Sequential recognition of discrete export signals in flagellar subunits during bacterial Type III secretion

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Abstract

The flagellar T3SS delivers proteins from the bacterial cytosol to nascent cell surface flagella. Early subunits of the flagellar rod and hook are unchaperoned and contain their own export signals. One export signal, the gate recognition motif (GRM) docks subunits at the export gate, which must then open for unfolded subunits to enter the flagellar channel. Here, we identify a second signal at the extreme N-terminus of flagellar rod/hook subunits and determine that key to the signal is its hydrophobicity. We show that the two export signals are recognised sequentially, with the N-terminal signal being recognised only after subunits have docked at the export gate. The position of the N-terminal signal relative to the GRM is important, as a FlgD deletion variant (FlgDshort), in which the distance between the N-terminal signal and the GRM was shortened, stalled at the export machinery and was not exported. The attenuation of motility caused by FlgDshort was suppressed by mutations that destabilised the closed conformation of the FlhAB-FlpQR flagellar export gate, suggesting that the hydrophobic N-terminal signal might trigger gate opening.

Introduction

Type III Secretion Systems (T3SS) are multi-component molecular machines that deliver protein cargo from the bacterial cytosol either to their site of assembly in cell surface flagella or virulence factor injectosomes, or directly to their site of action in eukaryotic target cells or the extracellular environment [1-5]. The flagellar T3SS (fT3SS) directs the export of thousands of structural subunits required for the assembly and operation of flagella, rotary nanomotors for cell motility that extend from the bacterial cell surface [1],[6]. Newly synthesised subunits of the flagellar rod,

hook and filament are targeted to the fT3SS, where they are unfolded and translocated across the cell membrane, powered by the proton motive force and ATP hydrolysis, into an external export channel that spans the length of the nascent flagellum [7],[8]. During flagellum biogenesis, when the rod/hook structure reaches its mature length, the fT3SS switches export specificity from recognition of ‘early’ rod/hook subunits to ‘late’ subunits for filament assembly [9],[10]. This means that early and late flagellar subunits must be differentiated by the fT3SS machinery to ensure that they are exported at the correct stage of flagellum biogenesis. This is achieved, in part, by targeting subunits to the export machinery at the right time using a combination of export signals in the subunit mRNA and/or polypeptide. T3SS substrates contain N-terminal signals for targeting to the export machinery, however these signals do not share a common peptide sequence [11-15]. In addition, some substrates are piloted to the T3SS machinery by specific chaperones [16-21].

The core export components of the fT3SS are evolutionarily related to those of the virulence injectosome, with which they share considerable structural and amino acid sequence similarity [22-25]. The flagellar export machinery comprises an ATPase complex (FliHJ) located in the cytoplasm, peripheral to the membrane. Immediately above the ATPase is a nonameric ring formed by the cytoplasmic domain of FlhA (FlhA_C), which functions as a subunit docking platform [20][21][26]. A recent cryo-ET map indicates that the FlhA family have a sea-horse-like structure, in which FlhA_C forms the ‘body’ and the FlhA N-terminal region (FlhA_N) forms the ‘head’, which is fixed in the plane of the membrane [27]. FlhA_N wraps around the base of a complex formed by FliPQR and the N-terminal sub-domain of FlhB (FlhB_N), and together

these form the FlhAB-FliPQR export gate that connects the cytoplasm to the central channel in the nascent flagellum, which is contiguous with the extracellular environment [22],[28]. FlhB_N is connected *via* a linker (FlhB_{CN}) to the cytoplasmic domain of FlhB (FlhB_C), which is thought to sit between the FlhA_N and FlhA_C rings, where it functions as a docking site for early flagellar subunits [22],[14],[28].

The ‘early’ flagellar subunits that assemble to form the rod and hook substructures are not chaperoned: instead, the signals for targeting and export are found within the early subunits themselves. We have shown that one of these signals is a small hydrophobic sequence termed the gate recognition motif (GRM), which is essential for early subunit export [14]. This motif binds a surface exposed hydrophobic pocket on FlhB_C [14]. Once subunits reach the export machinery, they must be unfolded before they can pass through the narrow channel formed by FliPQR-FlhB_N into the central channel of the nascent flagellum, through which the subunits transit until they reach the tip and fold into the structure [22],[6]. Structural studies suggest that FliPQR-FlhB_N adopts an energetically favourable closed conformation, possibly to maintain the membrane permeability barrier [22],[25],[29],[30]. This suggests that there must be a mechanism to trigger opening of the export gate when subunits dock at cytoplasmic face of the flagellar export machinery.

Here we sought to identify new export signals within flagellar rod/hook subunits, using the hook-cap subunit FlgD as a model export substrate. We show that the extreme N-terminus of rod/hook subunits contains a hydrophobic export signal and investigate its functional relationship to the subunit gate recognition motif (GRM).

Results

Identification of a hydrophobic export signal at the N-terminus of FlgD

The N-terminal region of flagellar rod and hook subunits is required for their export [12],[14]. Using the flagellar hook-cap protein FlgD as a model rod/hook subunit, we sought to identify specific export signals within the N-terminus. A screen of ten FlgD variants containing internal five-residue scanning deletions in the first 50 residues (though FlgD Δ 2-5 is a four-residue deletion, retaining the initial methionine) identified just two variants defective for export into culture supernatant (**Fig. 1A**). Loss of residues 2-5 caused a significant reduction in export, as did deletion of residues 36-40, though to a lesser extent (**Fig. 1A**; [14]). We have shown that FlgD residues 36-40 are the gate recognition motif (GRM) required for transient subunit docking at the FlhB_C component of the export gate [14]. The results suggest that the extreme N-terminus might also be important for interaction with the export machinery.

To gain insight into the putative new signal, we screened for intragenic suppressor mutations that could restore export of the FlgD Δ 2-5 variant. A *Salmonella flgD* null strain expressing *flgD* Δ 2-5 *in trans* was inoculated into soft-tryptone agar and incubated until 'spurs' of motile cell populations appeared. Sequencing of *flgD* Δ 2-5 alleles from these motile populations identified ten different intragenic gain-of-function mutations. These could be separated into two classes (**Fig. 1B**).

The first class of motile revertants carried *flgD* Δ 2-5 alleles with missense mutations that introduced small non-polar residues at the extreme N-terminus of FlgD Δ 2-5 (**Fig. 1B**). Deletion of residues 2-5 (²SI^{AV}⁵) had removed all small non-polar amino acids

from the first ten residue region of FlgD, effectively creating a new N-terminus containing a combination of polar, charged or large non-polar residues (**Fig. S1**). Analysis of other flagellar rod and hook subunit primary sequences revealed that in every case their native N-terminal regions contain small non-polar residues positioned upstream of the gate recognition motif (GRM residues 36-40; **Fig. S1**), indicating that hydrophobicity may be key to the function of the N-terminal export signal. Export assays performed with two representative motile revertant strains carrying *flgDΔ2-5* variants with gain-of-function point mutations, those encoding FlgDΔ2-5-N₈I and FlgDΔ2-5-T₁₁I, revealed that export of these subunits had recovered to ~50% of the level observed for wild type FlgD (**Fig. 1C**; **Fig. S2**; **Fig. S3**).

The second class of motile revertants carried *flgDΔ2-5* alleles that had acquired duplications or insertions introducing at least six additional residues between the FlgDΔ2-5 N-terminus and the gate recognition motif (GRM; **Fig. 1B**). It seemed possible that these insertions/duplications might have restored subunit export either by insertion of amino acids that could function as a 'new' hydrophobic export signal, or by restoring the position of an existing small hydrophobic residue or sequence relative to the GRM.

To assess these possibilities, we tested whether export of FlgDΔ2-5 could be recovered by inserting either polar (¹⁹STSTST²⁰) or small non-polar (¹⁹AGAGAG²⁰) residues in the FlgDΔ2-5 N-terminal region at a position equivalent to one of the suppressing duplications (¹⁹GSGSMT²⁰; **Fig. 1B and D**; **Fig. S3**). We reasoned that

if suppression by the additional sequence had been caused by repositioning an existing small hydrophobic amino acid relative to the GRM, then any insertional sequence (polar or non-polar) would restore export, while if suppression had resulted from insertion of a 'new' export signal, then either the polar STSTST or non-polar AGAGAG, but not both, could be expected to restore export.

We found that both the engineered FlgD variants (FlgD Δ 2-5-¹⁹AGAGAG²⁰ and FlgD Δ 2-5-¹⁹STSTST²⁰) were exported from a *Salmonella flgD* null strain as effectively as the gain-of-function mutant FlgD Δ 2-5-¹⁹GSGSMT²⁰ isolated from the suppressor screen (**Fig. 1D**). This suggests that the insertions had repositioned a sequence in the FlgD Δ 2-5 N-terminus relative to the GRM to overcome the loss of small hydrophobic residues.

The position of the hydrophobic export signal relative to the gate recognition motif is critical for rod and hook subunit export

The discovery of intragenic suppressors of the FlgD Δ 2-5 export defect indicated that FlgD export requires a hydrophobic signal towards the subunit N-terminus and that the position of this hydrophobic signal relative to the previously described GRM is important. Sequence analysis of the gain-of-function FlgD Δ 2-5 insertion variants revealed that the insertions were all located between the GRM and valine₁₅ (V₁₅; **Fig. 1B**). We reasoned that the insertions repositioned valine₁₅ relative to the GRM, such that it could perform the function of the N-terminal hydrophobic signal lost in FlgD Δ 2-5. To test this view, we replaced V₁₅ with alanine in the gain-of-function variant FlgD Δ 2-5-¹⁹(GSGSMT)²⁰ and assayed its export in the *Salmonella flgD* null (**Fig. 2A**;

Fig. S4). Unlike the *flgD* null strain producing either the parental FlgD Δ 2-5-¹⁹(GSGSMT)²⁰ or wild type FlgD, the *flgD* null carrying variant FlgD Δ 2-5-¹⁹(GSGSMT)²⁰-V₁₅A was non-motile, reflecting the variant's failure to export (**Fig. 2A**). This suggests that the V₁₅ residue had indeed compensated for the missing N-terminal hydrophobic signal.

By screening for intragenic suppressors of the motility defect associated with FlgD Δ 2-5-¹⁹(GSGSMT)²⁰-V₁₅A, four gain-of-function missense mutations were identified, M₇I, D₉A, T₁₁I and G₁₄V. All of these had introduced small hydrophobic residues, all positioned at least 27 residues upstream of the GRM. These FlgD Δ 2-5-¹⁹(GSGSMT)²⁰-V₁₅A gain-of-function variants restored motility to the *Salmonella flgD* null strain and were exported at levels similar to wildtype FlgD and FlgD Δ 2-5-¹⁹(GSGSMT)²⁰ (**Fig. 2A**). These data confirm the importance of small non-polar residues positioned upstream of the GRM.

Our results so far had indicated that the position of the FlgD N-terminal hydrophobic export signal relative to the GRM was critical and suggested that, for export to occur efficiently, at least 26 residues must separate the hydrophobic signal and the GRM (**Fig 1C**). In the primary sequences of all *Salmonella* flagellar rod/hook subunits the GRM is positioned ≥ 30 amino acids downstream of the subunit N-terminus (**Fig S1**), suggesting that separation of the two signals by a minimum number of residues might be a common feature among early flagellar subunits. To test this, a suite of engineered *flgD* alleles was constructed that encoded FlgD variants in which wildtype residues 9-32 were replaced with between one and four repeats of the six

amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser (GSTNAS). Swimming motility and export assays revealed that the minimum number of inserted GSTNAS repeats that could support efficient FlgD export was three, equivalent to separation of the hydrophobic N-terminal signal and the GRM by 24 residues (**Fig. 2B**; **Fig. S5**). Below this threshold, FlgD export and swimming motility were strongly attenuated (**Fig. 2B**). A further set of recombinant *flgD* alleles was constructed, which encoded FlgD Δ 9-32 variants carrying two GSTNAS repeats (hereafter termed FlgD_{short}) directly followed by between one and five additional residues (**Fig. 2C**; **Fig. S5**). Motility and FlgD export increased incrementally with the addition of each amino acid (**Fig. 2C**). The data indicate that a low level of FlgD export is supported when the hydrophobic N-terminal signal (₂SIAV₅) and the GRM (₃₆FLTLL₄₀) are separated by 19 residues, with export efficiency and swimming motility increasing as separation of the export signals approaches the optimal 30 residues.

To further establish the requirement for a minimum number of residues between the hydrophobic N-terminal signal and the GRM, we screened for intragenic suppressor mutations that could restore swimming motility in a *flgD* null strain producing FlgD_{short}. Sequencing of *flgD*_{short} alleles from motile revertant strains identified 12 gain-of-function mutations that introduced additional residues between the hydrophobic N-terminal signal and the GRM (**Fig. S6**). Swimming motility and FlgD export was assessed for three *flgD* null strains expressing representative *flgD*_{short} gain-of-function variants and all showed increased FlgD subunit export and swimming motility compared to the *flgD* null expressing *flgD*_{short} (**Fig. 2D**; **Fig. S6**).

The data confirm that the position of the hydrophobic N-terminal signal relative to the GRM is critical for efficient FlgD subunit export.

To establish that this is a general requirement for the export of other rod and hook subunits, engineered alleles of *flgE* (hook) and *flgG* (rod) were constructed that encoded variants in which FlgE residues 9-32 or FlgG residues 11-35 were either deleted (FlgE_{short} and FlgG_{short}) or replaced with four repeats of the sequence GSTNAS (**Fig. 3; Fig. S7**). As had been observed for FlgD_{short}, export of the FlgE_{short} and FlgG_{short} variants was severely attenuated compared to wild type FlgE and FlgG (**Fig. 3**). Furthermore, insertion of four GSTNAS repeats into FlgE_{short} and FlgG_{short} recovered subunit export to wild type levels, indicating that the minimum separation of the hydrophobic N-terminal signal and the GRM is a feature throughout rod and hook subunits (**Fig. 3**).

Sequential engagement of the subunit GRM and hydrophobic N-terminal export signal by the flagellar export machinery

Having identified a new hydrophobic N-terminal export signal and established that its position relative to the GRM was critical, we next wanted to determine the order in which the signals were recognised/engaged by the export machinery. The signals might be recognised simultaneously, with both being required for initial entry of rod/hook subunits into the export pathway. Alternatively, they might be recognised sequentially. If this were the case, then a subunit variant that possessed the ‘first’ signal but was deleted for the ‘second’ signal might enter the export pathway but fail to progress, becoming stalled at a specific step to block the pathway and prevent

export of wild type subunits. To test if FlgD Δ 2-5 or FlgD Δ GRM stalled in the export pathway, recombinant expression vectors encoding these variants or wild type FlgD were introduced into a *Salmonella* Δ recA strain that is wild type for flagellar export (**Fig. 4**). We could then assess whether the variant FlgD constructs could interfere *in trans* with the wild type flagellar export. We saw that FlgD Δ 2-5 inhibited motility and export of the FliK and FlgK flagellar subunits, whereas FlgD Δ GRM did not (**Fig. 4B and C**). The data indicate that FlgD Δ 2-5 enters the flagellar export pathway and stalls at a critical point, blocking export. In contrast, FlgD Δ GRM does not stall or block export.

To determine whether FlgD Δ 2-5 stalls at a point before or after subunit docking at the FlhB_C component of the flagellar export gate *via* the GRM, a recombinant vector encoding a FlgD variant in which both export signals were deleted (FlgD Δ 2-5 Δ GRM) was constructed. If loss of the hydrophobic N-terminal signal had caused subunits to stall after docking at FlhB_C, then additional deletion of the subunit GRM would relieve this block. Motility and subunit export assays revealed that the *Salmonella* Δ recA strain producing FlgD Δ 2-5 Δ GRM displayed swimming motility and levels of FliK and FlgK subunit export similar to cells producing FlgD Δ GRM (**Fig. 4B and C**). The data suggest that FlgD Δ 2-5 stalls after docking at the FlhB_C export gate, preventing docking of other early subunits.

It seemed possible that subunit docking *via* the GRM to the FlhB_C export gate might position the hydrophobic N-terminal signal in close proximity to its recognition site on the export machinery. If this were the case, ‘short’ subunit variants containing

deletions that decreased the number of residues between the hydrophobic N-terminal signal and the GRM might also stall at FlhB_C, and this stalling might be relieved by additional deletion of the GRM. To test this, recombinant expression vectors encoding 'short' subunit variants (FlgE_{short} or FlgD_{short}), 'short' subunit variants additionally deleted for the GRM (FlgE_{short}ΔGRM or FlgD_{short}ΔGRM) or wild type FlgE or FlgD were introduced into a *Salmonella* Δ*recA* strain (**Fig 5**). Compared to the wild type subunits expressed *in trans*, the 'short' subunits inhibited swimming motility and the export of other flagellar subunits (FliD, FliK, FlgK), whereas FlgE_{short}ΔGRM and FlgD_{short}ΔGRM did not (**Fig 5**). Taken together with the data presented in Figure 4, the results indicate that the subunit GRM and the hydrophobic N-terminal signal are recognised sequentially, with subunits first docking at FlhB_C *via* the GRM, which positions the hydrophobic N-terminal signal for subsequent interactions with the export machinery (**Fig 4D**).

Mutations that promote opening of the export gate partially compensate for incorrect positioning of the subunit N-terminal export signal

The accruing data indicated that subunit docking at FlhB_C might correctly position the hydrophobic N-terminal signal for recognition by the export machinery. To model the position of FlhB_C relative to other components of the export machinery, we placed the structures of FlhB_C and the FliPQR-FlhB_N export gate into the tomographic reconstruction of the *Salmonella* SPI-1 type III secretion system (**Fig 6B**; [31]). The model indicated that FliPQR-FlhB_N and the subunit docking site on FlhB_C are separated by a minimum distance of ~78 Å, and that FlhB_C is positioned no more than ~22-45 Å from FlhA (Fig 6A; [27]). Taking FlgD as a model early flagellar

subunit, the distance between the FlgD N-terminal hydrophobic signal and the GRM was found to be in the range of ~ 45 Å (α -helix) to ~ 105 Å (unfolded contour length), depending on the predicted structure adopted by the subunit N-terminus (Fig 6A). Based on these estimates, it seemed feasible that the hydrophobic N-terminal signal of a subunit docked at FlhB_C could contact either FlhA or the FlhPQR-FlhB_N complex, and that this interaction might trigger opening of the export gate [27],[30],[22]. If this were true, mutations that promote the open conformation of the export gate might compensate for the incorrect positioning of the hydrophobic N-terminal export signal in 'short' rod/hook subunits. One such export gate mutation, FlhP-M₂₁₀A, has been shown to increase ion conductance across the bacterial inner membrane, indicating that this gate variant fails to close efficiently [29].

To test whether the FlhP-M₂₁₀A variant gate could promote export of 'short' subunits, in which the distance between the hydrophobic N-terminal signal and the GRM was reduced, a recombinant expression vector encoding FlgD_{short} was introduced into *Salmonella flgD* null strains in which the *fliP* gene had been replaced with recombinant genes encoding either a functional FlhP variant with an internal HA-tag (designated wild type gate) or the equivalent HA-tagged FlhP-M₂₁₀A variant (designated M₂₁₀A gate; **Fig. 6C**; **Fig. S8**). The swimming motility of these strains was found to be consistently stronger in the strain producing the M₂₁₀A gate compared to the strain with the wild type gate, with the motility halo of the *fliP*-M₂₁₀A- Δ *flgD* strain expressing FlgD_{short} having a 50% greater diameter than that of the wild type *fliP*- Δ *flgD* strain expressing FlgD_{short} (**Fig. 6D**). This increase in motility indicated that the defect caused by incorrect positioning of the hydrophobic N-

terminal signal relative to the GRM in FlgD_{short} could indeed be partially compensated by promoting the gate open conformation.

Discussion

T3SS substrates contain N-terminal export signals, though these signals have not been fully defined and how they promote subunit export remains unclear. Here, we characterised a new hydrophobic N-terminal export signal in early flagellar rod/hook subunits and showed that the position of this signal relative to the known subunit gate recognition motif (GRM) is key to export.

Loss of the hydrophobic N-terminal signal in the hook cap subunit FlgD had a stronger negative effect on subunit export than deletion of the GRM that enables subunit docking at FlhB_C, suggesting that the hydrophobic N-terminal signal may be required to trigger an essential export step. A suppressor screen showed that the export defect caused by deleting the hydrophobic N-terminal signal could be overcome by mutations that either reintroduced small non-polar amino acids positioned 3-7 residues from the subunit N-terminus (e.g. M7I), or introduced additional residues between V₁₅ and the GRM. In such 'gain of function' strains containing insertions, changing V₁₅ to alanine abolished subunit export, which was rescued by re-introduction of small non-polar residues close to the N-terminus. These data point to an essential export function for small non-polar residues close to the N-terminus of rod/hook subunits.

It was fortuitous that we chose FlgD as the model for early flagellar subunit. All early subunits contain small hydrophobic residues close to the N-terminus, but FlgD is unique in that only four (I₃, A₄, V₅ and V₁₅) of its first 25 residues are small and non-polar (**Fig. S1**). Indeed, there are only three other small hydrophobic residues between the FlgD N-terminus and the GRM (**Fig. 1**). While deletion of residues 2-5 in FlgD removes the critical hydrophobic N-terminal signal, similar deletions in the N-terminal regions of other rod/hook subunits reposition existing small non-polar residues close to the N-terminus (**Fig. S1**). This is perhaps why previous deletion studies in early flagellar subunits have failed to identify the hydrophobic N-terminal signal [14],[37],[38].

The finding that subunits lacking the hydrophobic N-terminal export signal, but not the GRM, stalled during export suggested that these two signals were recognised by the flagellar export machinery in a specific order. Mutant variants of other flagellar export substrates or export components have been observed to block the export pathway. For example, a FlgN chaperone variant lacking the C-terminal 20 residues stalls at the Flil ATPase [39], while a GST-tagged FliJ binds FlhA but is unable to associate correctly with Flil so blocking wild type FliJ interaction with FlhA [40]. These attenuations can be reversed by further mutations that disrupt the stalling interactions. This was also observed for FlgD Δ 2-5. Loss of the hydrophobic N-terminal signal resulted in a dominant-negative effect on motility and flagellar export, but this was abolished by subsequent deletion of the GRM. This indicates that FlgD Δ 2-5 stalls in the export pathway at FlhB_C, blocking the binding site for early flagellar subunits. These data are consistent with sequential recognition of the two

export signals: the GRM first docking subunits at FlhB_C, and positioning the hydrophobic N-terminal signal to trigger the next export step.

The position of the subunit hydrophobic N-terminal export signal relative to the GRM appears critical for export. Engineering of *flgD* to encode variants in which the region between the N-terminus and the GRM was replaced with polypeptide sequences of varying lengths showed that these signals must be separated by a minimum of 19 residues for detectable export, with substantial export requiring separation by 30 residues (**Fig. 3**). When subunits dock at FlhB_C, which is likely situated within or just below the plane of the inner membrane, the hydrophobic N-terminal signal is positioned close to the FlhAB-FlhPQR export gate (**Fig. 6B**). Subunits in which the GRM and N-terminal signal are brought closer together stall at FlhB_C, suggesting that the hydrophobic N-terminal signal is unable to contact its recognition site on the export machinery (**Figs. 5, 6A and 6B**). In all flagellar rod/hook subunits, the GRM is positioned at least 30 residues from the N-terminus (**Fig. S1**). The physical distance between the two signals will depend on the structure adopted by the subunit N-terminus (**Fig. 6A**). The N-terminal region of flagellar subunits is often unstructured in solution [11-15],[32], and such disorder may be an intrinsic feature of flagellar export signals [11-15],[33],[34], as is typical in other bacterial N-terminal export signals such as those in substrates of the Sec and Tat systems [35],[36].

Unstructured signals may facilitate multiple interactions with different binding partners during export, and in the case of export systems that transport unfolded proteins they may aid initial entry of substrates into narrow export channels [35].

As yet, nothing is known about the structure of the subunit N-terminal domain upon interaction with the flagellar export machinery. Signal peptides in TAT pathway substrates switch between disordered and α -helical conformations depending on the hydrophobicity of the environment [41]. It therefore seems likely that local environments along the flagellar export pathway will influence the conformation of subunit export signals [42],[22]. In flagellar subunits, if the region between the hydrophobic N-terminal signal and the GRM is unstructured and extended, this would correspond to a polypeptide contour length of approximately 72-105 Å (where the length of one amino acid is ~ 3.6 Å). If the same region were to fold as an α -helix, its length would be approximately 30-36 Å (where one amino acid rises every ~ 1.5 Å). Without further structural information on subunit interactions with the flagellar export machinery and the precise position of FlhB_C within the machinery, it is difficult to determine precisely where the hydrophobic N-terminal signal contacts the machinery.

We speculate that one function of the subunit hydrophobic N-terminal signal might be to trigger opening of the FlhAB-FliPQR export gate, which rests in an energetically favourable closed conformation to maintain the permeability barrier across the bacterial inner membrane [29],[22],[30],[27]. The atomic resolution structure of FliPQR showed that it contains three gating regions [22]. FliR provides a loop (the R-plug) that sits within the core of the structure. Below this, five copies of FliP each provides three methionine residues that together form a methionine-rich ring under which ionic interactions between FliQ residues hold the base of the structure shut (Q-latch).

A recent structure of a substrate trapped within the vT3SS revealed conformational changes within the export gate [43]. These changes include a rearrangement of the M-gate methionine residues and folding up of the FliR plug to allow substrate passage. If the function of the subunit hydrophobic N-terminal signal was to trigger opening of this gate, we hypothesised that mutations which destabilised the gate's closed conformation would suppress the motility defect associated with FlgD_{short}, in which the distance between the N-terminal signal and the GRM is reduced. Introduction of the FliP-M₂₁₀A mutation, which partially destabilises the gate's closed state, did indeed partly suppress the FlgD_{short} motility defect. We did not find export gate variants that completely destabilised gate closure, but it may be that such mutations disrupt the membrane permeability barrier [29]. This could also explain why in screens for suppressors of FlgD Δ 2-5 or FlgD_{short} we did not isolate mutations in genes encoding export gate components (data not shown).

The surface-exposed hydrophobic GRM-binding pocket on FlhB_C is well conserved across the T3SS SctU family, to which FlhB belongs [14],[44],[45]. Furthermore, the GRM is conserved in all four injectosome early subunits (SctI, SctF, SctP, OrgC) and is located at least 30 residues away from small hydrophobic residues near the subunit N-terminus. It therefore seems plausible that the 'dual signal' mechanism we propose for early flagellar export operates in all T3SS pathways.

Based on the genetic and biochemical work presented here, we propose a model of sequential recognition of export signals at the T3SS export machinery. Early substrates engage with the GRM-binding pocket on FlhB_C, positioning the subunit N-

terminal hydrophobic signal to contact its recognition site. Given that FlhB_C is positioned directly below the FlpQR-FlhB_N gate, the N-terminal hydrophobic signal of substrates docked at FlhB_C would be suitably positioned to trigger export gate opening. Based on the location of FlhB_C and the relative position between export signals, the N-terminal hydrophobic signal could trigger gate opening by indirect interactions with FlhA/FlhB or *via* direct interactions with the FlpQR-FlhB_N export gate components.

In many other pathways, the presence of a substrate triggers opening or assembly of the export channel. The outer membrane chitin transporter in *Vibrio* adopts a closed conformation in which the N-terminus of a neighbouring subunit acts as a pore plug [46]. Chitin binding to the transporter ejects the plug, opening the transport channel and allowing chitin transport [45]. In the Sec pathway, interactions of SecA, ribosomes or pre-proteins with SecYEG can induce conformational changes that promote channel opening [47-49],[35]. In the TAT system, which transports folded substrates across the cytoplasmic membrane, substrate binding to the TatBC complex triggers association with, and subsequent polymerisation of, TatA, which is required for substrate translocation [50],[36]. All of these mechanisms serve both to conserve energy and prevent disruption of the membrane permeability barrier. Our data suggest that in a comparable way the signal of non-polar residues within the N-termini of early rod/hook subunits trigger export gate opening.

Methods

Bacterial strains, plasmids and growth conditions

Salmonella strains and plasmids used in this study are listed in **Supplementary**

Table 1. The $\Delta flgD::K_m^R$ strain in which the *flgD* gene was replaced by a kanamycin

resistance cassette was constructed using the λ Red recombinase system [58].

Strains containing chromosomally encoded FliP variants were constructed by aph-I-

Scel Kanamycin resistance cassette replacement using pWRG730 [59].

Recombinant proteins were expressed in *Salmonella* from the isopropyl β -D-

thiogalactoside-inducible (IPTG) inducible plasmid pTrc99a [60]. Bacteria were

cultured at 30–37 °C in Luria-Bertani (LB) broth containing ampicillin (100 μ g/ml).

Flagellar subunit export assay

Salmonella strains were cultured at 37 °C in LB broth containing ampicillin and IPTG

to mid-log phase (OD_{600nm} 0.6-0.8). Cells were centrifuged (6000g, 3 min) and

resuspended in fresh media and grown for a further 60 min at 37 °C. The cells were

pelleted by centrifugation (16,000g, 5 min) and the supernatant passed through a 0.2

μ m nitrocellulose filter. Proteins were precipitated with 10% trichloroacetic acid

(TCA) and 1% Triton-X100 on ice for 1 hour, pelleted by centrifugation (16,000g, 10

min), washed with ice-cold acetone and resuspended in SDS-PAGE loading buffer

(volumes calibrated according to cell densities). Fractions were analysed by

immunoblotting.

Motility assays

For swimming motility, cultures were grown in LB broth to A600nm 1. Two microliters of culture were inoculated into soft tryptone agar (0.3% agar, 10 g/L tryptone, 5g/L NaCl) containing ampicillin (100 µg/ml). Plates were incubated at 37 °C for between 4 and 6 hours unless otherwise stated.

Isolation of motile strains carrying suppressor mutations

Cells of the *Salmonella flgD* null strain transformed with plasmids expressing FlgD variants (FlgDΔ2-5, FlgDΔ2-5-¹⁹GSGSMT²⁰-V15A or FlgD_{short}) were cultured at 37 °C in LB broth containing ampicillin (100 µg/ml) to mid-log phase and inoculated into soft tryptone agar (0.3% agar, 10 g/L tryptone, 5g/L NaCl) containing ampicillin (100 µg/ml). Plates were incubated at 30 °C until motile ‘spurs’ appeared. Cells from the spurs were streaked to single colony and cultured to isolate the *flgD* encoding plasmid. Plasmids were transformed into the *Salmonella flgD* null strain to assess whether the plasmids were responsible for the motile suppressor phenotypes. Plasmids were sequenced to identify the suppressor mutations.

Quantification and statistical analysis

Experiments were performed at least three times. Immunoblots were quantified using Image Studio Lite. The unpaired two-tailed Student’s *t*-test was used to determine *p*-values and significance was determined as **p* < 0.05. Data are represented as mean ± standard error of the mean (SEM), unless otherwise specified and reported as biological replicates.

Author contributions

Owain J. Bryant: Conceptualization, Data curation, Investigation, Formal analysis, Methodology, Visualisation, Writing – original draft, Writing – review & editing.

Paraminder Dhillon: Conceptualization, Data curation, Investigation, Formal analysis, Methodology, Visualisation, Writing – review & editing. **Colin Hughes:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualisation, Writing – review & editing. **Gillian M. Fraser:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualisation, Writing – original draft, Writing – review & editing.

Competing interests

The authors declare no competing interests.

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Materials & Correspondence

Materials are available from the corresponding author upon request.

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Figure legends

Figure 1. Screening for export-defective FlgD variants

a. Whole cell (cell) and supernatant (sec) proteins from late exponential phase cultures of a *Salmonella flgD* null strain expressing wild type FlgD (FlgD) or its variants ($\Delta 2$ -5, $\Delta 6$ -10, $\Delta 11$ -15, $\Delta 16$ -20, $\Delta 21$ -25, $\Delta 26$ -30, $\Delta 31$ -35, $\Delta 36$ -40, $\Delta 41$ -45 or $\Delta 46$ -50) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera.

b. A schematic displaying the intragenic suppressor mutations within amino acids 1-40 of FlgD isolated from the FlgD $\Delta 2$ -5 variant. Small non-polar residues are highlighted in orange. All suppressor mutations were located between the gate-recognition motif (GRM, blue) and the extreme N-terminus, and can be separated into two classes: insertions or duplications that introduced additional sequence between valine-15 and the gate-recognition motif, or missense mutations that re-introduce small non-polar residues at the N-terminus.

c. Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of *Salmonella flgD* null strains expressing suppressor mutants isolated from the FlgD $\Delta 2$ -5 variant (FlgD $\Delta 2$ -5-N₈I or FlgD $\Delta 2$ -5-T₁₁I), FlgD $\Delta 2$ -5 variant (-) or wild type FlgD (FlgD) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera.

d. Whole cell (cell) and supernatant (sec) proteins from late exponential phase cultures of *Salmonella flgD* null strains expressing wild type FlgD (FlgD), FlgD $\Delta 2$ -5 ($\Delta 2$ -5) or variants of FlgD $\Delta 2$ -5 containing between residues 19 and 20 a six-residue insertion of either small non-polar (AGAGAG) residues (3x(AG)), polar

(STSTST) residues (3x(ST)), or the sequence from an isolated insertion suppressor mutant (GSGSMT), were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera.

Figure 2. Export of FlgD variants in which the position of the N-terminal hydrophobic export signal is varied relative to the gate recognition motif (GRM)

Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing suppressor mutants isolated from the FlgD Δ 2-5⁻¹⁹(GSGSMT)²⁰-V₁₅A variant (V₁₅A-M7I, V₁₅A-D9A, V₁₅A-T₁₁I, V₁₅A-G₁₄V), their parent FlgD variant FlgD Δ 2-5⁻¹⁹(GSGSMT)²⁰-V₁₅A (labelled as V₁₅A), FlgD Δ 2-5⁻¹⁹(GSGSMT)²⁰ (labelled as -) or wild type FlgD (FlgD) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains was assayed at 37°C for 4-6 hours.

b. Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgD (FlgD), FlgD Δ 9-32 or its variants in which residues 9-32 were replaced by between one and four six-residue repeats of Gly-Ser-Thr-Asn-Ala-Ser (Δ 9-32 4xRpt, Δ 9-32 3xRpt, Δ 9-32 2xRpt or Δ 9-32 1xRpt) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains was assayed at 37°C for 4-6 hours.

c. Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgD (FlgD), a FlgD variant in which residues 9-32 were replaced by two repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (labelled as 2xRpt) or its variants containing between one and five additional residues inserted directly after the two repeats (2xRpt+1, 2xRpt+2, 2xRpt+3, 2xRpt+4 or 2xRpt+5) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains was assayed at 37°C for 4-6 hours.

d. Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgD (FlgD), a FlgD variant in which residues 9-32 were replaced by two repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (FlgD_{short}) or suppressor mutants isolated from this strain (rev1, rev2 or rev3) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains were was assayed at 37°C for 4-6 hours.

e. N-terminal sequences of wild type FlgD, a FlgD variant in which residues 9-32 are replaced by two repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (yellow; FlgD_{short}) and its suppressor mutant revertants (rev 1-3) aligned at the gate recognition motif (GRM, blue). Suppressor mutants contained insertions (underlined) that introduced additional residues between the N-terminal hydrophobic signal (orange) and the gate recognition motif (blue).

Figure 3. Effect of the relative position of the N-terminus and GRM on the export of rod and hook subunits

a. Schematic representation of a wild type subunit (subunit_{wild type}), a subunit containing a deletion of sequence between the N-terminus and GRM (subunit_{short}) and a subunit in which the deleted sequence was replaced by four repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (yellow, subunit_{short+4Rpt}).

b. Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgE* null strain expressing wild type FlgG (FlgG_{wild type}), a FlgG variant in which residues 11-35 were deleted (FlgG_{short}) or a FlgG variant in which residues 11-35 were replaced by four repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (FlgG_{short+4Rpt}). All FlgG variants were engineered to contain an internal 3xFLAG tag for immunodetection. Proteins were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FLAG monoclonal antisera. Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains was assayed at 37°C for 4-6 hours.

c. Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgE (FlgE_{wild type}), a FlgE variant in which residues 9-32 were deleted (FlgE_{short}) or a FlgE variant in which residues 9-32 were replaced by four repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (FlgE_{short+4Rpt}). All FlgE variants were engineered to contain an internal 3xFLAG tag for immunodetection. Proteins were separated by SDS (15%)-

PAGE and analysed by immunoblotting with anti-FLAG monoclonal antisera.

Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains was assayed at 37°C for 4-6 hours.

Figure 4. Effect on subunit export of overexpression of FlgDΔ2-5 and variants.

a. Schematic representation of a FlgD subunit containing a N-terminal hydrophobic signal (orange, 2-5) and a gate-recognition motif (blue, GRM).

b. Swimming motility of a *Salmonella* Δ*recA* strain expressing wild type FlgD (FlgD), its variants (DΔ2-5ΔGRM, DΔ2-5 or DΔGRM) or empty pTrc99a vector (-). Motility was assessed in 0.25% soft-tryptone agar containing 100 μg/ml ampicillin and 100μM IPTG and incubated for 4-6 hours at 37°C.

c. Whole cell (cell) and secreted proteins (secreted) from late-exponential-phase cultures were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FliK, anti-FlgK, anti-FlgL, anti-FlhA or anti-FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).

d. A model depicting a FlgDΔ2-5 subunit (left) docked *via* its gate recognition motif (GRM, blue) at the subunit binding pocket on FlhB_C (PDB: 3B0Z[31], red), preventing wild type subunits (right) from docking at FlhB_C.

Figure 5. Effect on subunit export of overexpressed FlgE_{short}, FlgD_{short} and variants

a. Swimming motility of a *Salmonella* $\Delta recA$ strain expressing wild type FlgE (FlgE wild type), a FlgE variant in which residues 9-32 were deleted (FlgE_{short}), a FlgE variant in which residues 9-32 and residues 39-43 (corresponding to the gate-recognition motif) were deleted (FlgE_{short} Δ GRM), a FlgE variant in which residues 39-43 were deleted (FlgE Δ GRM) or empty pTrc99a vector (-). All FlgG variants were engineered to contain an internal 3xFLAG tag for immunodetection. Motility was assessed in 0.25% soft-tryptone agar containing 100 μ g/ml ampicillin and 100 μ M IPTG and incubated for 4-6 hours at 37°C (top panel). Whole cell (cell) and secreted proteins (secreted) from late-exponential-phase cultures were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FLAG monoclonal antisera or anti-FlgD, anti-FliD, anti-FlgK, anti-FliK, anti-FlhA or anti-FlgN polyclonal antisera (bottom). Apparent molecular weights are in kilodaltons (kDa).

b. Swimming motility of a *Salmonella* $\Delta recA$ strain expressing wild type FlgD (FlgD wild type), a FlgD variant in which residues 9-32 were replaced with two repeats of the six amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser (FlgD_{short}), a FlgD variant in which residues 9-32 were replaced with two repeats of the six amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser and residues 36-40 were deleted (FlgD_{short} Δ GRM), a FlgD variant in which residues 36-40 were deleted (FlgD Δ GRM) or empty pTrc99a vector (-). Motility was assessed in 0.25% soft-tryptone agar containing 100 μ g/ml ampicillin and 100 μ M IPTG and incubated for 4-6 hours at 37°C (top panel). Whole cell (cell) and secreted proteins (secreted) from late-exponential-phase cultures were

separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD, anti-FliD, anti-FlgK, anti-FliK, anti-FlhA or anti-FlgN polyclonal antisera (bottom). Apparent molecular weights are in kilodaltons (kDa).

Figure 6. Suppression of the FlgD_{short} motility defect by mutations in FliP

a. A model depicting subunits docked *via* their gate-recognition motif (GRM, blue) at the subunit binding pocket on FlhB_C (PDB: 3B0Z [31], red) with N-termini of early flagellar subunits adopting either an α -helical conformation separating the N-terminal hydrophobic signal (2-5, orange) and gate-recognition motif (GRM, blue) by ~40-60 Ångstrom (where each amino acid is on average separated by ~1.5Å, left) or an unfolded conformation where the polypeptide contour length separating the N-terminal hydrophobic signal (2-5) and gate-recognition motif (GRM) is ~90-150 Ångstrom (where each amino acid is on average separated by ~3.5Å, middle left). Values corresponding to the distance separating the N-terminal hydrophobic signal (2-5, orange) and gate-recognition motif (GRM, blue) of a FlgD subunit variant in which residues 9-32 are replaced with two repeats of the six amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser (FlgD_{short}) indicate that the N-terminal hydrophobic signal (2-5, orange) and gate-recognition motif (GRM, blue) are separated by ~29 Ångstrom (α -helical conformation, middle right) or ~67 Ångstrom (unfolded polypeptide contour length, right).

b. Placement of the crystal structure of FlhB_C (PDB:3B0Z [31]; red) and the cryo-EM structure of FliPQR-FlhB (PDB:6S3L[30]) in a tomographic reconstruction of the *Salmonella* SPI-1 injectisome (EMD-8544 [60]; grey). The minimum distance between the subunit gate recognition motif binding site on FlhB_C (grey) to FlhB_N

(defined as *Salmonella* FlhB residue 211 [62]; $\sim 78\text{\AA}$) was estimated by combining:
the value corresponding to the distance between the subunit binding pocket on
FlhB_C [14] (grey) and the N-terminal visible residue (D₂₂₉) in the FlhB_C structure
(PDB:3BOZ [31]; $\sim 52\text{\AA}$) with the value corresponding to the minimum distance
between FlhB residues 211 and 228 (based on a linear α -helical conformation;
 $\sim 26\text{\AA}$).

c. Swimming motility of recombinant *Salmonella flgD* null strains producing a
chromosomally-encoded FliP-M₂₁₀A variant (M₂₁₀A gate, left) or wild type FliP (wild
type gate, right). Wild type FliP and FliP-M₂₁₀A were engineered to contain an
internal HA tag positioned between residue 21 and 22 to allow immunodetection of
FliP. Both strains produced either a pTrc99a plasmid-encoded FlgD subunit variant
in which residues 9-32 were replaced with two repeats of the six amino acid
sequence Gly-Ser-Thr-Asn-Ala-Ser (FlgD_{short}; top panel) or a pTrc99a plasmid-
encoded wild type FlgD subunit (FlgD_{wild type}; bottom panel). Motility was assessed in
0.25% soft-tryptone agar containing 100 $\mu\text{g/ml}$ ampicillin and 50 μM IPTG and
incubated for 16 hours (top panel) or 4-6 hours at 37°C (bottom panel).

d. The mean motility halo diameter of a recombinant *Salmonella flgD* null strain
producing chromosomally-encoded FliP-M₂₁₀A (M₂₁₀A gate, left) and expressing the
FlgD short variant (left hand bar) was plotted as a percentage of the mean motility
halo diameter of the wild type FliP gate strain (wild type) producing FlgD_{short} (right
hand bar). Error bars show the standard error of the mean calculated from four
biological replicates. * indicates a p-value < 0.05.

Supplementary Figure Legends

Figure S1.

a. N-terminal sequences of all *Salmonella* flagellar rod and hook subunits aligned at their gate recognition motif (GRM, blue). Small non-polar residues upstream of the gate recognition motif are highlighted (yellow).

b. Hydrophobicity plots for the N-terminal 60 residues of each *Salmonella* flagellar rod and hook subunit were generated by ExPASy tools using the Kyte and Doolittle method [62]. The x axis of the plot indicates the amino acid position, starting from the N terminus. The y axis of the plot indicates the hydrophobicity of the amino acid sequence, where higher values represent higher hydrophobicity. Amino acid sequence corresponding to the gate-recognition motif of each subunit is highlighted in blue.

Figure S2

a. Swimming motility of a *Salmonella* $\Delta recA$ strain expressing suppressor mutants isolated from the FlgD Δ 2-5 variant (FlgD Δ 2-5 N₈₁ or FlgD Δ 2-5 T₁₁₁), the parent FlgD Δ 2-5 variant (D Δ 2-5) or wild type FlgD (FlgD). Motility was assessed in 0.25% soft-tryptone agar containing 100 μ g/ml ampicillin and 50 μ M IPTG and incubated 4-6 hours at 37°C.

b. Swimming motility of a *Salmonella* $\Delta recA$ strain expressing wild type FlgD (FlgD), FlgD Δ 2-5 (D Δ 2-5) or its variants containing a six-residue insertion between residues 19 and 20 of either small non-polar (AGAGAG) residues (D Δ 2-5 3x(AG)), polar

(STSTST) residues (DΔ2-5 3x(ST)), or the sequence from an isolated insertion suppressor mutant (GSGSMT) (DΔ2-5 GSGSMT). Motility was assessed in 0.25% soft-tryptone agar containing 100 µg/ml ampicillin and 50 µM IPTG and incubated 4-6 hours at 37°C.

Figure S3

a. Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of *Salmonella flgD* null strains expressing: suppressor mutants isolated from the FlgDΔ2-5 variant (FlgDΔ2-5 N₈I or FlgDΔ2-5 T₁₁I), FlgDΔ2-5 variant (-) or wild type FlgD (FlgD) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlhA or FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).

b. Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of *Salmonella flgD* null strains expressing wild type FlgD (FlgD), FlgDΔ2-5 (DΔ2-5) or its variants containing a six-residue insertion between residues 19 and 20 of either small non-polar (AGAGAG) residues (DΔ2-5 3x(AG)), polar (STSTST) residues (DΔ2-5 3x(ST)), or the sequence from an isolated insertion suppressor mutant (GSGSMT) (DΔ2-5 GSGSMT) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlgD polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).

Figure S4

a. Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing suppressor mutants isolated from a FlgD Δ 2-5-¹⁹(GSGSMT)²⁰-V₁₅A variant (V₁₅A-M₇I, V₁₅A-D₉A, V₁₅A-T₁₁I, V₁₅A-G₁₄V), their parent FlgD variant FlgD Δ 2-5-¹⁹(GSGSMT)²⁰-V₁₅A (V₁₅A), FlgD Δ 2-5-¹⁹(GSGSMT)²⁰ (-) or wild type FlgD (FlgD) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlhA and FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).

Figure S5

a. Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgD (FlgD), FlgD Δ 9-32 or its variants in which residues 9-32 were replaced by between one and four six-residue repeats of Gly-Ser-Thr-Asn-Ala-Ser (GSTNAS): (Δ 9-32 4xRpt, Δ 9-32 3xRpt, Δ 9-32 2xRpt, Δ 9-32 1xRpt) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlhA and anti-FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).

b. Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgD (FlgD), a FlgD variant in which residues 9-32 were replaced by two repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (2xRpt) or its variants containing between one and five additional residues inserted directly after the two repeats (2xRpt+ 1, 2xRpt+ 2, 2xRpt+ 3, 2xRpt+ 4 or 2xRpt+ 5) were separated by SDS (15%)-PAGE and

analysed by immunoblotting with anti-FlgD polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).

Figure S6

a. Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgD (FlgD), a FlgD variant in which residues 9-32 were replaced by two repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (FlgD_{short}) or suppressor mutants isolated from this strain (rev1, rev2 or rev3) were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlhA and anti-FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).

b. N-terminal sequences of wild type FlgD (wild type), a FlgD variant in which residues 9-32 are replaced by two repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (yellow; FlgD_{short}) and its suppressor mutants (rev 1-7) aligned to their gate-recognition motif (GRM, blue). Suppressor mutants contained insertions (underlined) that introduced additional residues between the N-terminal hydrophobic signal (orange) and the gate-recognition motif (blue).

Figure S7

a. Whole cell (cell) and supernatant (sec) proteins from late exponential-phase cultures of a *Salmonella flgE* null strain expressing wild type FlgG (FlgG_{wild type}), a FlgG variant in which residues 11-35 were deleted (FlgG_{short}) or a FlgG variant in which residues 11-35 were replaced by four repeats of a six-residue sequence Gly-

Ser-Thr-Asn-Ala-Ser (FlgG_{short}+4Rpt). All FlgG variants were engineered to contain an internal 3xFLAG tag for immunodetection. Proteins were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FlhA or anti-FlgN polyclonal antisera. Apparent molecular weights are in kilodaltons (kDa).

b. Whole cell (cell) and supernatant (secreted) proteins from late exponential-phase cultures of a *Salmonella flgD* null strain expressing wild type FlgE (FlgE_{wild type}), a FlgE variant in which residues 9-32 were deleted (FlgE_{short}) or a FlgE variant in which residues 9-32 were replaced by four repeats of a six-residue sequence Gly-Ser-Thr-Asn-Ala-Ser (FlgE_{short}+4Rpt). All FlgE variants were engineered to contain an internal 3xFLAG tag for immunodetection. Proteins were separated by SDS (15%)-PAGE and analysed by immunoblotting with anti-FLAG monoclonal antisera. Swimming motility (bottom panel; 0.25% soft tryptone agar) of the same strains were carried out at 37°C for 4-6 hours. Apparent molecular weights are in kilodaltons (kDa).

Figure S8

a. Whole cell (cell) proteins from late exponential-phase cultures of recombinant *Salmonella flgD* null strains producing a chromosomally-encoded FliP-M₂₁₀A variant (M₂₁₀A gate, left) or wild type FliP (wild type gate, right). Wild type FliP and FliP-M₂₁₀A were engineered to contain an internal HA tag positioned between residue 21 and 22 to allow immunodetection of FliP [29] (bottom panel). Both strains produced either a pTrc99a plasmid-encoded FlgD subunit variant in which residues 9-32 were replaced with two repeats of the six amino acid sequence Gly-Ser-Thr-Asn-Ala-Ser

976 (FlgD_{short}; top panel) or a pTrc99a plasmid-encoded wild type FlgD subunit (FlgD_{wild}
 977 type; middle panel). Proteins were separated by SDS (15%)-PAGE and analysed by
 978 immunoblotting with anti-FlgD polyclonal antisera or anti-HA monoclonal antisera.

Figure 1

Figure 2

Figure 3

Figure 5

Figure 6