Nonameric structures of the cytoplasmic domain of FlhA and SctV in the context of the full-length protein.

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Abstract
Type three secretion is the mechanism of protein secretion found in bacterial flagella and injectisomes. At its centre is the export apparatus (EA), a complex of five membrane proteins through which secretion substrates pass the inner membrane. While the complex formed by four of the EA proteins has been well characterised structurally, little is known about the structure of the membrane domain of the largest subunit, FlhA in flagella, SctV in injectisomes. Furthermore, FlhA/SctV is most often studied as a monomer and only a single structure of an SctV homologue assembled into the biologically relevant nonameric ring is available. FlhA has been shown to bind to chaperone-substrate complexes in an open state, but in the assembled ring structure SctV is in a closed state. Here, we identify FlhA and SctV homologues that can be recombinantly produced in the oligomeric state and study them using cryo-electron microscopy. The structures of the cytoplasmic domains from both FlhA and SctV are in the open state and we observe a conserved interaction between a short stretch of residues at the N-terminus of the cytoplasmic domain, known as $FlhA_L$/$SctV_L$, with a groove on the adjacent protomer's cytoplasmic domain, which stabilises the nonameric ring assembly. This represents the first structure of SctV in the open state, the first observation of the $SctV_L$ interaction with the adjacent protomer and confirms the importance of $FlhA_L$ for the stability of the FlhA nonameric ring.

Importance
Bacterial flagella are assembled from proteins secreted through a type III secretion system. A related type III secretion system is found in injectisomes, molecular syringes that bridge three membranes to secrete proteins directly from the bacterial cytoplasm into eukaryotic host cells. The major protein of the export apparatus of type III secretion is made up of a membrane and a cytoplasmic domain, which in the flagellar system can adopt an open or a closed state, is known to form a nonameric ring *in vivo*. We produced the full-length proteins from both injectisome and flagellar systems in the assembled state. The structures of the cytoplasmic domains demonstrate the conserved principle of the N-terminus of one subunit binding the membrane proximal face of the adjacent subunit to stabilise the assembled ring. Our structure of the homologue from the injectisome also demonstrates that the open state of the cytoplasmic domain is not unique to flagella.

Introduction
Type III secretion systems (T3SS) are bacterial macromolecular machines that facilitate transport of protein substrates across the bacterial cell envelope (Erhardt et al., 2010;

Wagner et al., 2018). The secretion machinery is conserved across at least two biological systems, the bacterial flagellum, which drives motility, and the virulence associated T3SS, which is an essential virulence factor for many bacterial pathogens due to the secretion of effector proteins directly into host cells (Galan, 2009). Most building blocks of the virulence associated T3SS or injectisome have a homologue in flagella and one of the most highly conserved parts of the system is a set of transmembrane proteins known collectively as the export apparatus (EA) (Deng et al., 2017).

The EA is made up of a core complex formed by the membrane proteins FliPQR (flagellar T3SS) or SctRST (virulence associated T3SS) which form a channel through which the unfolded protein substrates pass (Dietsche et al., 2016; Kuhlen et al., 2018). The regulatory subunit FlhB/SctU wraps around this core (Kuhlen et al., 2020) and the inner rod and needle assemble onto its periplasmic face (Hu et al., 2019; Torres-Vargas et al., 2019). Surprisingly, following assembly into the full T3SS, the core complex is not in the plane of the inner membrane. Instead, it is held in the plane of the periplasm by interactions with the surrounding basal body and its hydrophobic surface is covered by another membrane protein complex situated directly underneath it in the inner membrane. This protein complex is believed to be formed by the transmembrane domain of the fifth EA component, FlhA or SctV (Butan et al., 2019; Kuhlen et al., 2018). The FlhA/SctV cytoplasmic domain is known to form a nonameric ring underneath FliPQR-FlhB/SctRSTU (Abrusci et al., 2013), but the full-length FlhA/SctV complex remains poorly characterised. The entire EA is housed within the basal body of the T3SS and is thought serve as a nucleus for its assembly (Fan et al., 1997; Wagner et al., 2010).

FlhA/SctV has long been known to be the major component of the EA, but its stoichiometry was controversial for a long time (Li and Sourjik, 2011; Lilic et al., 2010) and may be dynamic *in vivo* (Li and Sourjik, 2011; Morimoto et al., 2014). Fluorescence based measurements showed that FlhA can form a large complex, but its exact stoichiometry couldn't be measured with high precision (Li and Sourjik, 2011; Morimoto et al., 2014). The crystallisation of the cytoplasmic domain of SctV from *Shigella flexneri*, $SctV_{Sf}$, as a nonameric ring matching the dimensions of a toroidal density in the cytoplasm, described by tomography, established the stoichiometry of FlhA/SctV (Abrusci et al., 2013) and the ability of the cytoplasmic domain to form a nonamer. This was later confirmed by high resolution AFM as the FlhA cytoplasmic domain also formed a nonameric ring on mica (Terahara et al., 2018) whose stability depended on the linker between cytoplasmic and membrane domains, $FlhA_L/SctV_L$. However, direct structural characterisation of the nonamer has so far been limited to the crystal structure of $SctV_{Sf}$, in which the crucial $SctV_L$ is not ordered, and full-length protein has been challenging to produce in an assembled state, precluding final confirmation that the full-length protein also forms a nonameric ring.

The lack of assembled ring structures of the FlhA/SctV cytoplasmic domain has also limited our understanding of the structural transitions this domain is thought to undergo in the secretion process. An open and closed state have been described, with the open state being competent for chaperone-substrate complex binding (Xing et al., 2018) while the closed state has been proposed to be responsible for binding early substrates (Inoue et al., 2019), but the open state has so far only been observed in protein that is not assembled into a ring. An interaction between $FlhA_L/SctV_L$ and the cytoplasmic domain of the neighbouring

subunit has been observed in crystal structures of the open state (Bange et al., 2010; Saijo-Hamano et al., 2010), but in the only structure of the assembled nonamer this interaction isn't seen. A structure of the assembled ring in the open state is needed to confirm observations that have been made with the monomeric cytoplasmic domain.

The transmembrane domain of FlhA/SctV is less well understood than the cytoplasmic domain. It is thought to contain approximately 8 transmembrane helices and conduct protons in order to use the pmf to power type III secretion (Minamino et al., 2016). Consistent with this function, the transmembrane domain contains a number of conserved charged residues essential for function (Erhardt et al., 2017). Structural studies of the membrane domain have been hampered by the difficulties in producing the full-length membrane protein in the assembled state. In addition to the predicted transmembrane helices the membrane domain also contains a short stretch of highly conserved soluble residues known as the FHIPEP domain (McMurry et al., 2004). It is known to be crucial for protein secretion, but its precise role in the secretion process remains to be elucidated (Barker et al., 2016; Erhardt et al., 2017).

We produced assembled rings of full-length FlhA/SctV from both flagellar and virulence associated systems and studied them using cryo-EM. Here, we present the structures of the nonameric cytoplasmic domain of both FlhA and SctV, the first structures of the assembled nonamer in the open state. The structures demonstrate the conserved interaction between $FlhA_L$/$SctV_L$ and the neighbouring subunit and further confirm the conserved stoichiometry of the complex.

Results

We screened a number of homologues of FlhA/SctV from many species for assembly in the *E. coli* membrane by employing GFP fusions and assaying fluorescent spot formation in the cell envelope, indicating membrane protein complex formation (Diepold et al., 2011; Li and Sourjik, 2011; Morimoto et al., 2014). FlhA from the lateral flagella of Vibrio parahaemolyticus ($FlhA_{Vp}$) and SctV from Yersinia enterocolitica ($SctV_{Ye}$) were found to assemble in *E. coli* (Fig 1a) and could be produced in large quantities at high purity (Fig 1b) using the gentle detergent LMNG. When the complex was subjected to gel filtration, a high molecular weight complex was isolated (Fig 1c).

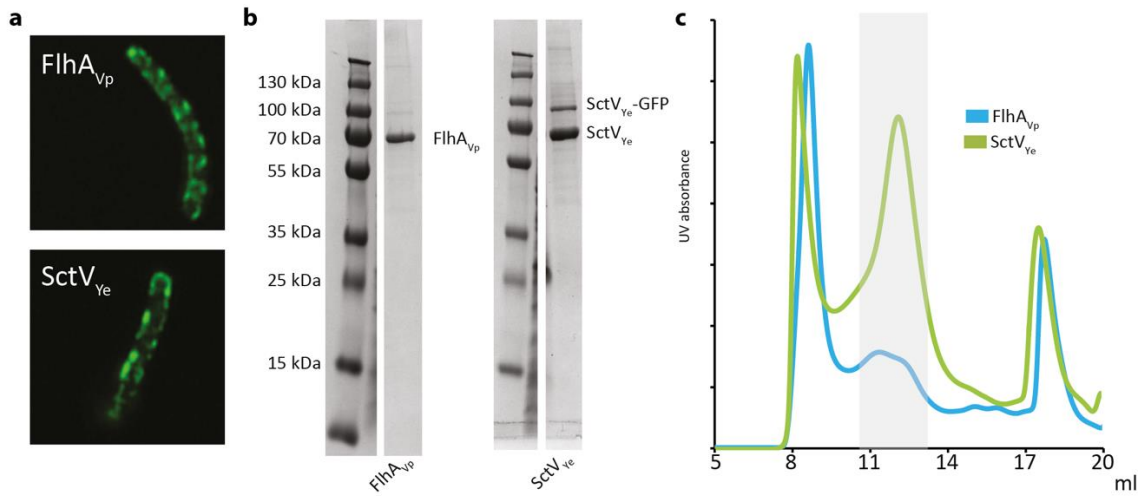

Figure 1 ***FlhA from Vibrio parahaemolyticus and SctV from Yersinia enterocolitica assemble into a large complex in the membrane of E. coli.***
a. Fluorescence images of *E. coli* cells expressing GFP tagged $FlhA_{Vp}$ or $SctV_{Ye}$ in the cell envelope. **b.** SDS-PAGE of purified $FlhA_{Vp}$ and $SctV_{Ye}$ following cleavage of GFP. **c.** Gel filtration traces of purified $FlhA_{Vp}$ and $SctV_{Ye}$. The grey shaded area indicates fractions collected to make cryoEM grids.

We imaged the purified $FlhA_{Vp}$ and $SctV_{Ye}$ complexes using cryo-EM and analysed them using single particle analysis in Relion (Zivanov et al., 2018). Particle classification revealed that surprisingly, $SctV_{Ye}$ had formed a dimer of nonamers and we imposed D9 symmetry for 3D refinement, resulting in a 3.7 Å reconstruction of the cytoplasmic domain double nonamer (Fig 2a). No high-resolution features of the transmembrane domain could be discerned. The $SctV_{Ye}$ cytoplasmic domain 18-mer is made up of two nonamers dimerising via the cytoplasmic face of the cytoplasmic domain, an interaction which is not consistent with tomographic studies of the injectisome (Hu et al., 2017). The 18-mer is most likely formed by a nonphysiological association of two nonamers, perhaps due to the absence of other proteins such as YscX and YscY that are known to bind to $SctV_{Ye}$ in the native T3SS (Diepold et al., 2012).

In contrast, $FlhA_{Vp}$ was found to form the expected nonameric complex and we imposed C9 symmetry for particle refinement, resulting in a 3.8 Å volume of the cytoplasmic domain but little detail in the transmembrane domain (Fig 2b). Both reconstructions were of sufficient quality to allow us to build a model of the structure of the cytoplasmic domain.

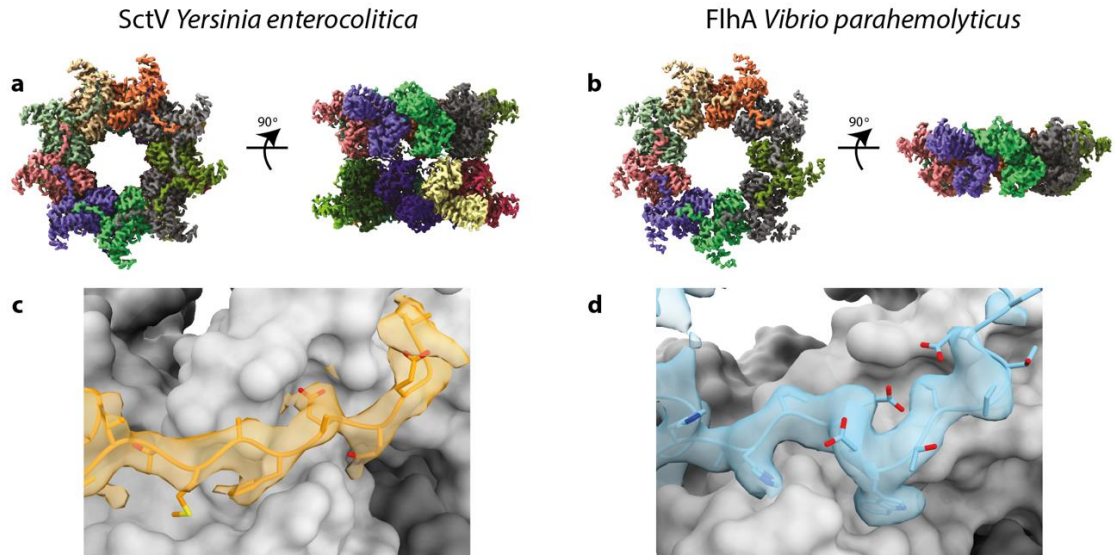

Figure 2 ***Cryo-EM volumes of the cytoplasmic domain of SctV and FlhA.***
a. 3D reconstruction of $SctV_{Ye}$ at a resolution of 3.7 Å using D9 symmetry. **b.** 3D reconstruction of $FlhA_{Vp}$ at a resolution of 3.8 Å using C9 symmetry. **c.** Model of the $SctV_{Ye}$ linker region in the cryo-EM volume (orange) in the context of the adjacent protomer (grey).

d. Model of the $FlhA_{Vp}$ linker region in the cryo-EM volume (blue) in the context of the adjacent protomer (grey).

A striking feature of both structures is the N-terminal density of the cytoplasmic domain, formed by $FlhA_L$/$SctV_L$, which reaches out to bind to the cytoplasmic face of the adjacent protomer (Fig 2c), demonstrating this well studied interaction for the first time in the context of the full-length protein in both flagellar and injectisome T3SS. Biochemical studies have indicated that the interaction between $FlhA_L$ and the neighbouring subunit is important for assembly of the nonameric ring (Terahara et al., 2018), however, the interaction has so far only been observed in crystal structures of monomeric protein (Bange et al., 2010; Saijo-Hamano et al., 2010) and $SctV_L$ is not ordered in the crystal structure of the assembled $SctV_{Sf}$ nonamer (Abrusci et al., 2013). In both of our structures a hydrophobic residue, Trp350 in $FlhA_{Vp}$ and Phe367 in $SctV_{Ye}$, sticks into a hydrophobic groove on the neighbouring subunit. This residue in FlhA has previously been shown to be involved in ring formation of the cytoplasmic domain of FlhA (Terahara et al., 2018).

The overall dimensions of the nonameric rings are similar to that of the crystal structure of $SctV_{Sf}$ (Fig 3a), but the individual subunits are in a different state. FlhA/SctV is known to be able to adopt at least two conformations, open and closed, and $FlhA_L$/$SctV_L$ is thought to bind to the membrane proximal face of the neighbouring subunit only in the open state (Inoue et al., 2019). Comparison of our structures with FlhA from *S.* Typhimurium, $FlhA_{ST}$, in the open state and $SctV_{Sf}$, which is in the closed state shows that both of our complexes are in the open state (Fig 3b), with $SctV_{Ye}$ being the first example of an SctV homologue in the open state. The demonstration of the open state opens up the possibility that SctV binds to substrate-chaperone complexes in an analogous manner to what has been described for FlhA (Xing et al., 2018), in which chaperones bind between SD4 and SD2 and are sterically unable to access the binding site in the closed state.

A conformational change from open to closed state would be expected to lead to further changes throughout the protein. We simulated the movement of $FlhA_L$ by overlaying one subunit of $FlhA_{Vp}$ onto the SD3 or SD2 subdomain of the closed $SctV_{Sf}$ structure (Fig 3c). This demonstrates that adoption of the closed state in one subunit would affect the neighbouring subunit, potentially propagating the conformational change in a wave around the ring. An additional effect on the transmembrane domain is possible.

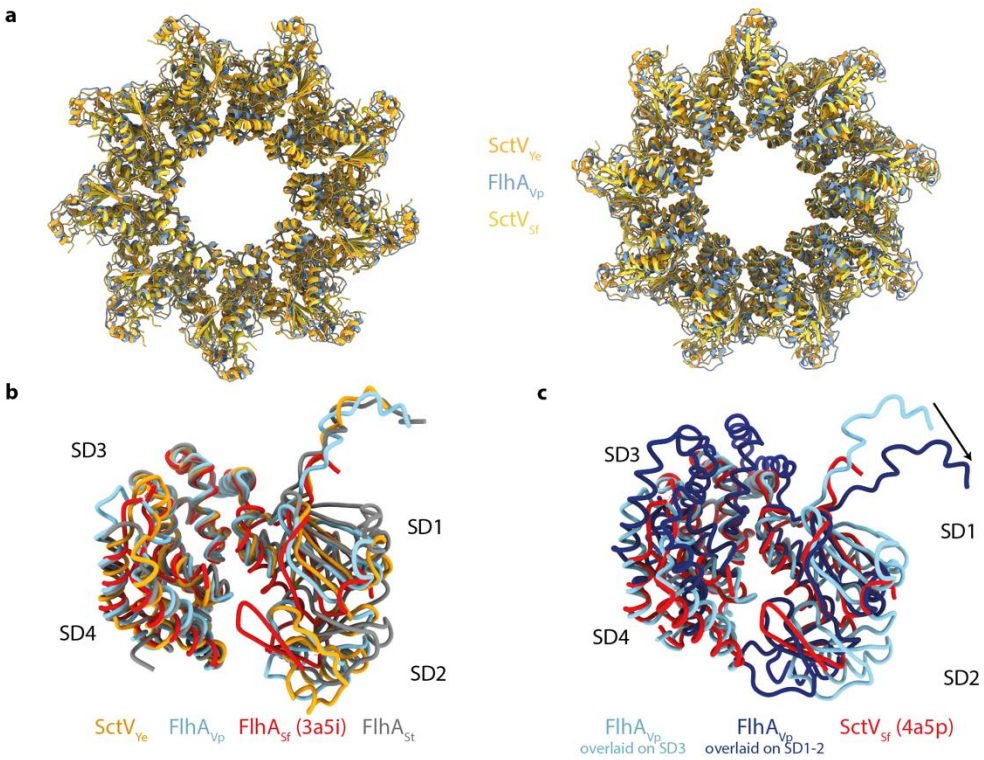

Figure 3 ***The cryo-EM structures of SctV$_{Ye}$ and FlhA$_{Vp}$ are in the open state.***
a. Overlay of the nonameric rings of the cytoplasmic domains of SctV$_{Ye}$ and FlhA$_{Vp}$ with the crystal structure of the SctV$_{Sf}$ nonameric ring (PDB: 4A5P). **b.** Overlay of a single subunit of SctV$_{Ye}$ and FlhA$_{Vp}$ with FlhA$_{ST}$ in the open state (grey, PDB: 3a5i) and SctV$_{Sf}$ in the closed state (red, PDB: 4a5p). **c.** Overlay of FlhA$_{Vp}$ (light blue) on the SD3 domain of SctV$_{Sf}$ (red) and overlay (dark blue) on the SD1 and SD2 domains of SctV$_{Sf}$ illustrating the movement of the linker (arrow) as the protein changes from the open to the closed state.

Discussion

Full-length FlhA/SctV forms a very fragile, detergent sensitive membrane protein complex. Here, we have identified particularly stable FlhA/SctV sequences and purification conditions that have allowed preparation of the assembled ring complex for structural studies. We have determined the first nonameric structures of the cytoplasmic domain of FlhA/SctV in the open state. Multiple studies have previously implicated the FlhA linker region, FlhA$_L$, in promoting complex formation. We provide the first structural view of this interaction in the assembled state in both FlhA and SctV, showing that SctV$_L$ is the functional equivalent of FlhA$_L$ in the stabilisation of the nonameric assembly.

It has been proposed that the closed state of the FlhA/SctV cytoplasmic domain interacts with early secretion substrates, while later substrates bind to the open state (Inoue et al., 2019) which is induced by completion of the first step of filament assembly. Both of our structures are in the open state. Our structures are not affected by crystallisation artefacts and are in the context of the full-length protein, suggesting that the open state is the default state in the absence of other factors. Our structures also suggest that at least in the open state the residues known as FlhA$_L$/SctV$_L$, which have previously been described as the linker between cytoplasmic and membrane domains, do not form part of this linker but are

part of the cytoplasmic domain. Instead, the residues N-terminal to $FlhA_L/SctV_L$ must be responsible for linking the membrane domain to the cytoplasmic domain.

Unfortunately, we were not able to determine the structure of the membrane domain of either complex, possibly due to damage to the fragile membrane domain at the air-water interface during cryo-EM sample preparation (D'Imprima et al., 2019). However, two features are clear in 2D averages of the side view of both complexes. The cytoplasmic and membrane domains are at a set distance from each other and this distance matches that observed in tomographic reconstructions of the T3SS (Fig 4a). This suggests that the linker between the domains is not flexible, which would be inconsistent with a previously suggested model of secretion according to which the cytoplasmic domain is tethered to the membrane by a flexible linker and moves towards the membrane domain and back into the cytoplasm in a cyclical fashion (Erhardt et al., 2017).

The methods developed here may be the basis of future studies of the FlhA/SctV transmembrane domain or interactions of the cytoplasmic domain with chaperones and substrates in the context of the assembled complex.

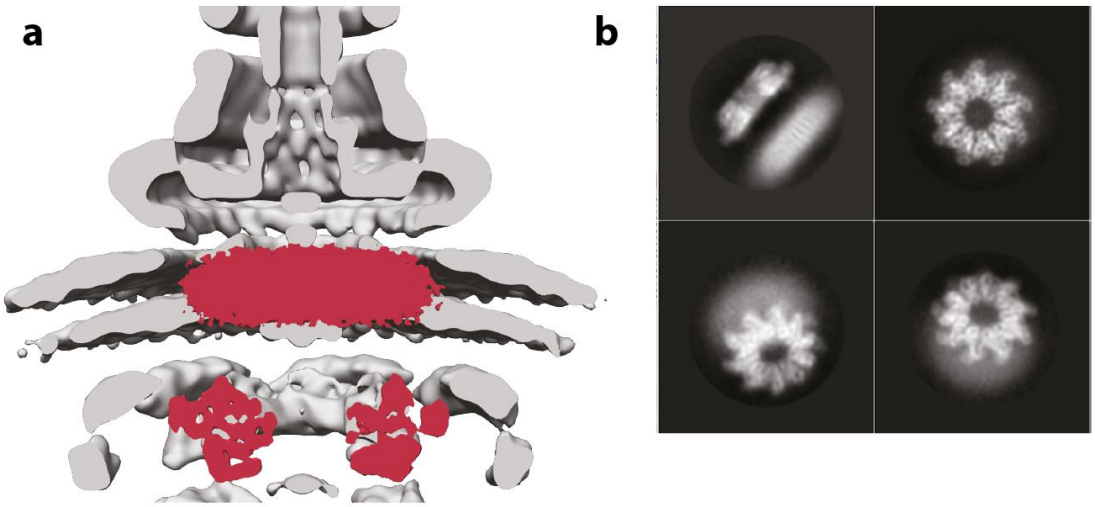

Figure 4 ***Position of the membrane domain of FlhA in the T3SS nanomachine.***
a. The volume of $FlhA_{Vp}$ is shown in red overlaid onto the tomographic reconstruction of the injectisome (EMD-8544). **b.** Selected 2D class averages of $FlhA_{Vp}$ show the high level of detail in the cytoplasmic domain and the lower information content in the presumed transmembrane, micelle-embedded, portion.

Methods

Protein purification
FlhA and SctV were produced as GFP fusion proteins in *E. coli* BL21 by expressing them from a pt12 plasmid (Dietsche et al., 2016). Cells were grown in terrific broth supplemented with rhamnose monohydrate (0.1%) and harvested after overnight growth. Cells were

resuspended in TBS (100 mM Tris, 150 mM NaCl, 1 mM EDTA) and lysed using an Emulsiflex homogeniser (Avestin). Membranes were purified by ultracentrifugation of the clarified lysate at 235,000g and solubilised in 1% LMNG using 0.1 grams of detergent per gram of membrane pellet. After gentle stirring for one hour aggregates were removed by centrifugation at 75,600g and the solubilised membrane proteins were applied to a StrepTrap column (GE healthcare) which was then washed in TBS supplemented with 0.01% LMNG. Pure protein was eluted in TBS containing 0.01% LMNG and 10 mM desthiobiotin. GFP was cleaved off by incubating with tev protease overnight. Next, the protein was concentrated and further purified by gel filtration using a superpose 6 increase (GE healthcare) in TBS containing 0.01% LMNG. The peak fraction, eluting close to 12 ml, was collected.

Cryo-EM analysis

3 µl of purified protein at a concentration between 0.8 and 1 mg/ml was deposited on a glow-discharged Quantifoil grid (2/2, 300 mesh for FlhA, 1.2/1.3, 300 mesh for SctV). Grids were blotted and plunged into liquid ethane using a Vitrobot Mark IV (FEI). The samples were imaged using a Titan Krios (FEI) equipped with a K2 detector (Gatan). Relion's implementation of MotionCor2 (Zivanov et al., 2019) was used for motion correction and SIMPLE's implementation of CTFFIND4 was used for CTF estimation (Reboul et al., 2018). Particles were picked using SIMPLE and classified in Relion3.0 (Zivanov et al., 2018). Atomic models were built in Coot (Emsley et al., 2010) and refined using phenix (Afonine et al., 2018).

Fluorescence imaging

8 µl of an overnight culture of *E. coli* BL21 expressing FlhA-GFP or SctV-GFP were applied to a glass slide and imaged using a Zeiss 880 inverted microscope equipped with a plan-apochromat 63x/1.4 NA objective and an Airyscan detector. GFP fluorescence was excited using a laser (488 nm).

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Data Availability

Coordinates and associated volumes and metadata have been deposited in the PDB and EMDB respectively. SctV EMD-11820 / PDB 7ALW, FlhA EMD-11827 / PDB 7AMY.

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	FlhA EMD- 11827 PDB 7AMY	YscV EMD-11820 PDB 7ALW
Data collection and processing		
Magnification	165,000	165,000
Voltage (kV)	300	300
Electron exposure (e−/Å2)	48	48
Defocus range (μm)	0.5–4	0.5–4
Pixel size (Å)	0.822	0.822
Symmetry imposed	C9	D9
Final particle images (no.)	9756	15913
Map resolution (Å)	3.75	3.7
FSC threshold	0.143	0.143
Refinement		
Initial model used	$FlhA_{ST}$ (3a5i)	$SctV_{Sf}$ (4a5p)
Model resolution (Å)	3.75	3.7
FSC threshold	0.143	0.143
Map sharpening B factor ($Å^2$)	-72	-58
Model composition		
Non-hydrogen atoms	24,849	50,148
Protein residues	3177	6192
Ligands	0	0
B factors ($Å^2$)		
Protein	64.56	57.65
R.m.s. deviations		
Bond lengths (Å)	0.0096	0.0099
Bond angles (°)	0.88	0.95
Validation		
MolProbity score	2.44	2.39
Clashscore	20.06	15.19
Poor rotamers (%)	0.66	0.98
Ramachandran plot		
Favoured (%)	85.94	82.37
Allowed (%)	13.78	17.33
Disallowed (%)	0.28	0.29

Table 1. CryoEM data collection, processing and model statistics