The substrate specificity switch FlhB assembles onto the export gate to regulate type three secretion

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Protein secretion through type-three secretion systems (T3SS) is critical for motility and virulence of many bacteria. Proteins are transported through an export gate containing three proteins (FliPQR in flagella, SctRST in virulence systems). A fourth essential T3SS protein (FlhB/SctU) functions to “switch” secretion substrate specificity once the growing hook/needle reach their determined length. Here, we present the cryo-electron microscopy structure of an export gate containing the switch protein from a Vibrio flagellar system at 3.2 Å resolution. The structure reveals that FlhB/SctU extends the helical export gate with its four predicted transmembrane helices wrapped around FliPQR/SctRST. The unusual topology of the FlhB/SctU helices creates a loop wrapped around the bottom of the closed export gate. Structure-informed mutagenesis suggests that this loop is critical in gating secretion and we propose that a series of conformational changes in the T3SS trigger opening of the gate through interactions between FlhB/SctU and FliPQR/SctRST.
Type-three secretion is a mechanism of bacterial protein secretion across both inner and outer bacterial membranes. It is found in the virulence-associated injectisome (vT3SS), a molecular syringe, and the bacterial flagellum (fT3SS), a motility organelle. Both families contribute in significant ways to bacterial pathogenesis. vT3SS facilitate secretion not only across the bacterial envelope but also insert translocon proteins at the tip of the needle into the eukaryotic host plasma membrane, allowing direct injection of virulence factors in the host cytoplasm. The fT3SS is responsible for construction of the flagellar filament in both Gram-negative and Gram-positive bacteria, and hence imparts pathogenicity, e.g., via the ability to swim towards favourable environments or sense environmental conditions.

Type-three secretion system (T3SS) are multi-megadalton protein complexes that are capable of bridging from the bacterial cytoplasm to the extracellular space. At the core of the secretion system is the highly conserved export apparatus (EA), which is made up of five predicted transmembrane (TM) proteins (SctR, SctS, SctT, SctU and SctV in the vT3SS; FliP, FliQ, FlhR, FlhB and FlhA in the fT3SS). FlhA/ScvT has been shown to form a nonameric ring, consisting of a large cytoplasmic domain situated below a hydrophobic domain predicted to lie in the inner membrane. Howver, our recently determined structures of the Salmonella enterica serovar Typhimurium FliPQR complex and the Shigella flexneri SctRST complexes demonstrated that the export gate is actually embedded within the proteinaceous core of the T3SS basal body, placing it above the inner membrane. Furthermore, the helical structure of the export gate makes it likely that it is responsible for nucleating the helical filaments that assemble above it. Interestingly, the EA has also recently been proposed to facilitate inward transport across the inner membrane associated with nanotubes. The final component of the EA, FlhB/ScvU, has long been known to be essential for all T3SS-mediated protein secretion. In addition, FlhB/ScvU has a major role in switching the specificity of secretion substrates, mediating the transition from the early components necessary to build the flagellar hook in fT3SS and injectisome needle in vT3SS, to the later subunits required for flagellar filament or injectisome translocon assembly. The FlhB/ScvU family of proteins all contain an N-terminal hydrophobic sequence that is predicted to form four TM helices (FlhB/ScvU) and a smaller cytoplasmic C-terminal domain (FlhB/ScvU). Crystal structures of the FlhB/ScvU cytoplasmic domain from a range of species and systems demonstrated a compact fold with an unusual autocatalytic cleavage site in a conserved NPTH sequence. Cleavage between the Asn and Pro residues, splitting FlhB into FlhBC and FlhBCO, is required for the switching event to occur and a variety of mechanisms have been proposed to explain the need for this unusual mechanism.

Little was known about the predicted TM portion of FlhB/ScvU. Co-evolution analysis and molecular modelling led to suggestions that it forms a four-helix bundle in the membrane, whereas crosslinks and partial co-purification of FlhB with FliPQRT were consistent with FlhB/ScvU interacting with the export gate via a conserved site on the FlpPQR complex. However, given the inaccuracy of the TM predictions for the other export gate components revealed by the FliPQRT structure, we sought to determine the molecular basis of the interaction of FlhB with FliPQRT. Here we present the structure of the TM region of FlhB bound to the FliPQRT complex, in addition to the structures of the FliPQRT complex from Vibrio mimicus and Pseudomonas savastanoi. The structure reveals a unique topology that presents a loop that wraps around the base of the closed export gate. Mutagenesis studies confirm the crucial role played by the FlhB loop in the export process and suggest potential mechanisms of regulation of opening of the assembly.

Results

Conservation of the FliPQR structure. Our previously determined structures of S. typhimurium FliPQR and the vT3SS homologue SctRST from S. flexneri demonstrated that the stoichiometry of the core structure (FlpPQR, R) is conserved between virulence and flagellar systems. However, classification of the SctRST data revealed variable occupancy of the SctS component (up to a maximum of four copies), consistent with our earlier native mass spectrometry measurements. Furthermore, our native mass spectrometry had also demonstrated that a small proportion of the P. savastanoi FliPQR complex contained five copies of FliQ, with the predicted fth FliQ-binding site beginning to encroach on the predicted FlhB interaction site. To further analyse the structural conservation and stoichiometry of the EA core FliPQR, we chose the homologous complexes from the fT3SS of two other bacterial species for structural studies: the V. mimicus polar flagellum FliPQR complex, which has a longer FlhB sequence including an N-terminal domain conserved in the Vibrionales order (Supplementary Fig. 1), and the P. savastanoi FliPQR complex, which is a mixture of FliPQR, R and FliPQQR complexes by native mass spectrometry. We determined the structures of both complexes using single-particle cryo-electron microscopy (cryo-EM) analysis to 4.1 Å and 3.5 Å, respectively (Fig. 1a, Table 1, Supplementary Fig. 2 and Supplementary Fig. 3). Both structures are similar to S. typhimurium FliPQR (root-mean-square deviation (RMSD) = 1.6 Å over all chains) and S. flexneri SctRST (V. mimicus FliPQR and SctRST RMSD = 1.9 Å and P. savastanoi FliPQR and SctRST RMSD = 2.3 Å).

Consistent with our previous native mass spectrometry data, the structure of P. savastanoi revealed an additional FliQ subunit in the complex. In the S. typhimurium and V. mimicus FliPQR structures, there are four FliP–FliQ units, each the structural equivalent of a FliR subunit, but the fifth FlhB is missing a FliQ. In the P. savastanoi structure, FlhQ binds the remaining FliQ subunit in the same way as in the other FliP–FliQ units. This FlhQ subunit is located close to the site on FliPQR we previously identified as important for interaction with FlhB/ScvU. We conducted an extensive in-vivo photocrosslinking analysis based on covariance between SctU, SctR, SctS and SctT, which supports a binding site for SctU that involves large parts of helix 2 of SctS and helix 4 of SctT (Fig. 1b, c and Supplementary Fig. 4). Mapping of the residues on the structure of S. typhimurium FliPQR and a model of FliPQR containing a fifth FliQ subunit reveals a large binding site in the complex containing four FliQ subunits and a more compact binding site when a fifth FlhB is modelled (Fig. 1d). In this way, FliQ/SctS might be compatible with FlhB/ScvU binding, depending on the unknown structure of the FlhB/ScvU TM domain (FlhB/ScvU10), but addition of a sixth FlhQ/ScvT using the same helical parameters would block this site.

Architecture of the FliPQR–FlhB export gate complex. We have observed four FliQ subunits in the S. typhimurium and V. mimicus FliPQR and the S. flexneri SctRST structures; however, as we have previously observed FliQ to be sensitive to dissociation by detergent in the purification process, it was possible that the fifth FliQ is a genuine component of the complex but was lost in the purification of less stable homologues. As the stoichiometry of FliQ has potentially large implications for the placement of FlhB in the system (Fig. 1d), we endeavoured to produce the more physiologically relevant FliPQR–FlhB complex.
After extensive screening of detergents, constructs with different placement of affinity tags and sequences from a variety of species for co-expression and co-purification of FlhB with FliPQR, we were able to prepare a monodisperse sample of the complex from V. mimicus (Fig. 2a). We analysed this sample by cryo-EM and determined the 3.2 Å structure of the complex (Fig. 2b, Table 1 and Supplementary Fig. 5), revealing a single copy of FlhB added to the previously observed FliP_5Q4R_1 complex. The FliPQR subcomplex in this structure is very similar to the structure of the FliPQR complex in the absence of FlhB (RMSD = 0.6 Å), whereas FlhB is observed to contain four long helices in the putative TM domain (FlhBTM), forming two distinct hairpins that are wrapped around the outside of the FliPQR complex. This extensive interaction surface between FlhB and FliPQR reveals FlhB to be an integral part of the core of the EA instead of an accessory factor. The opened out structure of the four predicted TM helices of the FlhBTM domain once again highlights the potential dangers in predicting complex structures in the absence of some of the subunits. The soluble, globular, cytoplasmic domain (FlhBC) is not visible, likely due to flexibility in the linker between the two domains. However, to assess whether the disorder was due to the presence of the detergent micelle in our sample, we also imaged FliPQR–FlhB in the amphipol A8-35, perhaps a better mimic of the proteinaceous environment relevant to the assembled T3SS. We did not observe any additional density resulting from ordering of FlhBC (Supplementary Fig. 6).

Intriguingly, the two helical hairpins of FlhB are joined by a loop (FlhBL) that literally loops around the (closed) entrance of the FliPQR gate (Fig. 2c). Consistent with our previous prediction and crosslinking analysis (Fig. 1), FlhB contacts the site across FliP5 and FliR, and it additionally contacts S. TyphimuriumΔsctRSTU, pT10-sctRSTU N269A FLAG SctS SctU SctS–SctU SctR SctT S189X SctT–SctU SctR

Fig. 1 Conservation of the structure of the FliPQR export gate in the closed state. a Cryo-EM volumes calculated in Relion using data from S. typhimurium FliPQR (left, EMD-4173), S. flexneri SctRST (centre left, SctR5S4T1 class10 (EMD-4734)), P. savastanoi FliPQR (centre right) and V. mimicus FliPQR (right). FliQ2 and FliQ4 are coloured orange and FliQ1, FliQ3 and FliQ5 are coloured red. b Immunodetection of SctUFLAG on western blottings of SDS-PAGE-separated crude membrane samples of the indicated S. typhimurium SctS pBpa mutants (denoted with X). Each sample is shown with and without UV-irradiation to induce photocrosslinking of pBpa to neighbouring interaction partners. c As in b, but testing interactions to SctU with pBpa in SctTand SctR. d Mapping of the confirmed contact points between FliPQR/SctRST and FlhB/SctU, including those previously identified on the structure of FliPQR (S. typhimurium) and a model with a fifth FliQ subunit, which is based on the structure of P. savastanoi FliPQR.
The density corresponding to FlhB was of sufficient quality to build an atomic model of the structure using only sequence information (Fig. 3a and Supplementary Fig. 5). The topology of FlhB is unusual; the helices 1 and 4 neighbour each other in the middle of the structure, whereas helix 2 and 3 flank the central pair on either side (Fig. 3b, c). To further validate the topology of FlhB, we compared our model with contacts derived from evolutionary co-variation (Fig. 3d and Supplementary Fig. 8). This shows strong contacts between helices 1 and 2, 3 and 4, and 1 and 4 but an absence of contacts between helices 2 and 3, which is inconsistent with a helical bundle but consistent with our more extended and topologically unusual structure.

Despite observing up to five FliQ subunits in different FliPQR structures, there are only four FliQ molecules in this structure. In fact, the hairpin composed of FlhB helices 1 and 2 is bound to the site occupied by FliQ3 in our P. savastanoi FliPQR structure, packing on FliP5, whereas helices 3 and 4 pack on FliR. Thus, the presence of FliQ3 would block binding of FlhB (Fig. 3e), suggesting that the fifth FliQ binds to the complex in a non-native manner due to the absence of FlhB in the overexpression system. This superposition of FlhB and FliQ also reveals that the hairpin of helices 1 and 2 of FlhB adopts a very similar structure to FliQ, whereas the helix 2 of FlhB being structurally equivalent to helix 1 of FliQ and vice versa, i.e., the directionality of the hairpin is reversed along the long axis. Modelling FliQ3 and FliQ5 using the same helical parameters by which FliQ1 to FliQ4 are related reveals that FlhB superposes is surprising. An evolutionary relationship between FlhB and FliQ is unlikely due to the topology differences, suggesting that the similarity of the structures is a result of convergent evolution and the need to form this helical assembly.

An extended loop between helices is essential for secretion. The unusual topology of FlhB means that a long loop, FlhB_{234}, between helix 2 and 3 (residues 110–139) connects the two hairpins of the structure. Most unexpectedly, this loop, which contains the most highly conserved residues within FlhB, is cross-linkable residues in the FliP4 subunit (Fig. 2d). Interestingly, both termini of FlhB are cytoplasmic, whereas the C terminus of FlhB is periplasmic. Perplexingly, the observation that an engineered fusion of FliR–FlhB in Salmonella weakly complements a double fliR/fliB knockout, in conjunction with the presence of FliQ5 and FliQ6 using the same helical parameters by which FliQ5 and FliQ6 are related reveals that FlhB superposes is surprising. An evolutionary relationship between FlhB and FliQ is unlikely due to the topology differences, suggesting that the similarity of the structures is a result of convergent evolution and the need to form this helical assembly.

**Table 1 Cryo-EM statistics.**

|                      | FlhB, V. mimicus (EMD-10093) (PDB 6535) | FlhB, V. mimicus (EMD-10093) (PDB 6535) | FliPQR–FlhB, V. mimicus (EMD-10653) | FliPQR–FliB, V. mimicus (EMD-10653) |
|----------------------|----------------------------------------|----------------------------------------|-------------------------------------|-------------------------------------|
| Magnification        | 165,000                                | 165,000                                | 165,000                             | 165,000                             |
| Voltage (kV)         | 300                                    | 300                                    | 300                                 | 300                                 |
| Defocus range (µm)   | 0.5–4                                  | 0.5–4                                  | 0.5–4                               | 0.5–4                               |
| Pixel size (Å)       | 0.82                                   | 0.82                                   | 0.82                                | 0.82                                |
| Symmetry imposed     | C1                                     | C1                                     | C1                                  | C1                                  |
| Initial particle images (no.) | 503,177                            | 1,050,955                              | 1,386,230                           | 677,403                             |
| Final particle images (no.) | 97,987                               | 243,489                                | 162,408                             | 137,136                             |
| Map resolution (Å)   | 3.5                                    | 4.1                                    | 3.2                                 | 4.0                                 |
| FSC threshold        | 0.143                                  | 0.143                                  | 0.143                               | 0.143                               |
| Map sharpening B factor (Å²) | -101                                | -214                                   | -97                                 | -97                                 |
| Model used           | EMD-4173                               | EMD-4173                               | EMD-4173                            | EMD-4173                            |
| Model resolution (Å) | 3.5                                    | 4.1                                    | 3.2                                 | 4.0                                 |
| FSC threshold        | 0.143                                  | 0.143                                  | 0.143                               | 0.143                               |
| Map sharpening B factor (Å²) | -101                                | -214                                   | -97                                 | -97                                 |
| Model composition    |                                        |                                        |                                     |                                     |
| Non-hydrogen atoms   |                                        |                                        |                                     |                                     |
| Protein residues     | 12,855                                 | 12,321                                 | 13,849                              | 13,049                              |
| Ligands              | 0                                      | 0                                      | 0                                    | 0                                    |
| B factors (Å²)       |                                        |                                        |                                     |                                     |
| Protein              |                                        |                                        |                                     |                                     |
| Ligand               |                                        |                                        |                                     |                                     |
| R.m.s. deviations    |                                        |                                        |                                     |                                     |
| Bond lengths (Å)     | 0.0058                                 | 0.0066                                 | 0.01                                | 0.01                                |
| Bond angles (°)      | 0.88                                   | 0.87                                   | 0.93                                | 0.93                                |
| Validation           |                                        |                                        |                                     |                                     |
| MolProbity score     | 2.54                                   | 2.25                                   | 2.45                                | 2.45                                |
| Clashscore           | 18                                     | 18.28                                  | 15.56                               | 15.56                               |
| Poor rotamers (%)    | 2.22                                   | 0.07                                   | 2.72                                | 2.72                                |
| Ramachandran plot    |                                        |                                        |                                     |                                     |
| Favoured (%)         | 90.82                                  | 91.86                                  | 93.64                               | 93.64                               |
| Allowed (%)          | 8.39                                   | 7.76                                   | 5.84                                | 5.84                                |
| Disallowed (%)       | 0.79                                   | 0.39                                   | 0.52                                | 0.52                                |
Mutations in either the conserved hydrophobic residues of FlhB, that insert between the FliQ subunits (Fig. 3f) or the highly conserved loop of FliQ severely reduced motility (Fig. 4a), protein secretion (Supplementary Fig. 11) and formation of flagella (Supplementary Fig. 13) without affecting EA assembly (Supplementary Fig. 14). Although substitution with the bulky, hydrophobic amino acid tryptophan and removal of bulky sidechains only reduced motility, introduction of charged residues completely abolished motility, suggesting that secretion can proceed at lower efficiency when the FliQ–FlhB interaction is only reduced rather than completely disrupted as in the aspartate and arginine mutants.

We performed an extensive in-vivo photocrosslinking analysis to validate the interactions and functional relevance of the corresponding SctU in the vT3SS-1 of S. typhimurium. Although no crosslinks to SctS could be identified with the artificial crosslinking amino acid p-benzoyl-phenylalanine (pBpa) introduced into SctU itself (Supplementary Fig. 15), numerous crosslinks were identified with pBpa in the lower part of SctS that faces SctU (Fig. 4b). Using two-dimensional blue native SDS-polyacrylamide gel electrophoresis (PAGE), we could show that the crosslink observed with SctS was not limited to the SctRSTU assembly intermediate but also in the assembled needle complex (Fig. 4c), adding further support to the idea that the structure of the isolated complex represents the structure of the complex in the full assembly. The observed crosslinks were independent of functional secretion of the vT3SS, indicating that assembly of needle adapter, the inner rod and needle filament does not lead to a conformational change of this part of the SctRSTU complex (Fig. 4b).

Our motility assays (Fig. 4a) could be influenced by multiple factors including growth of the cells, assembly of flagella, chemotaxis and secretion. In addition to assaying secretion of FliC directly in the flagellar strains (Supplementary Fig. 12), we also directly assayed secretion of the early secretion substrate SctP in the pBpa mutants. Strikingly, introduction of pBpa at several positions of SctS led to a strong defect in secretion but not SctS–SctU interaction, highlighting the relevance of this site for secretion function of T3SS (Fig. 4d), whereas pBpa substitutions within SctU were more functionally neutral. In total, we found a large number of residues at the FliQ/SctS–FlhB/SctU interface that are required for type-three secretion (Fig. 4e).

Strong crosslinking between SctS and SctU even in the assembled needle complex and loss of function in more disruptive mutations in which the interaction between FliQ and FlhB is altered through the introduction of charged residues suggest that this interaction is important for activity and FlhB is not simply one of the closure points of the complex in assembly intermediates. If this interaction is maintained in the open state of the export gate, FlhB would have to adopt a more extended conformation, requiring a minimum number of residues in the loop. Consistent with this idea, deletions of six or more residues in FlhB led to loss of motility, while duplication of six residues in FlhB was rather better tolerated, leading only to a reduction in motility (Fig. 4a).

We further investigated the function of FlhB through more targeted mutations. FlhB is a largely extended polypeptide with little secondary structure, but a short stretch at its C terminus is helical. Interestingly, a mutation of a glycine in this α-helix in the FlhB homologue YscU disrupted secretion in the Yersinia vT3SS. The equivalent mutation in E. coli FlhB, G133D (Supplementary Fig. 11), or mutation of the conserved positively charged residue R136 (Fig. 4a) had little effect assessed at the level of motility, although we cannot rule out more subtle effects on the efficiency with which secretion occurs.

**Fig. 2 Architecture of the FliPQR-FlhB export gate complex.** a SDS-PAGE gel of the V. mimicus FliPQR-FlhB complex. b Cryo-EM density map of the FliPQR-FlhB complex with the density corresponding to FlhB coloured in green. c Zoom of the FlhB loop at the bottom of the complex. d Model of FliPQR (surface) and FlhB (cartoon, green) with residues in FliP/SctR and FlhB/N terminus in contact with the binding sites on the opening FliQ subunits, reminiscent of a sphincter.
**Fig. 3 Structural analysis of the FlhB hydrophobic domain.**

(a) Quality of the cryo-EM volume corresponding to FlhB. Zoom box shows the fit to density of the model. (b, c) Rainbow colouring of the FlhB model with numbers indicating the N and C termini of the four helices. (d) Evolutionary co-variation within FlhB calculated using RaptorX. Only contacts with a probability >0.5 are plotted. Red boxes highlight the interaction between helix 1 and 2 (i), helix 1 and 4 (ii), helix 3 and 4 (iii), the N terminus and FlhBCN (iv), within the N terminus (v) and between FlhB and the N terminus (vi). (e) Overlay of FlhB (green) and a modelled FliQ5 and FliQ6 following the same helical parameters as FliQ1 to FliQ4 in *V. mimicus*. (f) Zoomed view of the interaction between the FlhB loop and FliQ, highlighting the intercalation of conserved hydrophobic residues in FlhB between the FliQ subunits.
Fig. 4 Functional analysis of FlhB. a Motility in soft agar of E. coli WΔflfOPQR complemented with plasmids expressing flfOPQR with the indicated mutations in FlfQ (left) and E. coli WΔflhB complemented with plasmids expressing FlhB with the indicated mutations (middle and right). b Immunodetection of SctUFLAG on western blottings of SDS-PAGE-separated crude membrane samples of the indicated S. typhimurium SctS 5bpa mutants (denoted with X). Each sample is shown with and without UV-irradiation to induce photocrosslinking of 5bpa to neighbouring interaction partners. Crosslinks between SctS5bpa and SctUFLAG are indicated. Crosslinking analysis was performed in the wild type and in an ATP hydrolysiss-deficient SctN37K mutant that is incapable of type III secretion. c Immunodetection of SctUFLAG on western blottings of 293 blue native/SDS-PAGE-separated crude membrane samples of the indicated S. typhimurium SctS 5bpa mutant. The sample is shown with and without UV-irradiation to induce photocrosslinking of 5bpa to neighbouring interaction partners. Crosslinks between SctS5bpa and SctUFLAG in the SctRSTU assembly intermediate and in the assembled needle complex (NC) are indicated. d Immunodetection of the early T3SS substrate Scp in western blottings of SDS-PAGE-separated culture supernatants of the indicated S. typhimurium SctS 5bpa mutants. e Structure of FliPQR-FlhB highlighting mutation sites that impaired motility or secretion in red and mutation sites that had no or only a small effect in yellow.

Discussion
In this study we show that FlhBTM is part of the export gate complex together with FliPQR. Two pairs of helices of FlhB bind to FliPQR through a structure mimicking the shape of FlfQ, despite topological reversal, an example of molecular convergent evolution. The unusual topology of FlhB places helices 2 and 3 apart from each other allowing them to mount a loop, FlhB1, onto the cytoplasm-facing surface of the export gate. Although the way in which FlhB1 wraps around the closed pore suggests a role in maintaining the closed state, our structures of FliPQR/SctRST in the absence of FlhB/SctU are also closed9,10, as is the complex in the context of the assembled T3SS23. This suggests FlhB may be involved in opening of the gate rather than locking it closed, although this would require the linker to be able to extend.

The location and the topology of FlhBTM place the N terminus, FlhBCN and FlhB1 in close proximity just underneath the aperture of the gate. Although the resolution of the map is poor in the region of the cytoplasmic face of the complex, it is possible to trace the approximate position of the FlhB N terminus and FlhBCN (Supplementary Fig. 10). The close association of the N terminus and FlhBCN is consistent with the strong contacts derived from evolutionary co-variation between the N terminus and the N-terminal part of the cytoplasmic domain (FlhBCN) (Fig. 3c), and a genetic interaction in S. typhimurium between E11 and E230, in the N terminus of FlhB and FlhBCN, respectively24. Furthermore, the direction in which FlhBCN leaves the export gate implies that, in the context of the assembled T3SS nanomachine, FlhBC could be located anywhere between the FliPQR–FlhB gate
and the nonameri c ring of the FlhA cytoplasmic domain below (Fig. 5a). It is conceivable that the conformational changes required for opening of the export gate are propagated via pulling forces imparted on helix 4 of FlhB\textsubscript{TM}, which is linked to the other helices of FlhB\textsubscript{TM} via a conserved network of buried charged residues in FlhA. As FlhB\textsubscript{C} (PDB: 3b0z)\textsuperscript{25} in yellow and light blue, and the density corresponding to the FlhA homologue is highlighted in blue. b Network of salt bridges formed by conserved charged residues in FlhB.

**Fig. 5 Position of FlhB\textsubscript{C} in the complete T3SS.** a Placement of FliPQR–FlhB in a tomographic reconstruction of the Salmonella SPI-1 V\textsubscript{T3SS} (EMD-8544)\textsuperscript{47}. FliPQR is shown in grey, FlhB\textsubscript{TM} in green, two possible positions of FlhB\textsubscript{C} (PDB: 3b0z)\textsuperscript{25} in yellow and light blue, and the density corresponding to the FlhA homologue is highlighted in blue. b Network of salt bridges formed by conserved charged residues in FlhB.

a strain deleted for the ATPase complex (AfliH)\textsuperscript{21,30}, has long been mysterious. Our structure, demonstrating the clustering of the N terminus, FlhB\textsubscript{CN} and FlhB\textsubscript{C}, at the cytoplasmic entrance of the gate, suggests that they may rescue function by altering the dynamics of the closure point to facilitate opening of the export gate. This notion is further supported by the interaction we observe of SctU\textsubscript{28pBpa} with SctS. This functional link between the ATPase and the gating mechanism, in conjunction with a host of other mutational data in FlhA and FlhB, suggests that cycles of ATP hydrolysis may induce conformational changes in the export gate. As the distance between the export gate and ATPase is large, these conformational changes would presumably be mediated via the FlhA ring that is positioned between them and, has been shown to interact with the FliJ stalk of the ATPase complex\textsuperscript{31,32}. As low levels of secretion are possible even in the absence of the ATPase complex or in mutants impaired in ATPase activity\textsuperscript{21}, low level, spontaneous, opening of the export gate complex must also be possible. Whether the gate subsequently stays open, e.g., due to the continued presence of substrate in the channel\textsuperscript{28}, or whether it opens and closes as one substrate molecule after the other is injected into the growing filament, as might be expected from the injection-diffusion model of flagellar growth\textsuperscript{25}, is unknown.

Finally, FlhB/SctU is known to play a key role in substrate switching, an event which requires autocatalytic cleavage of the NPTH sequence in FlhB\textsubscript{C}/SctU\textsubscript{C}\textsuperscript{16,17,28,34}. Although we do not observe the residues involved in switching in our export gate structure, the fact that we are able to crosslink SctU in fully assembled basal bodies, using the same residues as in the purified complex, suggests that the gating mechanism discussed here is likely applicable regardless of substrate. Clearly, future studies will need to focus on observing gating and switching events.

In summary, our structure of FlhB as part of the export gate complex extends our understanding of the regulation of the T3SS EA and suggests possible mechanisms of export gate opening.

**Methods**

**Materials.** Chemicals were from Sigma-Aldrich, unless otherwise specified. The detergents n-dodecylmaltoside (DDM) and lauryl maltose neopentyl glycol (LMNG) and the amphipol A8-35 were from Anatrace. pBPA was from Bachem. Primers are listed in Supplementary Table 2 and were synthesized by Invitrogen or Eurogentec.

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Supplementary Table 3. Plasmids were generated by Gibson assembly of PCR fragments using the NEBuilder HiFi Master Mix (NEB) or in vivo assembly\textsuperscript{35}. Fragments were created by PCR with the relevant primers using Q5 polymerase (NEB) and genomic DNA templates obtained from DSMZ (V. mimicus strain DSM 19130 and P. savastanoi pv. phaseolicola 1448A strain DSM 21482). Gibson assembly and PCR were carried out following the manufacturer’s recommendations. E. coli W for motility assays was obtained from DSMZ (DSM 1116). Bacterial cultures were supplemented as required with ampicillin (100 \(\mu\)g/mL) or kanamycin (30 \(\mu\)g/mL) or 60 \(\mu\)g/mL for large-scale expression in terrific broth (TB). S. typhimurium strains were cultured with low aeration at 37 °C in Luria Bertani (LB) broth supplemented with 0.3 M NaCl to induce expression of genes of SPI-1. As required, bacterial cultures were supplemented with tetracycline (12.5 \(\mu\)g/mL), streptomycin (50 \(\mu\)g/mL), chloramphenicol (10 \(\mu\)g/mL), ampicillin (100 \(\mu\)g/mL) or kanamycin (25 \(\mu\)g/mL). Low-copy plasmid-based expression of SctSTU\textsubscript{FLAG} was induced by the addition of 500 \(\mu\)M rhizmase to the culture medium.

**Generation of chromosomal deletion mutants.** Electrocompetent E. coli W expressing λ Red recombinase from plasmid pKD46 were transformed with DNA fragments containing a chloramphenicol resistance cassette surrounded by sequences homologous to the gene of interest as described in Supplementary Table 3. Colonies were selected on LB agar containing chloramphenicol (20 \(\mu\)g/mL) and transformed again with pCP20 and grown on LB agar containing ampicillin (100 \(\mu\)g/mL) at 30°C. Finally, clones were grown in LB media at 37 °C. Deletion mutations were confirmed by PCR. All Salmonella strains were derived from S. enterica serovar Typhimurium strain SL1344\textsuperscript{46} and created by allelic exchange as previously described\textsuperscript{47}. 

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Purification of export gate complexes. FliPQR or FliOPQR-FliHb were expressed in E. coli BL21 (DE3) as a single operon from a pT12 vector (Supplementary Table 3), as described previously18–20. Briefly, cells were grown at 37 °C in LB media containing rhamnose monohydrate (0.1%), collected by spinning at 4000 × g for 10 min in TBS (100 mM Tris, 150 mM NaCl, 1 mM EDTA pH 8) and lysed in an EmulsiFlex C5 homogenizer (Avestin). Membranes were prepared from the cleared lysate by ultracentrifugation at 125,000 × g for 3 h. Membranes were solubilized in 1% (w/v) LMNG in TBS and applied to a SuperTrap column (GE Healthcare). The resin was washed in TBS containing 0.01% (w/v) LMNG and proteins were eluted in TBS supplemented with 0.01% (w/v) LMNG and 10 mM dithioretilactin. Complexes were separated from aggregate by size-exclusion chromatography in TBS containing 0.01% (w/v) LMNG and 0.5 mM BSA. Proteins were eluted in 10% (v/v) increase or Superose 6 increase column using GE Healthcare.

For preparation of FliPQR-FliHb solubilized in the amphipol A8-35, the protein was purified as above using DDM (1% (w/v) for extraction from the membrane and 0.02% (w/v) subsequently) rather than LMNG. Eluate from the SuperTrap column was mixed with amphipol at a ratio of amphipol to protein of 10:1. After incubating for 1 h, the sample was dialyzed into TBS using a 10,000 MWCO Slide-A-Lyzer device (Thermo Fisher Scientific) overnight followed by size-exclusion chromatography on a Superose 6 increase column using TBS as the running buffer.

Sample preparation for cryo-EM. Purified complex (3 µl) at 1–3.6 mg/ml were applied to glow-discharged holey carbon-coated grids (Quantifoil 300 mesh, Au R12/1.3). Grids were blotted for 3 s at 50% humidity at 22 °C and frozen in liquid ethane using a Vitrobot Mark IV (FEI). For samples solubilized in detergent, blotting was preceded by a wait time of 5–10 s. V. mimosus FliPQR was supplemented with 0 mM, 0.05 mM, 0.5 mM or 3 mM LMNG (S200 10/300 increase or Superose 6 increase, GE Healthcare).

EM data acquisition and model building. All data contributing to the final models were collected on a Titan Krios (FEI) operating at 300 kV. All movies were recorded on a K2 Summit detector (Gatan) in counting mode at a sampling of 0.822 Å/px, 2.4 frames/s, 8 s exposure, total dose 48 e−/Å2, 20 fractions written. Motion correction and dose weighting were performed using MotionCor21. Contrast transfer functions were calculated using CTFIND4.18. Particles were picked in Simple and processed in Relion 2.117 and 3.18 as described in Supplementary Figs. 2, 3 and 5. Atomic models of FliPQR and FliHb were built using Coot22 and refined in Phenix23.

Motility assays. E. coli W strains W1L or W2L (Supplementary Table 3) were transformed with plasmids encoding FliPQR or FliHb containing the mutations to be tested. Saturated overnight cultures (3 µl) were injected into soft agar plates (0.28% agarose, 2YT media, containing ampicillin 100 µg/ml or kanamycin (50 µg/ml) and 0.1% arabinose or 0.5% rhamnose monohydrate as appropriate) and incubated at room temperature.

E. coli W FT3SS secretion assay. E. coli W strains W1L or W2L (Supplementary Table 3) transformed with plasmids encoding FliPQR or FliHb containing the mutations to be tested were grown overnight in 2YT media containing the appropriate antibiotics and 0.1% arabinose or 0.5% rhamnose monohydrate. Cells were pelleted and resuspended in fresh media and grown for another hour. Cells were pelleted again and the supernatant was filtered through a 0.22 µm filter. Proteins were bound to StrataClean beads (Agilent) and the beads were resuspended in SDS-PAGE buffer and run on a 4–20% polyacrylamide gel (Bio-Rad). FliC was detected by immunodetection using an antisera against S. typhimurium FlIC.

Imaging of flagella. E. coli W strains W1L or W2L (Supplementary Table 3) transformed with plasmids encoding FliPQR or FliHb containing the mutations to be tested were grown overnight in 2YT media containing the appropriate antibiotics and 0.1% arabinose or 0.5% rhamnose monohydrate. Cells were pelleted and resuspended in fresh media and grown for another hour. Cells were pelleted again and the supernatant was filtered through a 0.22 µm filter. Proteins were bound to StrataClean beads (Agilent) and the beads were resuspended in SDS-PAGE buffer and run on a 4–20% polyacrylamide gel (Bio-Rad). FliC was detected by immunodetection using an antisera against S. typhimurium FlIC.

In-vivo photocrosslinking. In-vivo photocrosslinking was carried out as described previously24–26 with minor modifications. To enhance expression of vT3SS-1, S. typhimurium strains expressed HilA, the master transcriptional regulator of SPI-1 T3SS, from a high copy plasmid under the control of an arabinose-inducible ParaBAD promoter27. Bacterial cultures were grown in LB broth supplemented with 0.3 M NaCl, 1 mM MgPta and 0.05% arabinose at 37 °C for 5 h. Bacterial cells (5 OD600) were collected and washed once with 5 ml chilled phosphate-buffered saline (PBS) to remove residual media. Bacteria were pelleted by centrifugation at 4000 × g for 3 min and 4 °C, and afterwards resuspended in 1 ml of chilled PBS. Bacterial suspensions were transferred into six-well cell culture dishes and irradiated for 30 min with UV light (λ = 365 nm) on a UV transilluminator table (UV). Subsequently, bacterial cells were pelleted by centrifugation at 10,000 × g for 2 min and 4 °C. Samples were stored at −20 °C until use.

Crude membrane preparation. Crude membranes were purified following the published protocol28. Bacterial cells (5 OD600) were resuspended in 750 µl lysis buffer (50 mM triethanolamine pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM MgCl2, 10 µg/ml DNAase, 2 µg/ml lysozyme, 1:100 protease inhibitor cocktail) and incubated on ice for 30 min. Cell slurries were lysed via continuous bead milling. Intact cells, beads and debris were removed by centrifugation for 10 min at 10,000 × g and 4 °C. Supernatants were centrifuged for 50 min at 32,000 × g and 4 °C in a Beckman TLS 55 rotor to pellet bacterial membranes. Pellets containing crude membranes were stored at −20 °C until use. Samples were analysed by SDS-PAGE, western blotting and immunodetection.

Western blotting and immunodetection. Samples were loaded onto SERVA GeT™ TG PRIME 18–20% gels and transferred on polyvinylidene difluoride (PVDF) membranes (Bio-RAD). Proteins were detected with primary antibodies anti-Stx3T (Inv)17 (1: 1000) or M2 anti-FLAG (1: 10,000) (Sigma-Aldrich, F3165). Secondary antibodies (ThermoFisher, SAS-35571) were goat anti-mouse IgG Dynolyt 800 conjugate (1:5000). Scanning of the PVDF membranes and image analysis was performed with a Li-Cor Odyssey system and Image Studio 3.1 (Li-Cor).

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Cryo-EM volumes and atomic models have been deposed to the EMDB (accession codes EMID-10095, EMID-10096, EMID-10093 and EMID-10653) and PDB (accession codes 6S3R, 6S3S and 6S3L), respectively. The source data underlying Figs. 1b, c, 2a, 3d and Supplementary Figs. 2b, 3b, 4, 5c, 11, 12 and 13 are provided as a Source Data file. Other data are available from the corresponding author upon reasonable request.

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27. Author contributions L.K. performed experiments, did strain and plasmid construction, complex purification, native mass spectrometry, cryo-EM grid optimization, cryo-EM data analysis, and model building and analysis. J.D. performed experiments, cryo-EM grid optimization, cryo-EM data analysis, and model building and analysis. J.D. performed experiments, cryo-EM grid optimization, cryo-EM data analysis, and model building and analysis. A.Z., S.B. and R.D. generated pBpa mutants, performed crosslinking experiments and secretion assays, and analysed data. S.W. designed functional experiments and analysed data. S.J. and S.M.L. supervised experimental work and wrote the first draft of the paper with L.K. All authors contributed to and commented on the final manuscript.

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