FliC into filaments.

Keywords

flagellin; chaperone; *Salmonella*; FliS; filament polymerisation

Introduction

The principal component of the bacterial flagellum is the long helical filament that rotates as a propeller when torque is transmitted from a motor housed in the basal body.¹ Each filament comprises ~20,000 subunits of monomeric flagellin (FliC) under the filament cap FliD.² FliC and the other axial proteins making up the contiguous flagellar substructures are exported sequentially by a specialised type III export apparatus in the cytoplasmic membrane, and polymerise at the distal end of the growing cell-surface structure.³⁻⁵ They are assumed to move through the flagellum central channel of 25-30 Å as partially unfolded monomers.⁶⁻⁸

It seems probable, therefore, that premature folding and interaction of these monomers in the cytosol would have to be actively prevented. This is particularly so for flagellin, which is synthesised in such large amounts and has strong oligomerisation potential. Indeed, FliC polymerises readily into filaments *in vitro*.^{9,10} It is likely that such a cytosolic control mechanism would be targeted especially at the N and C-terminal regions of monomeric

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Note added in proof

After submission of this manuscript, 2.0 Å resolution crystal structure of *Salmonella* flagellin has been reported (Samatey, F. A. *et al.* (2001) *Nature*, 410, 331-337). This provides insights into the structural mechanism of flagellin polymerisation and the mechanical switch between filament helical states. However, the crystallised flagellin lacks the N terminal 52 amino acids and the C-terminal 44 amino acids, so it is not known whether the crystal structure contains the FliS binding site.

FliC, as they are solvent-exposed and unfolded, and their removal hinders filament assembly.¹¹⁻¹³ Upon polymerisation at the distal end of the filament, they become organized into a compact structure and are thought to play a role in quaternary interactions between subunits, maintaining the overall stability and conformation of the axial structure.¹³⁻¹⁶

A possible effector of cytosolic polymerisation control would be a dedicated export chaperone, especially as such substrate-specific chaperones bind to the hook-filament junction proteins FlgK and FlgL and the FliD cap, also called the hook associated proteins or HAPs.^{17,18} A candidate chaperone for the flagellin subunit is FliS, which is encoded by a flagellar operon but does not have a known structural or regulatory function in flagellar biogenesis. FliS is small (15 kDa) and predicted to be predominantly α -helical with an acidic pI (4.9), characteristics common to the HAP chaperones and the chaperones of the type III virulence secretion systems thought to have evolved from the “archetypal” flagellar export machinery.^{19,20} Compatible with this possibility, a *Salmonella typhimurium* *fliS* mutant has been reported to produce short flagella.²¹ Here, we provide evidence that FliS is an export chaperone specific for FliC, and that it binds to flagellin *in vitro* and prevents its polymerisation.

Results

FliS is specifically required for the export of flagellin subunits

The *fliS* gene of motile *S. typhimurium* SJW1103 was mutated by inserting a chloramphenicol-resistance cassette. Because of polarity exerted on *fliT* expression (not shown) in the *fliDST* operon,²² this mutant was named *fliST*. Loss of FliT specifically impairs export of its substrate, the filament cap FliD, causing the release of unpolymerised, low molecular mass FliC into the extracellular medium.^{23,24} Coomassie blue staining of FliC precipitated from culture supernatant shows that the *fliT* effect was reversed in the *fliST* double mutant (Figure 1(a), compare lane 3 to lane 2), and restored by complementation of *fliST* with plasmid pBAD-FliS expressing FliS (Figure 1(a), lane 4). This confirms the report by Yokoseki *et al.*²¹ that FliS is required for FliC export. That loss of FliS was specifically inhibiting FliC export was shown by assaying export of other flagellar proteins by the *fliST* mutant, alone or complemented with plasmid pBAD-FliT, compared to the wild-type. Western blotting indicated that secretion of the HAPs, FlgK, FlgL and FliD, and the anti-sigma factor FlgM was unaffected by the absence of FliS (Figure 1(b)).

FliS is cytosolic and forms stable homodimers

Polyclonal antiserum raised against His-tagged FliS recognised a 15 kDa polypeptide in whole-cell extract of the wild-type *S. typhimurium* but not the *fliST* mutant (Figure 2(a), left panel). FliS was present only in the cytosol of the wild-type strain, not in the periplasmic or membrane fractions, or the culture supernatant (Figure 2(a), right panel). To estimate the size of native FliS, total cytosolic proteins from late exponential phase cultures were separated by gel-filtration chromatography and the eluted fractions immunoblotted with the anti-FliS antiserum. FliS did not elute in fractions corresponding to its predicted monomeric molecular mass (14.7 kDa), but rather eluted as a single peak at ca 30 kDa (Figure 2(b)), suggesting that the cytosolic FliS exists as a stable homodimer. It indicates also that FliS does not form a stable complex with its FliC substrate *in vivo*, as has been established for the HAP chaperones FlgN and FliT.²⁴ This was confirmed by immunoblotting the same fractions with anti-FliC antiserum, which showed that FliC eluted in fractions that corresponded to its expected monomeric molecular mass (50-60 kDa, Figure 2(b)).

FliS binds specifically to flagellin

Affinity blotting of putative chaperones to immobilised substrates has been used to demonstrate *in vitro* binding of substrate-specific chaperones to flagellar HAPs¹⁸ and virulence proteins secreted by the type III mechanism.^{20,25} Proteins of whole-cell lysates from wild-type *S. typhimurium* and a derivative *fliC* mutant²⁶ were separated by SDS-PAGE and either immunoblotted with anti-FliC antiserum (Figure 3, left) or affinity blotted with crude soluble extracts of *Escherichia coli* BL21(DE3) (pET-FliS) expressing ³⁵S-labelled FliS (Figure 3, right). The radiolabelled FliS bound to a single polypeptide present in the wild-type cell lysate (Figure 3, right) and migrating to the same position as FliC (Figure 3, left). However, it did not bind to any protein in the lysate of the *fliC* mutant (Figure 3, right), which, as expected, does not produce any FliC protein (Figure 3, left). An identical result was seen when FliS was affinity blotted to concentrated culture supernatants (Figure 3, right), which provide an enriched source of exported flagellar axial proteins.²⁷ No signal was detected when the immobilised substrates were affinity blotted with soluble extracts of radiolabelled *E. coli* BL21(DE3) transformed with the plasmid vector pET-15b (data not shown). The data indicate that FliS binds specifically to FliC.

In vitro assembly of a stable FliS-FliC complex

To assess whether FliC is able to form a stable complex with FliS in the absence of other flagellar components, the two proteins were purified. FliC was obtained from *S. typhimurium* flagella (see Materials and Methods), while FliS was artificially expressed in *E. coli* BL21(DE3) cells. It was purified with an in-frame N-terminal hexahistidine affinity tag from inclusion bodies by nickel-affinity chromatography under denaturing conditions (not shown). The purified FliS was refolded by 1:15 dilution into 20 mM Tris-HCl (pH 7.4), with or without FliC, followed by dialysis (see Materials and Methods). Insoluble aggregates were removed by centrifugation and the soluble proteins analysed by gel-filtration chromatography. Immunoblotting of the eluted fractions with anti-FliS antiserum showed that FliS refolded in the absence of FliC eluted as a single peak of ca 30 kDa (Figure 4; -C). This is consistent with the assembly of stable homodimers, as indicated earlier by the gel filtration of wild-type *S. typhimurium* cell extract (Figure 2(b)). However, when FliS was refolded in the presence of FliC, immunoblotting showed that FliS eluted at a higher molecular mass, ca 80 kDa (Figure 4; +C), suggesting that FliS formed a stable complex *in vitro* with FliC. This possibility was supported by immunoblotting of the same fractions with anti-FliC antiserum, which showed that FliC co-eluted with FliS in a single peak at ca 80 kDa (Figure 4; +S). As expected, FliC monomers were eluted at ca 51 kDa in the absence of FliS (Figure 4; +S). These data are compatible with a dimer of FliS (ca 30 kDa) binding to a monomer of FliC (ca 51 kDa).

FliS binds to the helical C-terminal region of flagellin

The N and C-terminal regions of flagellin contain heptad repeats of hydrophobic amino acid residues that potentially form amphipathic helices involved in quaternary interactions between the subunits.⁶ N and C-terminal truncates of FliC were constructed by inserting *fliC* and its deleted derivatives, amplified by PCR, into a T7 expression vector (Figure 5(a), Table 1). SDS-PAGE of cell lysates from *E. coli* BL21(DE3) expressing full length FliC or one of its derivatives confirmed production of stable polypeptides with molecular masses consistent with their expected sizes (Figure 5(b), left panel). Affinity blot analysis of these lysates showed that removal of the 85 amino acid residue C-terminal helical region of FliC abolished binding by radiolabelled FliS (Δ 411-495; Figure 5(b), right panel), while FliS still bound to a 25 kDa polypeptide containing only the C-terminal 186 residues of FliC (C186).

FliS inhibits *in vitro* polymerisation of FliC

The possibility that FliS prevents premature polymerisation of FliC was investigated. The formation of flagellar filaments can be monitored *in vitro* by mixing a solution of monomeric FliC with a solution of short flagellar fragments or “seeds” that nucleate polymerisation.⁹ Monomeric FliC and seeds were obtained by heat treatment or sonication of purified *S. typhimurium* flagellar filaments, respectively (see Materials and Methods). Filament assembly was assayed by sedimentation at 600,000 *g*, which separates flagellin polymers from the monomeric subunits. Upon addition of seeds, FliC monomers polymerised into filaments, so that FliC was found almost exclusively in the pellet fraction (Figure 6, lane 3P). However, when the FliC monomers were pre-incubated with increasing concentrations of FliS (Figure 6, lanes 4-7), there was a shift of flagellin from the pellet to the supernatant fraction. Filament assembly was abolished at a 2:1 FliS to FliC molar ratio (the residual FliC in the pellet most likely corresponds to the seeds added to the reaction; compare lane 6P to lane 1P). The inhibitory effect of FliS was specific, neither bovine serum albumin (BSA) nor the HAP chaperone FlgN prevented FliC polymerisation and there was no effect when FliS was heat-denatured prior to its addition (data not shown). These experiments indicated that FliS inhibited flagellin polymerisation into filaments.

This effect was visualised by electron microscopy of polymerisation reactions. Upon addition of FliC monomers to seeds, flagellar filaments up to 10 μm long were assembled (Figure 7, top right). The length of the seeds added to the reaction did not exceed 1 μm (Figure 7, top left). The addition of FliS at a 2:1 FliS to FliC molar ratio inhibited filament formation completely (Figure 7, bottom right), with only filament fragments corresponding to the seeds observed. FliC polymerisation was prevented only partially at a lower, 1:1, FliS to FliC ratio (Figure 7, bottom left), with filaments of a length higher than 1 μm observed less frequently than in the reaction performed in the absence of FliS.

Discussion

Flagellin translocates through the hollow core of the growing flagellum and polymerises at the distal end of the filament under the FliD cap.²⁸ The N and C-terminal regions of flagellin have been suggested to be responsible for the regulation of FliC polymerisation.^{8,16,29} They are solvent-exposed and have no ordered tertiary structure in the monomeric form, but become folded and ordered upon incorporation in the filament.¹¹⁻¹³ It has been reasonably assumed that the presence of unfolded termini prevents spontaneous nucleation of flagellin monomers in the cytosol, but here we provide evidence that the cytosolic FliS protein is involved in controlling flagellin polymerisation prior to export and assembly. We found that FliS binds to the C-terminal helical region of FliC, and inhibits its polymerisation into filaments *in vitro*. FliS specifically bound a FliC truncate containing the 85 residue C-terminal α -helical region but not a truncate lacking it, suggesting that the C-terminal amphipathic helix is likely to be central to FliS interaction, as reported for the recognition of the HAPs by the related flagellar export chaperones FlgN and FliT.¹⁸ FliS formed homodimers and a chaperone-substrate complex, apparently composed of a FliS dimer and a FliC monomer, could be assembled *in vitro*. A 2:1 stoichiometry was further indicated by the requirement for a 2:1 FliS to FliC molar ratio for complete inhibition of FliC polymerisation, while a lower ratio prevented polymerisation only partially.

We propose that FliS acts as a specific chaperone or “bodyguard” for FliC, preventing premature folding or inappropriate association of newly synthesised flagellin subunits by binding to their C-terminal interactive region prior to translocation by the export machinery. This view is in agreement with the observation that spontaneous mutations causing flagellin accumulation in the cytoplasm map to the C-terminal region of FliC.³⁰ By binding to the FliC C-terminal region, FliS might prevent the premature direct interaction of flagellin

termini, which are known to be involved in inter-subunit interactions and which form a single structure, the inner tube present at the filament core.^{15,16} When a *S. typhimurium* cell extract was subjected to gel-filtration chromatography, no stable FliS-FliC complex could be detected. This has been observed also for the HAP chaperones and substrates.²⁴ These combined results indicate that the interactions occurring *in vivo* between the flagellar chaperones and their corresponding substrates may be transient. Rapid chaperone dissociation from the flagellar axial proteins could facilitate subsequent export *via* the type III flagellar apparatus.

Accumulation and premature oligomerisation of flagellar axial proteins in the cytosol would be wasteful and detrimental to the cell. This is especially so in the case of flagellin, which is by far the most abundant, with ~20,000 monomers per structure,² compared to ~140 FlgE hook protein monomers³¹ and 5 to 20 HAP monomers per flagellum.³² FliC would potentially have the greatest requirement for a chaperone, and database searches reveal that FliS is the most widely conserved flagellar chaperone (F. Auvray, unpublished data).³³ In contrast to the HAP chaperones FlgN and FliT, FliS homologs are found in the hyperthermophilic bacteria *Aquifex aeolicus*³⁴ and *Thermotoga maritima*,³⁵ two species suggested to be the deepest-branching lineages within the Eubacteria, having protein sequences and gene structures that are considered primitive and ancestral. It is thus tempting to propose that the family of flagellar chaperones, such as FlgN and FliT, has evolved from FliS.

Materials and Methods

Bacterial strains and recombinant DNA manipulations

Wild-type *S. typhimurium* SJW1103 is motile,³⁶ its derivatives are mutated in flagellar genes *flhDC* (SJW1368) or *fliC* (SJW2536).²⁶ Generation of the *fliS* null mutant was achieved as described for *flgN* and *fliT* mutants.²⁴ Briefly, the *fliDST* operon was amplified by PCR using primers D1 and T2 (Table 2) and cloned into plasmid pBlueScript II SK. The *fliS* gene was then inactivated by insertion of a chloramphenicol omega cassette after codon 37, and the disrupted locus subcloned into suicide vector pCVD442,³⁷ which was then transferred by conjugation into *S. typhimurium* SJW1103. Specific disruption of the *fliS* gene was verified by PCR amplification using primers S1 and S2 (Table 2) located in the 5' and 3' ends of the gene respectively. Immunoblotting with anti-FliS antiserum confirmed disruption of *fliS*. Bacteria were grown at 37 °C in Luria-Bertani (LB) broth or on LB agar, supplemented with ampicillin (100 µg/ml), chloramphenicol (25 µg/ml) or spectinomycin (50 µg/ml) as necessary. Routine DNA manipulation and electroporation were carried out as described,³⁸ using *E. coli* *recA1* XL1 Blue (Stratagene).

Plasmids and oligonucleotides are listed in Tables 1 and 2. The *fliS* and *fliC* genes and the *fliC* derivatives were amplified by PCR using *S. typhimurium* SJW1103 chromosomal DNA template (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 Wizard PCR Preps (Promega), digested with *NdeI* and *BamHI*, and ligated to *NdeI*/*BamHI*-digested T7 expression vector pET15b (Novagen).³⁹ To construct pBAD-FliS, a *fliS*-containing fragment was excised from pET-FliS by *XbaI*/*EcoRI* digestion, blunted and ligated into the *SmaI* site of the arabinose-inducible expression vector pBAD18.⁴⁰

Isolation of proteins from cells and culture supernatants

Wild-type *S. typhimurium* cells from late-exponential phase culture were fractionated using a modification of a previous protocol.⁴¹ To create spheroplasts and release periplasmic

contents, 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 µg/ml) and one volume of sterile, distilled water. To stabilise the cytoplasmic membranes, 20 mM MgCl₂ was added to the suspension of spheroplasts before being pelleted by centrifugation at 60,000 *g* for 20 minutes (the supernatant being the periplasmic fraction). The pellet was resuspended in a hypotonic solution of 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM Pefabloc, and 50 µg/ml DNase I was added. The suspension was agitated until viscosity decreased. 20 mM MgCl₂ was added to pellet membranes and the suspension was centrifuged (the supernatant being the cytosolic fraction). The membrane pellet was resuspended in urea/SDS loading buffer. Proteins in culture supernatants were concentrated as described,¹⁸ prior to SDS-PAGE. Efficient release of periplasmic content was confirmed by immunoblotting proteins from cell fractions with an antiserum raised against a periplasmic protein (maltose-binding protein, New England Biolabs).

Affinity blotting with FliS

E. coli BL21(DE3) cells carrying recombinant plasmid expressing FliC or its truncated derivatives (Table 1), were grown at 37 °C to mid-exponential phase (A_{600} of 0.7) and T7-controlled expression of protein was induced by the addition of 1 mM IPTG.³⁹ Cultures were incubated further for three hours, after which cells were harvested and resuspended in SDS-PAGE loading buffer. Proteins were separated by SDS-15 % PAGE and transferred to nitrocellulose membranes. Expression of radiolabelled FliS in *E. coli* BL21(DE3) carrying pET-FliS was induced in the presence of 10 mM IPTG and 200 µg/ml rifampicin, followed by incubation of the cells with [³⁵S]methionine (100 µCi/ml; Amersham). Labelled cells were fractionated and soluble extracts (16,000 *g*, ten minutes) were added to the membranes, which had been preincubated for two hours at room temperature in PBS containing 5 % (w/v) skimmed milk. Membranes were further incubated for 16 hours, washed three times for 15 minutes in PBS, and dried. Binding of radiolabelled FliS was detected by autoradiography.

Isolation of FliS, FliC and FliS-FliC complex

FliC was isolated from *S. typhimurium* SJW1103 as described.¹² To purify FliS, exponentially growing *E. coli* BL21(DE3) cells (A_{600} 0.6) carrying pET-FliS were induced with 1 mM IPTG for three hours. Cells were harvested, resuspended in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Pefabloc, and disrupted by three passages through a French pressure cell (82,800 kPa; Aminco). Following centrifugation (43,000 *g*, 30 minutes), inclusion bodies containing His-tagged FliS were washed with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 % (v/v) Triton X-100. Non-soluble protein aggregates were pelleted (43,000 *g*, 30 minutes), washed with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and resuspended in binding buffer (6 M guanidinium hydrochloride, 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 5 mM imidazole). Soluble material was collected after centrifugation (20,000 *g*, 15 minutes). Binding and elution from nickel-nitrilotriacetic acid agarose resin was carried out according to the manufacturer's instructions (Qiagen). Purified His₆-FliS was diluted 15-fold in 20 mM Tris-HCl (pH 7.4) and dialysed overnight against 20 mM Tris-HCl (pH 7.4), 0.4 M NaCl, followed by four hours against 20 mM Tris-HCl (pH 7.4), 150 mM NaCl. Insoluble aggregates were removed by centrifugation at 600,000 *g* for 20 minutes and soluble His₆-FliS was further concentrated to a final concentration of 1 mg/ml using a centricon-3 concentrator (Amicon).

To isolate a soluble FliS-FliC complex, His₆-FliS solubilised in 6 M guanidinium hydrochloride and purified by nickel chromatography (see above) was diluted 15-fold with 20 mM Tris-HCl (pH 7.4), containing monomeric FliC, to obtain a 1:1 FliS to FliC molar ratio. Following dialysis overnight against 20 mM Tris-HCl (pH 7.4), 0.4 M NaCl, and four

hours against 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, insoluble protein was removed by centrifugation at 600,000 *g* for 20 minutes.

Gel-filtration chromatography

Purified FliS, FliC, or the preformed FliS-FliC complex were loaded on to a pre-equilibrated Superose 12 HR10/30 column (Pharmacia) developed at 0.5 ml/minute by Fast Protein Liquid Chromatography (FPLC; Pharmacia) in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl. Proteins in eluted fractions were precipitated by adding 10 % (w/v) trichloroacetic acid, resuspended in SDS buffer and separated on a SDS-15 % PAGE followed by immunoblotting. Soluble cell extract (200 µl; in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Pefabloc), from 2.5 ml of LB broth culture of *S. typhimurium* (A_{600} 1.2) was analysed by gel-filtration chromatography under equivalent conditions. The column was size-calibrated using bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

In vitro polymerisation of FliC onto flagellin seeds

In vitro assembly of flagellar filaments from monomeric FliC and flagellin seeds was carried out as described.⁹ Flagellar filaments were prepared from *S. typhimurium* SJW1103 by shaking the cells for 40 minutes, and were dissolved at 4 mg/ml in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl following centrifugation at 600,000 *g* for 30 minutes. Purified flagella were either depolymerised into monomeric flagellin by heating at 60 °C for 20 minutes, or fragmented into short pieces (seeds) by sonication for two minutes (Ultrasonic Processor, Heat Systems-Ultrasonics Inc). FliC polymerisation reactions were performed in 60 µl of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and contained 5 µg of flagellin seeds and 15 µg of monomeric flagellin. Following incubation for 15 hours at room temperature, FliC polymerisation was monitored by sedimentation or electron microscopy. Seed solutions and monomer solutions incubated separately under the same conditions were examined as controls. The effect of the presence of increasing concentrations of FliS on FliC polymerisation was analysed by preincubating the FliC monomers for 30 minutes with FliS. The effect of FlgN, BSA or heat-inactivated FliS was analysed as for FliS.

Assay of FliC filament polymerisation

Polymerisation reaction mixtures were centrifuged in a Beckman TLA100.2 rotor at 600,000 *g* for ten minutes. Acid-precipitated proteins from the supernatant fraction and proteins from the pellet fraction were resuspended in an equivalent volume of SDS loading buffer and analysed by SDS-12.5 % PAGE and Coomassie blue staining. Aliquots withdrawn from polymerisation reaction mixtures were diluted 1:10, adsorbed onto freshly glow-discharged, carbon-coated copper grids for one minute and washed in water before negative staining with 2 % (w/v) phosphotungstic acid (pH 7.0). Grids were then examined using a Philips CM100 transmission electron microscope (at a magnification of 6600×).

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Figure 1.

Effect of FliS loss on the secretion of flagellar filament subunit FliC. (a) Secreted proteins in culture supernatants of *S. typhimurium* SJW1103 wild-type (*wt*), the mutants *fliT* and *fliST*, and the *fliST* mutant transformed with plasmid pBAD-FliS (*fliST* + S), were precipitated with trichloroacetic acid, separated by SDS-12.5 % PAGE, and stained with Coomassie blue. Molecular mass markers are in kDa. (b) Secreted flagellar proteins in culture supernatants of SJW1103 (*wt*), the mutants SJW1368 (*flhDC*) and *fliST*, and the *fliST* mutant transformed with pBAD-FliT (*fliST* + T), were separated by SDS-15 % PAGE, and immunoblotted with anti-FlgK, anti-FliD, anti-FlgL and anti-FlgM antisera. The *flhDC* mutant, which expresses no flagellar proteins, was used as a negative control.

Figure 2.

Cell location and size of FliS. (a) Left panel: Proteins from whole-cell lysates of *S. typhimurium* SJW1103 (*wt*) and the *fliST* mutant were separated by SDS-15 % PAGE and immunoblotted with anti-FliS antiserum. Right panel: Cells from *wt* were fractionated and proteins from cytoplasmic (cyt), periplasmic (peri), membrane (mem) and supernatant (snt) fractions were separated by SDS-15 % PAGE and immunoblotted with either anti-FliS antiserum (FliS, upper) or anti-maltose-binding protein antiserum (MBP, lower) as a control for periplasm release. (b) Cytoplasmic cell extract was loaded onto a Superose 12 10/30 HR gel-filtration column. Eluted fractions were separated by SDS-15 % PAGE and immunoblotted with anti-FliS or anti-FliC antisera. Size markers (kDa) eluted as shown.

Figure 3.

Affinity blot of FliS to proteins from cells and culture supernatants. Proteins from total cell lysates (cell) and culture supernatants (snt) of *S. typhimurium* SJW1103 (*wt*) and the derived *fliC* mutant SJW2536 (*fliC*) were separated by SDS-15 % PAGE and either immunoblotted with anti-FliC antiserum (anti-FliC, left panel) or affinity blotted with radiolabelled FliS (^{35}S -FliS, right panel). Molecular mass markers are in kDa.

Figure 4.

In vitro complex assembly by FliS and FliC. Gel-filtration chromatography of purified FliS, FliC, and the FliS-FliC complex assembled *in vitro*. Protein samples were loaded separately onto a Superose 12 HR 10/30 column, and elution fractions were subjected to SDS-15 % PAGE and immunoblotted as indicated. Protein markers (kDa) for gel filtration are as for Figure 2. Upper panel: FliS alone (-C), and after incubation with FliC (+C), immunoblotted with anti-FliS antiserum. Lower panel: FliC alone (-S), and after incubation with FliS (+S), immunoblotted with anti-FliC antiserum. (note: the lower immunoblot in each shows the same fractions).

Figure 5.

Affinity blot of FliS to truncated FliC derivatives. (a) Representation of full-length FliC and truncated derivatives, with N and C-terminal α -helical regions indicated as shaded boxes. (b) Proteins from *E. coli* BL21 (DE3) artificially overexpressing FliC or truncated derivatives ($\Delta 411-495$, $\Delta 1-71/\Delta 411-495$ and C186) were separated by SDS-15 % PAGE and either stained with Coomassie blue (left panel) or affinity blotted with ^{35}S -labelled FliS (right panel). Molecular mass markers are in kDa.

Figure 6.

Effect of FliS on *in vitro* FliC polymerisation. *In vitro* polymerisation reactions containing flagellin seeds (5 μ g) and monomeric flagellin (15 μ g), in the absence (-) or presence (+) of increasing amounts of FliS (2.5 μ g, 5 μ g, 10 μ g or 20 μ g, corresponding to 0.5:1, 1:1, 2:1 or 4:1 FliS to FliC molar ratio, respectively, as indicated at the bottom) were centrifuged at 600,000 *g* for ten minutes, and the pellets (P) and supernatants (S) analysed by SDS-12.5 % PAGE and Coomassie blue staining. Molecular mass markers (M) are in kDa.

Figure 7. Inhibition of FliC filament polymerisation by FliS. Electron micrographs (6600×) of *in vitro* polymerisation reactions shown in Figure 6, i.e. reaction 1, FliC seeds; reaction 3, FliC monomers + seeds; reaction 5, FliC monomers + seeds + FliS (1:1 FliS to FliC molar ratio); reaction 6, FliC monomers seeds + FliS (2:1 FliS to FliC molar ratio).

Table 1

Plasmids	
pET15b	Amp ^r , T7 expression vector, 6×His translational fusion (a)
pBAD18	Amp ^r , P _{ara} expression vector (b)
pCVD442	Amp ^r , suicide vector (c)
pET-FltS	pET15b <i>fltS</i> (<i>fltS</i> amplified using S1 and S2)
pETC	pET15b <i>fltC</i> (<i>fltC</i> amplified using C1 and C4)
pETC(Δ411-495)	pET15b <i>fltC</i> (<i>fltC</i> Δ411-495 amplified using C1 and C5)
pETC(Δ1-71/Δ411-495)	pET15b <i>fltC</i> (<i>fltC</i> Δ 1-71/Δ 411-495 amplified using C2 and C5)
pETC(C186)	pET15b <i>fltC</i> (<i>fltC</i> C186 amplified using C3 and C4)
pBAD-FltS	pBAD18 <i>fltS</i> (<i>Xba</i> I- <i>Eco</i> RI <i>fltS</i> -containing fragment from pET-FltS, blunted and subcloned into pBAD18 <i>Sma</i> I site)
pBAD-FltT	pBAD18 <i>fltT</i> (<i>Xba</i> I- <i>Hind</i> III <i>fltT</i> -containing fragment from pET-FltT, blunted and subcloned into pBAD18 <i>Sma</i> I site (d))
(a) Novagen	
(b) 40	
(c) 37	
(d) 24	
all other material, this study.	

Table 2

Oligonucleotides

Primer	Sequence (5'-3')	Site
D1	GGAAGAACA <u>TATGGCTTCAA</u> TTTCATCATTAGG	<i>NdeI</i>
T2	GAGACGGGATCCTA <u>TAT</u> TGAGGCGCCAGGCGC	<i>Bam</i> HI
S1	GAGGTCCATATGTACACCGCGAGCGGGTATCAAAG	<i>NdeI</i>
S2	CCACGGATCCGGTCA <u>TTAACGAGACTCCT</u> TGGAA	<i>Bam</i> HI
C1	GATAAGGAAAGCA <u>TAT</u> TGGCACAAAGTCATT	<i>NdeI</i>
C2	CGTAACGCTAACGACCA <u>TAT</u> GTCCATTGCGCAGACC	<i>NdeI</i>
C3	GCATCTGTGTTCAT <u>TAT</u> GTCTTATACTGAT	<i>NdeI</i>
C4	CACGTGTCGGTGGATCCATCGCCGGATTAAAC	<i>Bam</i> HI
C5	CAAAAGCAGGATCCATTTA <u>CTG</u> CAGCGGGTTTTC	<i>Bam</i> HI

Mismatch nucleotides are shown in bold; start or termination codons are underlined.