A chain mechanism for flagellum growth

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Bacteria swim by means of long flagella extending from the cell surface. These are assembled from thousands of protein subunits translocated across the cell membrane by an export machinery at the base of each flagellum. Unfolded subunits then transit through a narrow channel at the core of the growing flagellum to the tip, where they crystallize into the nascent structure. As the flagellum lengthens outside the cell, the rate of flagellum growth does not change. The mystery is how subunit transit is maintained at a constant rate without a discernible energy source in the channel of the external flagellum. We present evidence for a simple physical mechanism for flagellum growth that harnesses the entropic force of the unfolded subunits themselves. We show that a subunit docked at the export machinery can be captured by a free subunit through head-to-tail linkage of juxtaposed amino (N)- and carboxy (C)-terminal helices. We propose that sequential rounds of linkage would generate a multisubunit chain that pulls successive subunits into and through the channel to the flagellum tip, and by isolating filaments growing on bacterial cells we reveal the predicted chain of head-to-tail linked subunits in the transit channel of flagella. Thermodynamic analysis confirms that links in the subunit chain can withstand the pulling force generated by rounds of subunit crystallization at the flagellum tip, and polymer theory predicts that as the N terminus of each unfolded subunit crystallizes, the entropic force at the subunit C terminus would increase, rapidly overcoming the threshold required to pull the next subunit from the export machinery. This pulling force would adjust automatically over the increasing length of the growing flagellum, maintaining a constant rate of subunit delivery to the tip.

The structural subunits of the sequential rod, hook and flagellin filament of the bacterial flagellum (Extended Data Fig. 1) are unfolded and translocated across the cell membrane by a type III export machinery, energized by ATP hydrolysis and the proton motive force. They pass into a central channel that extends the length of the growing flagellum outside the cell. The channel has a diameter of around 20 Å, so subunits must remain unfolded as they transit to the flagellum tip up to 15–20 μm, a distance that is 10 cell lengths away, where they crystallize into the growing structure. Theoretical models describing the physical challenges presented by assembling flagella outside the living cell are set out in Supplementary Information section 1, but how this feat is achieved remains a mystery. Models based on pushing or pumping mechanisms are unlikely, since the channel resides in the unfolded subunits themselves as they move from the export machinery at the base of the flagellum. We reasoned that such movement might be achieved by capture of each docked subunit by the preceding subunit already in the channel. Direct support for this possibility is provided by challenge experiments (Fig. 1) in which free flagellar hook subunit FlgE effected concentration-dependent release of another subunit docked at the export machinery gate component (that is, in preformed subunit-gate complexes, FlgD–[GST–FlhBC]; FlhBC stands for the C-terminal cytoplasmic domain of FlhB). Subunits docked at the gate component were captured, not displaced, by free subunit as the challenge subunit FlgE was engineered such that it was unable to bind the export gate (gate-blind; Extended Data Figs 2 and 3), and heteromeric FlgD–FlgE subunit–subunit capture complexes were confirmed by affinity chromatography (Fig. 1c). Further capture assays established that gate-docked FlgD can be captured by other hook subunits, but not by the filament cap subunit that is assembled later (Extended Data Fig. 4). We supposed that in the subunit docked at the export machinery, the extreme N terminus would be available to the free C terminus of the preceding subunit already in the channel, and that linkage of these juxtaposed termini could effect capture. Evidence for this was obtained by showing that C-terminally truncated gate-blind FlgE challenge subunit was unable to release gate-docked subunit in a concentration-dependent manner from the FlgD–FlhBC subunit-gate complex (Fig. 1b). We reasoned that links between N and C termini of unfolded adjacent subunits might adopt a parallel coiled-coil conformation (Fig. 1d) and using unique cysteine–cysteine crosslinks (FlgE-Ct V409C with FlgE-Nt A41C, D43C or A42C), we trapped a subunit dimer compatible with just such a head-to-tail arrangement (Fig. 1e). Weaker crosslinking was detected between FlgE-Ct V400C and FlgE-Nt A41C, which are at opposite ends of the predicted coiled-coil, and no complexes were detected for FlgE-Nt A41C, which lies out with the subunit extreme N-terminal helix (Fig. 1e). This conformation is different from that observed in assembled flagella, in which subunit termini fold as antiparallel coiled-coils that line the channel. The 8 Å cysteine–cysteine crosslinks could not form between subunits in the assembled hook, as the cysteines are too far apart (Extended Data Table 1). The crosslinking data show that subunit capture is achieved by subunits linking head-to-tail through their terminal helices, predicted to form a parallel coiled-coil, and indicate that each subunit terminus contributes 14–25 residues to each coiled-coil link.

We suggest that successive rounds of such linkage could generate a chain of subunits that extends through the flagellar channel. However, although a single unfolded hook subunit could span the channel in the rod/hook structure (~90 nm), crystallizing into the tip of the hook as it simultaneously recruits the next subunit from the export machinery, the flagellar filament grows up to 20 μm (ref. 2). Our proposed chain mechanism would therefore require many flagellin (FlhC) subunits to link in the same way to span the transit channel of the flagellar filament. We therefore set out to visualize the linked flagellin subunits in vivo by isolating part of the predicted flagellin chain from within the flagellar filament growing on the surface of living cells. First, again using in vitro cysteine–cysteine crosslinking, we confirmed the same type of head-to-tail linkage between flagellin subunits that we had shown between hook subunits (Fig. 2a, FlhC-Ct Q453C or N453C with FlhC-Nt S127C, L135C, L135C, N453C).
in vitro

that each flagellin terminus could contribute 21–32 residues to the terminal helix; Extended Data Table 1). The crosslinking distances indicate at least three times and were biological replicates. Engineered cysteines (Extended Data Fig. 5e). All experiments were carried out at least three times and were biological replicates.

Our data show that there are multiple linked subunits in the channel, incompatible with the recent theoretical model of filament growth in that a force of approximately 11 nN would be required to break the anchor. The proposed mechanism requires strong anchoring of the subunit at the flagellum tip. In this mechanism, depicted in Fig. 3 and energized by the thermal motion of a chain of linked unfolded subunits anchored at the flagellum tip, the proposed mechanism provides an increasing pulling force on the next linked subunit as it leaves the export machinery.

The proposed mechanism requires strong anchoring of the subunit at the tip, and breaking this anchor would require an estimated force of approximately 11 nN. Strong anchoring (FA of ~6–6.7 nN) could also be achieved in the absence of subunit assembly into the crystallographic structure, explaining how some flagellar proteins, for example, filament cap and hook-filament junction proteins, are exported even when no longer required for flagellum assembly.
**METHODS SUMMARY**

*Salmonella enterica* serovar *Typhimurium* (*S. typhimurium*) strains and plasmids used in this study are described in Extended Data Table 3. Bacteria were cultured at 30–37 °C in Luria-Bertani broth containing, where appropriate, antibiotics and chemical inducers. Protein–protein interactions were assessed by ITC and affinity chromatography using purified proteins or lysates of cells expressing recombinant proteins. Interaction surfaces in protein complexes were identified by photo-crosslinking of unnatural amino acids or chemical crosslinking of site-specific engineered cysteines. Covalently linked complexes from crosslinking experiments exhibited a higher apparent molecular mass than the free proteins when samples were separated by SDS–PAGE. Capture assays were performed by challenging preformed subunit FlhBC gate complexes with increasing concentrations of free subunit, and released subunit was subsequently affinity purified to assess the stability of capture complexes. *In vivo* flagellar subunit export assays were performed at mid-log growth in fresh warm media for 40 min, as previously described. Proteins separated by SDS–PAGE were visualized by either Coomassie staining or immunoblotting using specific antibodies, either to an epitope tag or the recombinant protein. All experiments were carried out at least three times and were biological replicates.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information** is available in the online version of the paper.

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METHODS
Bacterial strains, recombinant plasmids and antibodies. Wild-type Salmonella enterica serovar Typhimurium (S. typhimurium) SJW1103 is motile,\(^1\) derived mutants carry stable lesions in flagellar genes (flhDC) SJW11368. Bacteria were cultured at 30–37 °C in Luria-Bertani (LB) broth containing (where appropriate) ampicillin (100 μg ml\(^{-1}\)), tetracycline (3 μg ml\(^{-1}\)), chloramphenicol (20 μg ml\(^{-1}\)) and/or amino acid p-benzoyl-l-phenylalanine (pBpa, 1 mM) and collected by centrifugation (8000 g for 15 min). Recombinant proteins were expressed from isopropylthiolgalactoside (IPTG) (0.8 mM) inducible plasmids pT15b, pET20b, pGEX-4T-3 (ref. 34), pDULE\(^{2}\) or pACT7 (ref. 36) or from l-arabinosine (0.0002–0.2% (w/v)) inducible pBAD18 (ref. 37). To construct recombinant plasmids encoding wild-type, derivative and fusion genes (a full list and description of the constructs used in this study can be found in Extended Data Table 3), S. typhimurium flhB, flagD, flagE, flagK, flc, fltD and spdB\(^2\) were each amplified from chromosomal DNA by PCR or overlap-extension PCR using Pfu Ultra DNA polymerase. PCR products were inserted XbaI–HindIII/Sall into pBAD18 with or without 3× Flag or C-terminal 6His tags. For GST fusion constructs, gene products were inserted BamHI/Xhol into pGEX-4T-3. For C-terminal His-tagged proteins, gene products were inserted NcoI/BamHI into pET15b or NdeI/BamHI into pET20b and pACT7. Inserts were verified by DNA sequencing. Analysis by immunoblotting was carried out using the following antibodies: Flag antibody (Sigma, suitable for immunoblotting); His antibody (Qiagen, suitable for immunoblotting); Flc antibody (non-commercial, suitable for immunoblotting); FlgD antibody (non-commercial, suitable for immunoblotting); FliD antibody (non-commercial, suitable for immunoblotting).

Purification of proteins. Proteins were purified as described\(^{20,38,39}\), recombinant proteins engineered to possess affinity tags are listed in Extended Data Table 3. Briefly, recombinant cells were resuspended in buffer, GST–FlhB derivatives in buffer A (50 mM Tris-HCl pH 7.4, 200 mM NaCl), C-terminally 6His tagged derivatives in buffer B (50 mM Tris-HCl pH 7.4, 400 mM NaCl, 5–10 mM imidazole), derivatives to be purified by ion exchange in 50 mM sodium phosphate pH 7.0 to pH 7.8,5–50 mM NaCl and lysed by cell disruption (Constant Systems). Membranes, unlysed cells and insoluble proteins were removed (40,000 × g, 1 h), and the cleared cell lysate was incubated with affinity resin (Glutathione Sepharose (GSH-resin, GE Healthcare), Ni\(^2\+)–nitrilotriacetic acid agarose (Ni\(^2\+)–affinity) or anion exchange resin (Q HP (GE Healthcare)). After washing (>10 column volumes buffer), complexes were eluted using buffer containing 10 μM glutathione (GST–resin), 600 mM imidazole (Ni\(^2\+)–affinity) or an increasing NaCl gradient (Q HP), dialysed against an appropriate buffer.

Isothermal titration calorimetry. ITC was performed at 20 °C using a VP-ITC system (MicroCal). Purified proteins were dialyzed into 50 mM Tris-HCl (pH 7.4), 100 mM NaCl. The calorimetry cell was filled with 1.4 ml of GST–FlhB\(_C\) at 58 μM before making sequential injections of between 0.5 μl and 4.0 μl of 1,600 μM FlgD wild-type at 200-s intervals from the syringe to a final volume of 116.3 μl. Controls contained titrant or titrand against buffer alone. The heat due to the binding reaction was monitored from the time of injection to the time of reaction stabilization. The difference between the heat of reaction and the reaction heat due to the binding reaction was calculated from the integral of the calorimetric signal using Origin software (MicroCal).

Affinity chromatography co-purification. Co-purification of protein complexes was achieved with either Glutathione Sepharose 4B or Ni\(^2\+)–affinity.\(^3\) Purified proteins or cleared cell lysates (input) were incubated together for 1 h with affinity resin. After extensive washing with buffer A (GST–resin) or buffer B containing 10–50 mM imidazole (Ni\(^2\+)–affinity) proteins were eluted in buffer containing either 20 mM glutathione (GST–resin) or 600 mM imidazole (Ni\(^2\+)–affinity) or in SDS loading buffer. Fractions were analysed by immunoblotting.

Flagellar subunit export. S. typhimurium harbouring plasmids encoding flagellin subunit-challenged unbound/released fractions were adjusted to contain 5 mM imidazole, 0.5% w/v BSA and then Ni\(^2\+)–affinity purified, samples were washed extensively with buffer (50 mM sodium phosphate pH 7.4, 250 mM NaCl, 10 mM imidazole, 0.5% w/v BSA), co-purified captured samples were eluted in SDS loading buffer. Released, bound and captured samples were analysed by immunoblotting.

Rationale for choice of residues in crosslinking experiments. To distinguish subunit parallel-coiled-coil termini in the assembled structure from those comparable with subunits linked by alternative parallel coiled-coils, we used short range (8 Å) crosslinking of engineered cysteine pairs in juxtaposed subunits. Residue pairs (for replacement with cysteines) were chosen that would not form crosslinks between/corresponding subunits (we intended this would not interfere with the interface). This was determined by measuring lateral and axial distances between cysteine in adjacent subunits in the assembled structure. For in vivo assays in which two cysteines were introduced into each subunit, intra-subunit distances were also measured. This was achieved by analysing atomic models of the assembled hook and filament structures, generated by fitting the atomic resolution data of hook and filament subunits into their respective electron cryomicrographs\(^{40–42}\) (Extended Data Fig. 5 and Extended Data Table 1). This crosslinking strategy was also designed to examine interactions with all faces of the helical termini.

In vitro site-specific cysteine crosslinking. Purified N- and C-terminally truncated hook subunits (either FlgE or FliC, see Extended Data Table 3 for full descriptions) containing engineered cysteine in their remaining termini were dialysed into buffer C (50 mM sodium phosphate pH 7.0, 200 mM NaCl, 1 mM β-mercaptoethanol) and mixed (1:1 molar ratio), with or without 150 μM bismaleimidoethane (BMOE)\(^{43}\), then incubated at room temperature for 40 min before the reaction was quenched using 4 mM N-ethylmaleimide. Samples were then Ni\(^2\+)–affinity purified and washed extensively with buffer C containing 10 mM imidazole, isolated protein complexes were eluted in SDS loading buffer and analysed by immunoblotting.

In vivo site-specific cysteine crosslinking. Flagellin subunits subjected to in vivo cysteine crosslinking were isolated by adapting the method described in ref. 42. S. typhimurium AflhC::kmR harbouring pBAD18 plasmids encoding flagellin subunit derivatives for 20 min at room temperature (A10,000 g for 1.0). Cultures were centrifuged (16,000 g, 3 min), the supernatant discarded and the cell pellet resuspended in 37 °C in LB broth containing 1-arabinosine (0.0002% w/v) and 1 mM dithiothreitol to late-log phase (A600nm~1.0). Cultures were centrifuged (16,000 g, 3 min), the cell pellet was resuspended in M9 media containing 1-arabinosine (0.2% w/v), with or without BMOE (0.1 mM), and cultures were incubated at 37 °C for a further 30 min. To remove free exported subunit monomers, cultures were centrifuged (16,000 g, 3 min), the supernatant discarded and the cell pellet resuspended in 50 mM sodium phosphate (pH7.0), 200 mM NaCl with or without BMOE (0.1 mM), then shaken vigorously for 45 min at 4 °C. The crosslinking reaction was then quenched using 4 mM N-ethylmaleimide. To remove samples, cultures were centrifuged (12000 g, 4 min) and the supernatant containing sheared flagellar filaments was further decanted, this step was repeated. To pellet flagellar filamentous the supernatant was centrifuged (108,000g, 40 min) and the resulting pellet was resuspended in 50 mM sodium phosphate (pH7.0), 200 mM NaCl and incubated for 20 min at 65 °C to depolymerize flagellin subunits. Subunit concentration was assessed and the samples were precipitated by 10% (v/v) TCA on ice for 1 h. Crosslinking subunit samples were resuspended in LDS sample buffer (volume calibrated to protein concentration) and visualized by electrophoresis on 3–8% Tris-acetate PAGE (Invitrogen) followed by immunoblotting.

Fluorescence microscopy of S. typhimurium cells and flagella. S. typhimurium were cultured at 37 °C in LB broth to late-log phase (~1.0 A600). Cultures were centrifuged (16,000 g, 3 min), the cell pellet was resuspended, without vigorous mixing, in buffer A and incubated for 20 min with 2% (w/v) paraformaldehyde (in 20 mM PIPES, pH 7.4). Cells were centrifuged (16,000 g, 3 min). The resulting cell pellet was resuspended in buffer A and spread on a lysine coated glass coverslip, incubated at 25 °C for 5 min. Cells fixed on coverslips were probed\(^\text{44}\) using flagellin anti-sera followed by AlexaFluor488/594-conjugated secondary antibody, cell membranes were stained for 10 min with SynaptRed, and visualized using a fluorescence microscope (Leica DM IRBE). Images were captured by a CCD digital camera (Hamamatsu) and processed using OpenLab (Improvement).

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Extended Data Figure 1 | The bacterial flagellum. The cell surface bacterial flagellum has three contiguous hollow substructures: the rod, hook and filament, which are assembled sequentially. The ‘drive-shaft’ rod (FliE, FlgB, FlgC, FlgF and FlgG), which together with a series of rings comprises the basal body, extends from the inner membrane (IM) to span the periplasm, peptidoglycan (PG) cell wall and outer membrane (OM). The cell surface hook (FlgE) is a flexible universal joint with a defined length of ~55 nm that is controlled by the exported protein FliK. The hook–filament junction proteins (FlgK and FlgL) connect the hook to the external flagellar filament (flagellin FliC). An export machinery at the base of the flagellum translocates subunits across the cytoplasmic membrane. Subunits then transit through the external flagellar central channel to the distal tip where they crystallize beneath cap foldases (FlgJ for rod, FlgD for hook and FlID for filament subunit assembly). During the early stages of flagellum assembly, rod and hook proteins are exported as a single class of early subunits (FliE, FlgB, FlgC, FlgF, FlgG, FlgJ, FlgK, FlgD, FlgE and FliK). Upon hook completion the export machinery switches subunit specificity to allow export of a single class of late filament subunits (FlgK, FlgL, FliC, FliD). Only proteins that remain in the mature structure are shown in the figure.
Extended Data Figure 2 | Flagellar export gate FlhBC$_C$ hydrophobic pocket is essential for subunit docking (in support of Fig. 1). a, Isothermal titration calorimetry (ITC) of hook cap subunit FlgD binding to the C-terminal domain of the export machinery gate FlhB$_C$ (FlhBC$_C$) reveals low-affinity binding ($K_D$ 39 µM), compatible with transient association$^{20}$ and a 1:1 stoichiometry. b, Binding of FlgD subunit (input, middle panel) to purified wild-type FlhB$_C$ and its variants containing five-residue deletions (input, lower panel; for example, Δ278–282), assessed by affinity chromatography. Subunit binding (upper panel, immunoblot) was abolished by deletion of FlhB$_C$ residues 278–282, 283–287, 333–337, 338–342 or 343–347 identifying a single contiguous on FlhB$_C$ subunit-binding site containing a hydrophobic pocket. Glutathione S-transferase (GST) does not bind FlgD. c, Binding of FlgD hook and FlgG rod subunits (input, middle panel, immunoblot) to purified FlhB$_C$ wildtype and variants containing non-conservative point mutations within the hydrophobic pocket (input, lower panels, Coomassie stained; Δ286E, P287E, A341E and L344E). Binding was assessed by affinity chromatography (bound, upper panels immunoblot). d, Mapping subunit binding (b, c) on the FlhB$_C$ export gate structure (PDB 3B0Z)$^{44}$ reveal a contiguous subunit docking surface (blue) containing the hydrophobic pocket. Mutations in the pocket (Δ286E, P287E, A341E and L344E; orange) abolish binding of FlgD subunit. All experiments were carried out at least three times and were biological replicates.
Extended Data Figure 3 | A conserved recognition motif determines subunit docking at the flagellar export gate (in support of Fig. 1).

a, Identification of the subunit region that recognizes the export gate. FlhBC export gate binding (upper panels, immunoblot) by N-terminal (residues 1–100) subunit (FlgG, FlgD and FliK) regions, and FlgD subunit N-terminal peptides (residues 1–10, 1–50, 1–70) fused to SptP reporter (detected using anti-SptP sera). These data locate the gate-recognition region of FlgD to residues 10–50, and lead to the in vivo identification of the gate-recognition motif (e, below). The SptP reporter alone (−) did not bind FlhBC, nor did glutathione S-transferase (GST) alone bind the SptP reporter or subunit fusions (middle panels). Input samples are shown in the lower panels.

b, Identification of an essential short conserved motif near the N terminus FlgD36–40 by assay of in vivo subunit export (exported) by cell cultures expressing wild-type FlgD hook cap subunit or recombinant five amino acid deletion (Δ) variants (cell). c, Alignment of S. typhimurium FlgD essential sequence motif 36–40 with homologous regions in FlgD subunits from other bacterial species.

d, Conservation of the short motif comprising a phenylalanine followed by three residues and a hydrophobic residue (FxxxFw), revealed by alignment of S. typhimurium FlgD residues 36–40 with homologous N-terminal regions of all S. typhimurium rod and hook subunits. e, Subunits lacking the N-terminal conserved FxxxFw motif are severely attenuated for docking to the FlhBC export gate (these are termed gate-blind in Fig. 1). FlhBC binding assay of wild-type (+) subunits (FlgG, FlgD, FlgE and FliK) and their FxxxFw motif deletion (−) variants. FlhBC gate-bound subunits (upper panel; FlgD lanes) and subunit input fractions (lower panel) were immunoblotted with anti-Flag sera.

f, Direct binding of the subunit gate-recognition motif to FlhBC, shown by the formation of covalent complexes using engineered ultraviolet-activated (+) site-specific crosslinking residues (FlgD V 5X, L39Xo r L40X). g, Binding of FlgD subunit to autocleavage-defective FlhBc (N269A40) used in site-specific crosslinking assays (f) is comparable to wild-type FlhBc (upper panel; immunoblot, anti-FlgD sera). Input samples (middle and lower panels) of FlgD subunit (immunoblot, anti-FlgD sera) and FlhBc (Coomassie stain). All experiments were carried out at least three times and were biological replicates.
Extended Data Figure 4 | Capture of hook cap subunit docked at the export gate by free hook subunits and identification of stable capture complexes (in support of Fig. 1). a–d. Docked Flag-tagged FlgD hook cap subunit was challenged (as in Fig. 1a) by increasing concentrations of free His-tagged FlgE (a expanded from Fig. 1b), FliD (b), FlgD (c) or FliK (d) subunit, either wild-type or gate-blind (that is, unable to recognize the gate). e. Summary of capture (+) by challenge subunit of FlgD hook cap subunit docked at FlhBc (Fig. 1b, and Extended Data Fig. 4a–d). Capture assays establish that gate-docked FlgD can be captured by other hook subunits, but not by filament cap subunit that is only exported once the hook is complete 45. These data could reflect the well-defined classing of subunits as either ‘early’ (subunits of the rod and hook) or ‘late’ (subunits of the filament, filament cap and hook-filament junction, which are only exported and assembled once the hook is complete). f. Subunit capture complexes released from the gate (generated in c and d, indicated by asterisks) were isolated using affinity chromatography confirming that captured FlgD is linked to wild-type challenge subunits (FlgD or FliK) and their gate-blind variants, supporting the evidence that docked subunits are captured (not displaced) from the export gate by free subunit (Fig. 1b, c). g. In vivo FlgE hook subunit export is attenuated by deletion of C-terminal helix involved in subunit capture from the gate (ΔCt; Fig. 1b) and/or the motif required for gate recognition (gate-blind) assessed in the S. typhimurium flgD null by collecting culture supernatants. All experiments were carried out at least three times and were biological replicates.
Extended Data Figure 5 | Analysis of interactions between subunit terminal helices within the crystallized mature hook (in support of Fig. 1). a, Subunits crystallized in a lattice as they would be in the flagellum, with the terminal helices that line the channel forming intra-subunit antiparallel coiled-coils and inter-subunit lateral and axial packing interactions. Distances between engineered cysteine residues (Extended Data Table 1) were assessed between intra, axial and lateral subunits within the lattice, indicated by the open circles and dashed lines. This analysis allows the identification of crosslink residue pairs within subunit terminal helices that would form in an alternative coiled-coil conformation (parallel; Fig. 1d), distinct from subunits crystallized in the hook (antiparallel). b, Electron cryomicroscopy of the flagellar hook (EMD 1647) carved to show four subunits within a lattice. Terminal helices are coloured as in a. c, Atomic model of four subunits, coloured as in a, assembled as they would in the flagellar hook (PDB 3A69), derived from high-resolution electron cryomicroscopy and the atomic resolution structure of the hook subunit (PDB 2BGZ). Subunit N-terminal helices and the C-terminal helix (light blue) of the upper central subunit are indicated by a dashed box. d, A magnification of c, showing N-terminal helices of four adjacent subunits (coloured as in a) and the C-terminal helix (light blue) of the upper central subunit (a). Distances (dashed lines) between specific selected engineered cysteine residues (yellow) are shown in Extended Data Table 1. e, Control crosslinking assay showing that FlgE-Ct without engineered cysteines, incubated with (+) or without (−) BMOE cross-linker (x-link), cannot form complexes with FlgE-Nt (no cysteine) or its mutated variants A6C, A14C, D18C, A25C or A40C. All experiments were carried out at least three times and were biological replicates.
Extended Data Figure 6 | In vitro and in vivo head-to-tail linkage of flagellin subunits (in support of Fig. 2). a, Isolation of flagellin linked (dimer) in vitro by site-specific cysteine–cysteine crosslinks. Flagellin (FliC) and its variants containing unique cysteines (S11C, L13C, L18C, R31C or K178C; Extended Data Table 1) within and adjacent to the N-terminal helix predicted to generate a coiled-coil were incubated, with (+) or without (−) BMOE cross-linker (x-link), with a FliC variant containing a unique cysteine (N489C) within the C-terminal helix (upper panel). Flagellin derivatives were engineered to lack either their C or N termini (FliC-Nt, FliC-Ct) to preclude self-interaction. Control crosslinking (lower panel) assay showing that FliC-Ct without engineered cysteines cannot form crosslinked complexes.

b, Trapping of flagellin subunits linked head-to-tail in chains within flagella growing on S. typhimurium cells using in vivo site-specific cysteine–cysteine crosslinking. Cells expressing recombinant, export-competent full-length flagellin containing engineered cysteines (lanes from left to right: negative control L13C; L13C and Q488C predicted to trap chain; negative control Q488C alone; negative control wild type; negative control vector) were incubated with (+) or without (−) BMOE cross-linker (x-link). Flagellar filaments were then isolated, depolymerized and resolved (immunoblot, panel exposure times decrease from top to bottom) to reveal monomer (×1), dimer (×2) and higher order head-to-tail chains of flagellin.

c, Depiction of the in vivo trapping of flagellin subunit chain in the transit channel of growing flagellar filaments. Panels show a series of magnifications of a flagellated S. typhimurium cell, flagellar filament containing flagellin subunit chain linked head-to-tail by N- and C-terminal helices (blue and red cylinders, respectively) and the flagellar transit channel containing the subunit–subunit link and figurative positions of engineered cysteine residues (yellow segment of cylinder). Engineered cysteine residues in C-terminal helices of assembled flagellin subunits that line the channel could form crosslinked dimers with subunits in transit. All experiments were carried out at least three times and were biological replicates.
Extended Data Figure 7 | Generation of entropic force at the end of an unfolded polypeptide chain anchored to, and confined in, a narrow channel (in support of Supplementary Information section 3). a, Depiction of the transit of an unfolded polypeptide chain (blue) anchored through its N terminus at the tip of a narrow channel (with a diameter of $d$ and length of $R$). The pulling force ($F$, red arrow) at the C terminus of the polypeptide chain is generated by the total number ($N$) of free unfolded residues of length ($a$).

b, Depiction of the increase in pulling force $F(R,Na)$ as residues of the polypeptide chain crystallize at the tip of the channel, reducing the number of free residues within the channel, resulting in an increase in the ratio ($R/Na$) between channel length and number of free unfolded residues. This leads to an overstretched regime where the pulling force rapidly increases to reach the critical force ($F_M$, dashed line) required to capture the awaiting subunit docked at the export machinery.
Extended Data Table 1 | Distance between engineered cysteine pairs in the terminal helices of crystallized subunits (in support of Figs 1 and 2)

| Hook subunit pairs | Distance (Å) between cysteines in crystallized subunits |
|--------------------|--------------------------------------------------------|
|                    | intra | lateral 1 | lateral 2 | axial |
| FigE-Nt            |       |           |           |       |
| FigE-Ct            |       |           |           |       |
| **A**<sub>4</sub>C | V<sub>402</sub>C | 18 | 19 | 15 | 32 |
| A<sub>13</sub>C   | V<sub>406</sub>C | 27 | 12 | 15 | 21 |
| D<sub>14</sub>C   | V<sub>402</sub>C | 33 | 16 | 21 | 19 |
| A<sub>32</sub>C   | V<sub>406</sub>C | 43 | 23 | 27 | 14 |

**Flagellin L-form**

| FiIC-Nt | FiIC-Ct |       |           |           |       |
|---------|---------|-------|-----------|-----------|-------|
| S<sub>11</sub>C | Q<sub>406</sub>C | 11 | 33 | 32 | 55 |
| L<sub>13</sub>C | Q<sub>406</sub>C | 11 | 29 | 32 | 52 |
| L<sub>13</sub>C | Q<sub>416</sub>C | 15 | 25 | 22 | 44 |
| R<sub>32</sub>C | Q<sub>416</sub>C | 31 | 19 | 19 | 27 |
| K<sub>17</sub>C | Q<sub>416</sub>C | 122 | 99 | 102 | 77 |
| S<sub>17</sub>C | N<sub>406</sub>C | 15 | 31 | 33 | 55 |
| L<sub>17</sub>C | N<sub>406</sub>C | 18 | 29 | 34 | 52 |
| L<sub>17</sub>C | N<sub>416</sub>C | 19 | 24 | 24 | 44 |
| R<sub>32</sub>C | N<sub>416</sub>C | 35 | 21 | 23 | 29 |
| K<sub>17</sub>C | N<sub>416</sub>C | 129 | 103 | 105 | 82 |

In vivo crosslinking

| FiIC-Nt | FiIC-Ct |       |           |           |       |
|---------|---------|-------|-----------|-----------|-------|
| L<sub>13</sub>C | L<sub>13</sub>C | - | 29 | 33 | 53 |
| R<sub>32</sub>C | R<sub>32</sub>C | - | 30 | 34 | 53 |
| K<sub>17</sub>C | K<sub>17</sub>C | - | 45 | 47 | 53 |
| Q<sub>416</sub>C | Q<sub>416</sub>C | - | 27 | 31 | 53 |
| N<sub>416</sub>C | N<sub>416</sub>C | - | 25 | 30 | 53 |

**Flagellin R-form**

| FiIC-Nt | FiIC-Ct |       |           |           |       |
|---------|---------|-------|-----------|-----------|-------|
| S<sub>11</sub>C | Q<sub>406</sub>C | 13 | 32 | 30 | 53 |
| L<sub>13</sub>C | Q<sub>406</sub>C | 12 | 28 | 30 | 50 |
| L<sub>13</sub>C | Q<sub>416</sub>C | 15 | 23 | 19 | 41 |
| R<sub>32</sub>C | Q<sub>416</sub>C | 31 | 20 | 18 | 26 |
| K<sub>17</sub>C | Q<sub>416</sub>C | 127 | 106 | 106 | 84 |
| S<sub>17</sub>C | N<sub>406</sub>C | 17 | 30 | 29 | 51 |
| L<sub>17</sub>C | N<sub>406</sub>C | 18 | 27 | 32 | 50 |
| L<sub>17</sub>C | N<sub>416</sub>C | 19 | 21 | 21 | 40 |
| R<sub>32</sub>C | N<sub>416</sub>C | 35 | 22 | 22 | 28 |
| K<sub>17</sub>C | N<sub>416</sub>C | 123 | 101 | 78 | 119 |

In vivo crosslinking

| FiIC-Nt | FiIC-Ct |       |           |           |       |
|---------|---------|-------|-----------|-----------|-------|
| L<sub>13</sub>C | L<sub>13</sub>C | - | 29 | 32 | 52 |
| R<sub>32</sub>C | R<sub>32</sub>C | - | 29 | 34 | 52 |
| K<sub>17</sub>C | K<sub>17</sub>C | - | 42 | 42 | 52 |
| Q<sub>416</sub>C | Q<sub>416</sub>C | - | 26 | 31 | 52 |
| N<sub>416</sub>C | N<sub>416</sub>C | - | 25 | 29 | 52 |
Extended Data Table 2 | Length of flagellar substructures and component subunits used to estimate the minimum value of the entropic pulling force of the subunit chain

|                                | Rod     | Hook    | Filament |
|--------------------------------|---------|---------|----------|
| Length of the element          | ~35 nm  | ~55 nm  | up to 20 μm |
| Distance R from the export gate along the channel | ~35 nm  | ~90 nm  | Min. 90 nm |
| Average number N of subunit residues (amino acids) | 208     | 403     | 495      |
| Maximum unfolded length in the channel (N minus overlap of 36 for rod/hook; N minus overlap of 54 for flagelin) | 172     | 367     | 405      |
| Minimum pulling force of one subunit at full extension | ~42 pN  | ~134 pN | ~31 pN   |
Extended Data Table 3 | Strains and recombinant constructs

| Plasmids (continued) | Description |
|----------------------|-------------|
| pACT7 FdBΔras | 1:232aa, Ct-FLAG*C3 |
| pET20b FgE | 1:403aa, Ct-His6x |
| pET20b FgEΔ350-43 (ΔGRM) | 1:38-44, 403aa, Ct-His6x |
| pET20b FgEΔ350-43, Δ350-403 (ΔGRM, ΔCt) | 1:38-44, 350-354aa, Ct-His6x |
| pET20b FID | 1:467, Ct-His6x |
| pET20b FgDΔ350-40 (ΔGRM) | 1:35, 41-232aa, Ct-His6x |
| pET20b FIK | 1:402aa, Ct-His6x |
| pET20b FIKΔ30-34 (ΔGRM) | 1:29, 35-402aa, Ct-His6x |
| pBAD18 FgEΔ350-43, Δ350-403 (ΔCt) | 1:234, FLAG*C3, 235-403aa |
| pBAD18 FgEΔ350-43, Δ350-403 (ΔGRM, ΔCt) | 1:234, FLAG*C3, 235-354aa |

His × 6: 6 histidine epitope tag; Nt, amino terminus; Ct, carboxy terminus; Flag × 3, 3 Flag epitope tag; aa, amino acid; GST, glutathione S-transferase; GRM, gate-recognition motif.