Bacteria share their ecological niches with other microbes. The bacterial type VI secretion system is one of the key players in microbial competition, as well as being an important virulence determinant during bacterial infections. It assembles a nano-crossbow-like structure in the cytoplasm of the attacker cell that propels an arrow made of a haemolysin co-regulated protein (Hcp) tube and a valine–glycine repeat protein G (VgrG) spike and punctures the prey’s cell wall. The nano-crossbow is stably anchored to the cell envelope of the attacker by a membrane core complex. Here we show that this complex is assembled by the sequential addition of three type VI subunits (Tss)—TssJ, TssM and TssL—and present a structure of the fully assembled complex at 11.6 Å resolution, determined by negative-stain electron microscopy. With overall C2 symmetry, this 1.7-megadalton complex comprises a large base in the cytoplasm. It extends in the periplasm via ten arches to form a double-ring structure containing the carboxy-terminal domain of TssM (TssMct) and TssJ that is anchored in the outer membrane. The crystal structure of the TssMct–TssJ complex coupled to whole-cell accessibility studies suggest that large conformational changes induce transient pore formation in the outer membrane, allowing passage of the attacking Hcp tube/VgrG spike.

Localization, dynamics and biogenesis of the T6SS membrane core complex

We first sought to determine the assembly pathway of the enteric-aggregative Escherichia coli (EAEC) T6SS membrane core complex. Strains producing fluorescently labelled T6SS membrane subunits were engineered. The sequence encoding the super-folder green fluorescent protein (sfGFP) was inserted upstream of the stop codon of the tssL gene or downstream of the start codon of the tssL and tssM genes. In these constructs, the fusion proteins were produced from their native chromosomal loci. Hcp release and anti-bacterial assays demonstrated that the sfGFP–TssL and sfGFP–TssM fusion proteins were functional (Extended Data Fig. 1a). By contrast, strains producing TssJ–sfGFP or TssJ–mCherry had a non-functional T6SS. Co-localization experiments with strains producing TssJ–sfGFP or TssJ–mCherry had a non-functional T6SS. These proteins are connected through a network of interactions between TssM and TssL, and TssM and TssJ. Although the localization and topology of these subunits, their interactions and the crystal structures of the soluble domains of TssJ and TssL have been described, we still lack crucial information on the biogenesis and overall architecture of this complex and how it is used as a channel during T6SS action.

Location, biogenesis and structure of a type VI secretion membrane core complex

The T6SS is composed of 13 different proteins, encoded by genes that are usually clustered. It assembles a tubular puncturing device that is evolutionarily, structurally and functionally similar to the tail of contractile bacteriophages. Its sheath is a tubular structure, hundreds of nanometres long, that extends in the cytoplasm and is built by the polymerization of TssBC building blocks. It is assembled on an assembly platform, the baseplate, and maintained in an extended, metastable conformation. The attacking arrow, wrapped by the sheath, comprises an inner tube that is built by stacked Hcp hexameric rings and tipped by a puncturing spike composed of VgrG. Upon contact with the prey, structural rearrangements of the sheath subunits induce its contraction and propulsion of the Hcp tube/VgrG spike towards the target cell, allowing toxin delivery. The phage-like T6SS tail is anchored to the attacker cell membrane by a trans-envelope complex. This membrane complex not only serves as a docking station but has been proposed as a channel for the passage of the inner tube after sheath contraction, thereby preventing membrane damage in the attacker. The membrane core complex of the T6SS (that is, the minimal module required to function and conserved in all T6SS) is composed of the TssL and TssM inner-membrane proteins and the TssJ outer membrane lipoprotein. These proteins lack crucial information on the biogenesis and overall architecture of this complex and how it is used as a channel during T6SS action.

In the environment, bacteria have evolved collaborative or aggressive mechanisms to communicate, exchange information and chemicals, or compete for space and resources. One of the main weapons of bacterial conflicts is a multi-protein device called the type VI secretion system (T6SS) that is assembled in the attacker bacterium. The T6SS bacterial activities allow the bacterium to destroy competitors and to have a privileged access to the niche, to nutrients or to new DNA. These authors contributed equally to this work.

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**ARTICLE**

**Biogenesis and structure of a type VI secretion membrane core complex**

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Figure 1 | Biogenesis of the T6SS membrane-associated core complex. a, Time-lapse fluorescence microscopy recordings showing localization and dynamics of the sfGFP–TssM and sfGFP–TssL fusion proteins. Individual images were taken every 30 s. The positions of the foci are indicated by arrowheads. Scale bars, 1 μm. Larger fields are presented in Extended Data Fig. 1c. b, Statistical analysis of sfGFP–TssM and sfGFP–TssL localization. Shown are box-and-whisker plots of the measured number of sfGFP–TssM and sfGFP–TssL foci per cell for each strain with the lower and upper boundaries of the boxes corresponding to the 25% and 75% percentiles respectively. The black horizontal bar represents the median values for each strain and the whiskers represent the 10% and 90% percentiles. The number of cells studied per strain is indicated above the bars. c, Spatial repartition of the sfGFP–TssM and sfGFP–TssL foci. Shown is a superposition of the different foci analysed in a single cell. d, sfGFP–TssM and mCh–TssL proteins co-localize. Fluorescence microscopy recordings showing co-localization between sfGFP–TssM and mCh–TssL fusion proteins. The positions of the foci are indicated by the arrowheads. Scale bars, 1 μm. e, The membrane complex serves as a docking site for tail sheath polymerization. Time-lapse fluorescence microscopy recordings showing co-localization between sfGFP–TssL and TssB–mCh fusion proteins. Individual images were taken every 30 s. Assembly/contraction of the sheath and TssL localization events is schematized in the bottom row of panels. Scale bars, 1 μm. f, Assembly pathway of the T6SS TssJLM membrane complex. Fluorescence microscopy recordings showing sfGFP–TssM and sfGFP–TssL localization in the absence of the TssJ or TssL and TssJ or TssM proteins respectively. The positions of the foci are indicated by the arrowheads. Scale bars, 1 μm. The quantification of the sfGFP–TssM and sfGFP–TssL clusters per cell is presented in Extended Data Fig. 1g. g, Schematic representation of the sequential biogenesis of the T6SS membrane complex. The names of the proteins, their localizations and topologies are shown.

Architectural evidence for the T6SS membrane complex core

To gain further insights on the architecture of the T6SS membrane complex core, the tssJ, tssL and tssM genes were co-expressed in E. coli BL21(DE3). Constructs were designed to add StrepII, Flag and 6×His tags at the carboxy (C) terminus of TssM, amino (N) terminus of TssL and N terminus of TssM, respectively (Extended Data Fig. 2). Total membranes were isolated and solubilized using detergents. Two-step affinity chromatography followed by gel filtration resulted in the purification of a complex containing TssJ, TssL and TssM (Fig. 2a and Extended Data Fig. 2f–h). In this complex, we determined the TssM–TssL stoichiometry as 1 to 1 (Extended Data Fig. 2h). Purified complexes were visualized by negative-stain electron microscopy (EM) (Fig. 2b and Extended Data Fig. 3a). A data set was collected, and reference-free classification and averaging revealed characteristic views of the complex (class averages) (Fig. 2b). We observed rocket-shaped and ring-shaped views corresponding to side...
To define how the core complex is inserted in the cell envelope, we first performed differential solubilization of the inner and outer membranes. The total membrane fraction was solubilized with N-lauryl sarcosyl, a detergent that preferentially solubilizes inner-membrane proteins. This differential solubilization resulted in the fractionation of the core complex in both inner and outer membrane fractions (Extended Data Fig. 4b), indicating that this complex resides in both membranes. To determine its orientation in the cell envelope, the purified core complex was incubated with anti-StrepII antibodies or Ni-NTA-coated gold particles targeting the TssJ C-terminal StrepII and TssM N-terminal 6×His tags respectively (Extended Data Fig. 4c), before EM analyses. Anti-StrepII antibodies labelled the tip complex/cap while the base was labelled by the Ni-NTA gold particles (Extended Data Fig. 4c). When the N-terminal cysteine residue of the TssJ lipoprotein was substituted by Ser (C1S) to prevent its acylation, an intact TssJCIS–L–M core complex was formed (Extended Data Fig. 4b), but differential solubilization proved the complex mis-localized to the inner membrane fraction only (Extended Data Fig. 4b). Hence, TssJ acylation tethers the apex of the complex to the outer membrane whereas the base of the complex is located in the cytoplasm.

We next analysed the EM reconstruction to assign the different regions of the core complex to its components. The volume corresponding to one arch and the corresponding pillar within the tip complex (Extended Data Fig. 4a) is comparable in size and shape to that of the isolated TssM periplasmic domain (amino acids 386–1129; TssMp) in complex with TssJ obtained by small-angle X-ray scattering (SAXS) (Extended Data Fig. 4d, e). Segmentation of this volume yielded five different sub-volumes (Fig. 3a). We propose that the sub-volume closest to the cap corresponds to TssJ, in agreement with its location close to the outer membrane. The other four sub-volumes would correspond to sub-domains of TssMp. Sub-volume 4 is in close contact with TssL, suggesting that it corresponds to the C-terminal domain of TssM domain, which was previously shown to mediate contact with TssJ25. With sub-volume 3, it forms the tip complex while sub-volumes 1 and 2 correspond to the arches (Fig. 3a). Interestingly, the last TssM transmembrane segment crossing the inner membrane is located just upstream of TssMp. This would place the inner membrane at the bottom of the arches or at the top of the base. The volume of the base (1,450 Å3) is much bigger than the estimated volume occupied by ten copies of the cytoplasmic domains of TssM and TssL (825 Å3). The crystal structure of the TssL cytoplasmic domain dimer28,29 could be fitted in the hooks with 88% correlation (Extended Data Fig. 4f). This indicates that the remainder of the base could correspond to the cytoplasmic domain of TssM and the 40 transmembrane segments bound to detergent (Extended Data Fig. 4f).

To gain more insight into the structure of the TssMp–TssJ complex, TssMp was produced and purified as described previously23. To help crystallization, TssMp complex was subjected to controlled proteolytic digestion24. A protease-resistant fragment of an apparent size of ~32 kDa (called hereafter TssM32Ct; residues 836–1129; Extended Data Fig. 5a) was further purified and co-crystallized with nb25, a specific camelid single-chain nanobody31,32. The structure of the TssM32Ct–nb25 complex was solved by molecular replacement using the X-ray structure of nb25 reported previously32 (Extended Data Fig. 5b and Extended Data Table 1). In the complex, the TssM32Ct amino-acid chains are defined in the electron density map between residues 868 and 1107. We therefore purified a new TssMp fragment (TssM26Ct) encompassing the crystallographic visible chain. This shorter fragment crystallized readily alone as well as in complex with the unacylated TssJ protein (Extended Data Table 1). The structure of TssM26Ct is composed of two domains. The N-terminal domain (residues 870–974) is a bundle of four α-helices, covered on one side by a β-hairpin (Fig. 3b) and on the other by the C-terminal elongated stretch of the protein. The C-terminal domain (residues 975–1085) is a nine-stranded β-sandwich.
that contacts nb25 or TssJ (Fig. 3b and Extended Table 2a, b). This C-terminal domain is followed by a stretch of residues (1086–1107) comprising helix α5 (Fig. 3b). Tss binds to the apex of the C-terminal domain, and the 590-Å² interaction area involves contacts between TssJ loops L1–2, L3–4 and L5–6 with TssM26Gct loops L3–4 and L5–6 (Extended Data Fig. 5c and Extended Data Table 2b), in agreement with a previous study demonstrating the importance of TssJ loop L1–2 for TssM–TssJ complex formation35. Superimposition of the structures of TssM26Gct–nb25 and TssM26Gct–TssJ shows that nb25 and TssJ cannot bind simultaneously to TssM (Extended Data Fig. 5d), explaining the nb25 in vivo inhibitory effect on T6SS function14. The comparison between TssM26Gct–TssJ crystal structure and the volume proposed to correspond to TssJ and domains 3 and 4 of TssMp determined by EM resulted in 95% correlation between the two structures (Fig. 3c). This confirms the location of TssM26Gct–TssJ in the tip complex (Fig. 3d).

Cell surface accessibility and transient pore formation

The orientation of the TssJ N terminus places the outer membrane above TssJ, where the cap is located (Figs 2c and 4a and Extended Data Fig. 5e). Accordingly, close inspection of the proposed oligomeric structure of the TssM26Gct–TssJ complex could not reveal any obvious transmembrane region (Extended Data Fig. 5f). To test this, we engineered functional cysteine derivatives between the β-strands of the C-terminal domain of TssM (Extended Data Fig. 6a). The extracellular accessibility of these residues was assessed by incubating whole cells with an outer membrane-impermeant cysteine-reactive maleimide. We observed that positions 989, 1005, 1035, 1075 and 1109 were labelled whereas positions 972, 1019, 1062 and 1092 were not (Extended Data Fig. 6b and Extended Data Table 2c). With the exception of position 1092, all other positions were labelled when cell lysates were used instead of intact cells (Extended Data Table 2c). The labelled cysteine substitutions are on the tip of TssM facing the outer membrane (Fig. 4a). Interestingly, residues 989 and 1005 are buried at the interface with TssJ (Extended Data Fig. 6c). Therefore, for these residues to be labelled, the TssM–TssJ complex has to dissociate. This result also suggests that the tip of TssM26Gct is exposed to the cell exterior. To test whether TssM stably crosses the outer membrane or accesses the cell exterior temporarily, similar experiments were conducted in a tssBC-deleted background. In the absence of the TssB and TssC sheath components, the TssJLM membrane complex is properly assembled but the T6SS is inactive as no sheath assembly or contraction could occur. In tssBC cells, only position 1109 was labelled (Extended Data Fig. 6b and Fig. 4a). These results suggest that the TssM α5-helix crosses the outer membrane permanently, exposing the C-terminal extension to the extracellular medium whereas part of TssM26Gct domain is exposed transiently at the cell surface during the T6SS mechanism of action.

Closing remarks and outlook

The data presented here allow an unprecedented understanding of the biogenesis, architecture and role of the T6SS TssJLM membrane core complex. This complex anchors the phage tail-like structure to the cell envelope and is thought to serve as conduit to guide the Hcp tube/VgrG spike upon sheath contraction15–17. Using fluorescence microscopy, we demonstrate that the three subunits are recruited in a specific order, starting from the outer membrane TssJ lipoprotein and pursued by the sequential addition of TssM and TssL, a hierarchy in agreement with previously published localization and interaction studies17,22–27. Therefore, T6SS biogenesis is initiated by an outer membrane lipoprotein nucleation factor and progresses inwards, like the assembly mechanisms of other bacterial secretion systems13–19. Our fluorescence microscopy analyses also showed that the T6SS membrane core complex assembles randomly in the cell envelope, without specific localization. The complex is stable and can be used for several events of sheath assembly/contraction, increasing the amount of toxin effectors delivered to the target cell.

The TssJLM complex has a five-fold symmetry and is composed of ten copies of each component that assemble a 1.7-MDa structure crossing the inner membrane, the periplasm and anchored to the outer membrane via the TssJ N-terminal lipid moiety. Its architecture is unique compared with other trans-envelope bacterial secretion systems13–19. Our fluorescence microscopy analyses also showed that the T6SS membrane core complex assembles randomly in the cell envelope, without specific localization. The complex is stable and can be used for several events of sheath assembly/contraction, increasing the amount of toxin effectors delivered to the target cell.
Figure 4 | Cell surface accessibility and mechanism of action of the T6SS membrane core complex during secretion. a. Cell surface accessibility studies. Crystal structure of the TssM26Ct, represented as ribbons, coloured cyan (α-domain) and blue (β-domain). The C-terminal α5-helix and the extended stretch are coloured magenta. The C terminus (lacking in the crystallized fragment) is represented as a random structure beyond the last residue in the crystallographic model. The cysteine substitutions (in sphere representation) used for labelling experiments are positioned in the TssM26Ct crystal structure. Cysteines with extracellular accessibility when the T6SS is active are coloured yellow, while the unlabelled ones are coloured grey. b. Model of action. The proposed mechanism of action involves five sequential stages. Stage 1: the assembled TssLM complex is not integrally inserted in the outer membrane, but anchored to it by the TssJ N-terminal lipid moiety. This stage corresponds to the ‘resting’ state of the T6SS machinery (Fig. 4b, stage 2). Ten arches cross the periplasm and are followed by ten pillars positioned in two concentric layers in the tip complex. The inner pillars define a channel of 15–20 Å in diameter that is not large enough to allow the passage of the ~110 Å Hcp tube16–18 (Extended Data Fig. 7b). Interestingly, it was previously shown that TssM undergoes large conformational changes during secretion26. Therefore, we propose that the inner TssM pillars are pushed outwards to define a wider TssM ring with internal dimensions compatible with the passage of the Hcp tube/VgrG spike (Fig. 4b, stages 3 and 4, and Extended Data Fig. 7b, c). In other secretion systems, specific components are dedicated to assemble the outer membrane pore. No obvious transmembrane region could be found in the TssM C-terminal domain or in TssJ. It is unlikely that the C-terminal portion of TssM would form a pore of sufficient dimension by itself. Therefore, we propose that the stroke of the Hcp–VgrG arrow would mechanically push the C-terminal TssM domain towards the cell exterior, allowing the transient formation of a pore through the outer membrane (Fig. 4b, stage 4). To avoid deleterious effects for the bacterium, one may expect that the C-terminal domain of TssM returns to its initial ‘resting’ conformation at the periplasmic face of the outer membrane once the Hcp tube has been released, closing the outer membrane channel (Fig. 4b, stage 5). Overall, the membrane core complex appears to act like a docking station for the phage-like T6SS device. It nucleates the assembly of the rest of the secretion system and then guides the Hcp tube/VgrG spike through the bacterial cell envelope upon sheath contraction. Further studies will be necessary to fully understand the complete assembly process of the T6SS, the trigger that releases sheath contraction and how the Hcp tube/VgrG spike crosses both bacterial and host membranes.

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

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Strains, media and chemicals. The strains, plasmids and oligonucleotides used in this study are listed in Supplementary Table 1. The E. coli K-12 DH5s strain was used for cloning steps whereas E. coli K-12 BL21(DE3) and T7-1q-plys strains were used for protein purification. The enterogaugregative E. coli AEAC strain 17-2 was used to engineer gene knockouts and fusions with fluorescent labels. Strains were routinely grown in lysogeny broth (LB) rich medium (or Terrific broth medium for protein purification) or in Sci-1-inducing medium (SIM; M9 minimal medium, glycerol 0.2%, vitamin B1 1 μg ml−1, casaminoacids 100 μg ml−1, LB 10%, supplemented or not with bacitracin 1.5%) with shaking at 37°C. Plasmids were maintained by the addition of ampicillin (100 μg ml−1 for E. coli K-12, 200 μg ml−1 for AEAC) or kanamycin (50 μg ml−1). Expression of genes from pET28a and pRSF vectors was induced with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG, Eurobio) for 16 h.

Strain construction. Gene deletion into the enterogaugregative E. coli 17-2 strain was achieved by using a modified one-step inactivation procedure4 as previously described23 using plasmid pKOBEG24. Briefly, a kanamycin cassette was amplified from plasmid pKD43 using oligonucleotide pairs carrying 5’-3’ nucleotide extensions homologous to regions adjacent to the gene to be deleted. After electroporation of 600 ng of column-purified PCR product, kanamycin-resistant clones were selected and verified by colony-PCR. The kanamycin cassette was then excised using plasmid pCP20 (ref. 41). Gene deletions were confirmed by colony-PCR. The same procedure was used to introduce the mCherry- or sfGFP-coding sequences downstream from the stop codon (vector pKD4-sfGFP or pKD4-mCherry) or the mCherry-coding sequence upstream from the stop codon (vector pwtCherry-KD4). This procedure yields strains producing fusion proteins from their original chromosomal loci.

Plasmid construction. PCRs were performed using the Phusion DNA polymerase (Thermo Scientific). Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer’s instructions. Custom oligonucleotides were synthesized by Sigma Aldrich and are listed in Supplementary Table 1. Enterogaugregative E. coli 17-2 chromosomal DNA was used as a template for all PCRs. E. coli strain DH5s was used for cloning procedures. The pET28CA vector derivative encoding the periplasmic domain of the TssM periplasmic domain (TssMp, residues 386–1129) or the TssJ soluble domain have been previously described23. The fragment encoding TssMp3 (residues 869–1107) was cloned into the pET22A vector by restriction-free cloning45. The pRSF–TssJ intermediate plasmid was constructed by restriction cloning. Briefly, the sequence encoding the full-length tssJ gene (residues 1–178) was PCR-amplified using primers RSF-sJ-LF and RSF-sJ-R. The PCR product was subsequently cloned into the pRSF-Duet (Novagen) MCS2 corresponding restriction sites. The pRSF–TssJ–3’-L23M (encoding C-terminally StopII-tagged TssJ, N-terminally Flag-tagged TssJ and N-terminally 6×His-tagged TssM) was constructed by restriction-free cloning as previously described23. Briefly, the sequence encoding the full-length tssJ (residues 1–217) and full-length tssM (residues 1–1129) genes were PCR-amplified using the primer pairs RSF-L-F/RSF-L-R and RSF-M-F/RSF-M-R, respectively. The two PCR products (tssJ and tssM) were synthesized with 30-base-pair overhangs, from both 5’ and 3’ ends, corresponding to the designed integration sites into the pRSF–TssJ plasmid. The double-stranded product of the first PCR was then used as oligonucleotides for a second PCR using the target vector as template. The introduction of the Cis mutation in TssJ was performed by QuikChange mutagenesis of the pRSF–TssJ–3’-L23M plasmid using oligonucleotides Jcs-F and Jcs-R. Plasmid pBRA37-TssM was constructed by restriction-free cloning and cytochrome derivatives were obtained by QuikChange mutagenesis using pBRA37-TssM-C757S mutant as template.

Hcp release assay. Cells producing Flag- or HA-tagged Hcp from plasmids pUCHCpHA or pDCKPHA42,43 were grown in SIM to an absorbance A600 nm ~ 0.8. Supernatant were harvested and centrifuged at 10,000 × g for 5 min. The supernatant fraction was then subjected to a second low-speed centrifugation and then at 16,000 × g for 15 min. The supernatant was filtered on sterile polyester membranes with a pore size of 0.2 μm (Membrex 25 PET, membraPure) before overnight precipitation with trichloroacetic acid 15% on ice. Cells and precipitated supernatants were resuspended in loading buffer and analysed by SDS-PAGE and immunoblotting with the anti-Flag or anti-HA antibody. As control for cell lysis, western blots were probed with antibodies raised against the periplasmic TolB protein. The assays were performed from three independent cultures, and a representative experiment is shown.

Interbacterial competition assay. The antibacterial growth competition assay was performed as described for the studies on the Citrobacter rodentium and EAEC Sci-2 T6SS7,8,9 with modifications. The wild-type E. coli strain W3110 bearing the Kan’ pU666-ramB plasmid23 was used as prey in the competition assay. Attacker and prey cells were grown for 16 h in LB medium, then diluted in SIM to allow maximal expression of the sci-I gene cluster40. Once the culture reached A600 nm ~ 0.8, the cells were harvested and normalized to A600 nm = 0.5 in SIM. Attacker and prey cells were mixed to a 4:1 ratio and 20-mm drops of the mixture were spotted in triplicate on a pre-warmed dry SIM agar plate supplemented or not with anhydrotetracyclin 0.02 μg ml−1. After overnight incubation at 37°C, the bacterial spots were then cut off, and cells were resuspended in SIM to A600 nm = 0.5. Two hundred microliters of serial dilutions were plated on kanamycin LB plates and the number of colonies was scored after overnight incubation at 37°C. The assays were performed from at least three independent cultures, with technical triplicates and a representative technical triplicate shown.

Fluorescence microscopy, image treatment and statistical analyses. Fluorescence microscopy experiments were performed essentially as described23,35,36. Briefly, cells were grown overnight in LB medium and diluted to A600 nm ~ 0.04 in SIM. Exponentially growing cells (A600 nm = 0.8–1) were harvested, washed in phosphate buffered saline buffer (PBS), resuspended in PBS to A600 nm ~ 50, spotted on a thin pad of 1.5% agarose in PBS, covered with a cover slip and incubated for 1 h at 37°C before microscopy acquisition. For each experiment, ten independent fields were manually defined with a motorized stage (Prior Scientific) and stored (x, y, z, Perfect Focus System (PFS) offset) in our custom automation system designed for time-lapse experiments. Fluorescence and phase contrast micrographs were captured every 30 s using an automated epifluorescence microscope TE2000-E-PFS (Nikon) equipped with PFS. PFS automatically maintains focus so that the point of interest within a specimen is always kept in sharp focus at all times despite mechanical or thermal perturbations. Images were recorded with a CoolSNAP HQ 2 (Roper Scientific) and a x100/1.4 DII objective. The excitation light was emitted by a 120 W metal halide light. All fluorescence images were acquired with a minimal exposure time to reduce bleaching and phototoxicity effects. The sfGFP images were recorded by using the ET-GFP filter set (Chroma 49002) with an exposure time of 200–400 ms. The mCherry images were recorded by using the ET-mCherry filter set (Chroma 49008) using an exposure time of 100–200 ms. Slight movements of the whole field during the time of the experiment were corrected by registering individual frames using StackReg and Image Stabilizer plugins for ImageJ. sfGFP and mCherry fluorescence channels were adjusted and merged using ImageJ (http://rsb.info.nih.gov/ij/). sfGFP fluorescence sets of data were treated to monitor foci detection. Noise and background were reduced using the ‘Subtract Background’ (20 pixels Rolling Ball) plugin from Fiji (ImageJ/National Institutes of Health). The sfGFP foci were automatically detected by simple image processing: (1) create a mask of cell surface and dilate, (2) detect the individual foci using Analyze Particles plugin of Fiji and (3) apply the ‘Find Maxima’ process in Fiji. To avoid false positive results, each event was manually controlled in the original data. Microscopy analyses were performed at least six times, each in technical triplicate, and a representative experiment is shown. Box-and-whisker plots representing the number of detected foci for each strain were made with R software. To compare each population, t-tests were performed in R. Sub-pixel resolution tracking of fluorescent foci: Fluorescent foci were detected using a local and sub-pixel resolution maxima detection algorithm and tracked over time with a specifically developed plug-in for ImageJ. The x and y coordinates were obtained for each fluorescent focus on each frame. The mean square displacement was calculated as the distance of the foci from its location at t0 at each time using R software and plotted over time. For each strain tested, the mean square displacement of at least ten individual focus trajectories was calculated. Inner and outer membrane separation. Cells were broken using an Emulsiflex-C5 (Avestin) and the crude membrane fraction was isolated by ultracentrifugation at 100,000 g for 45 min. Outer and inner membranes were separated by differential solubilization. Inner membranes were solubilized by 0.5% sodium N-lauroyl sarcosyl in 50 mM Tris-HCl pH 8.0 for 30 min at 20°C. The insoluble material containing the outer membrane fraction was isolated by ultracentrifugation at 100,000 g for 20 min. The outer membrane pellet was then solubilized in SDS-loading buffer. The assay was performed in triplicate, from three independent cultures and a representative experiment is shown. TssJLM complex production and purification. The pRSF–TssJ–3’-L23M plasmid was transformed into the E. coli BL21(DE3) expression strain (Invitrogen). Cells were grown at 37°C in lysogeny broth (LB) to A600 nm ~ 0.7 and the expression of the tssJLM genes was induced with 1.0 mM IPTG for 16 h at 16°C. Cell pellets were resuspended in ice-cold 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA.
and 1 mM TCEP, supplemented with 100 μg ml⁻¹ of DNPase I, 100 μg ml⁻¹ of lysozyme and EDTA-free protease inhibitor (Roche). After sonication, MgCl₂ was added to the final concentration of 10 mM and the cell suspension was further broken using an Emulsiflex-C5 (Avestin). The broken cell suspension was clarified by centrifugation at 38,500g for 20 min. The membrane fraction was then recovered by centrifugation at 98,000g for 45 min. Membranes were mechanically homogenized and solubilized in 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.5% (w/v) n-dodecyl-b-D-maltopyranoside (DDM, Anatrace), 0.75% (w/v) decyl maltose non-entrainer (DM-NGP, Anatrace), 0.5% (w/v) digitonin (Sigma-Aldrich), 100 mM TCEP and 1 mM EDTA at 22 °C for 45 min. The suspension was clarified by centrifugation at 98,000g for 20 min. The supernatant was loaded onto a 5-ml StrepTrap HP (GE Healthcare) column and then washed with 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.05% (w/v) DM-NGP (Affinity buffer). The flow-through was then washed in the affinity buffer supplemented with 200 mM imidazole and the TssJ/L core complex was eluted in the same buffer supplemented with 500 mM imidazole. Peak fractions were pooled and loaded onto a Superose 6 10/300 column (GE Healthcare) equilibrated in 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.025% (w/v) DM-NGP. The TssJ/L core complex eluted as a single monodisperse peak close to the void volume of the column. The sample was used immediately for EM sample preparation.

Stoichiometry analyses. The purified TssJ/L core membrane complex was diluted to a final concentration of 0.1 mg ml⁻¹ and denatured at 100 °C for 10 min after the addition of 1% sodium dodecyl sulfate. The denatured sample was incubated in presence of 40 μM of Alexa Fluor 550 CS-maleimide (Invitrogen) and 1 mM TCEP (Pierce) for 2 h at room temperature. The labelled proteins were separated by SDS–PAGE and protein-bound fluorescence was visualized and quantified using FujiFilm FLA-3000 scanner. The assay was performed in triplicate, from three independent TssJ/L core complex preparations, and a representative experiment is shown. The quantification is expressed with the standard deviation from the three biological replicates.

EM and image processing. Determination of the TssJ/L core membrane complex structure was achieved by negative-stain EM. Nine microtiter plates of suitably diluted (0.01 mg ml⁻¹) TssJ/L complex sample was spotted to glow-discharged carbon-coated copper grids (Agar Scientific). After 30 s of aspiration, the sample was blotted, washed with three drops of water and then stained with 2% uranyl acetate. Images were recorded automatically using the EPU software on an FEG microscope operating at a voltage of 200 kV and a defocus range of 0.6–25 nm, using an FEI Falcon-II detector (Gatan) at a nominal magnification of 50,000, yielding a pixel size of 1.9 Å. A dose rate of 25 electrons per square ångström per second, and an exposure time of 1 s, were used. A total of 72,146 particles were automatically selected from 1,200 independent images and extracted within boxes of 280 pixels × 280 pixels using EMAN2/BOXER. The defocus value was estimated and the contrast transfer function was corrected by phase flipping using EMAN2 (ezctf). All 2D and 3D classifications and refinements were performed using RELION 1.3 (ref 48, 49). We used three rounds of phase flipping using EMAN2 (e2ctf). All 2D and 3D classifications and refinements were performed using RELION 1.3. The resolution of the final model was estimated with BoxCUBE tool, yielding radially averaged normalized intensities as a function of the momentum transfer q, with q = 4πsin(θ)/λ, where 2θ is the total scattering angle and λ is the X-ray wavelength. Data were collected in the range q = 0.04–6 nm⁻¹. The ten frames were combined to give the average scattering curve for each measurement. Data points affected by aggregation, possibly induced by radiation damage, were excluded. Scattering from the buffer alone was also measured before and after each sample analysis and the average of these buffer measurements was used to correct the data. The scattering background was estimated using the program PRIMUS5 from the ATSAS package54. PRIMUS was also used to perform Guinier analysis of the low q data, which provides an estimate of the radius of gyration (Rg). Regularized indirect transforms of the scattering data were performed with the program GNOM54 to obtain P(r) functions of interatomic distances. The P(r) function has a maximum at the most probable intermolecular distance and goes to zero at Dmax, the maximum intramolecular distance. The values of Dmax were chosen to fit with the experimental data and to have a positive P(r) function. Three-dimensional bead models that fitted with the scattering data were built with the program DAMMIF57. Twenty independent DAMMIF runs were performed using the TssJ–TssL–TssM–TssF complexes, with data extending up to 0.35 nm⁻¹, using slow mode settings, assuming no symmetry and allowing for a maximum 500 steps to grant convergence. The models resulting from independent runs were superimposed using the DAMAVER suite58. This yielded an initial alignment of structures based on their axes of inertia followed by minimization of the normalized spatial discrepancy59. The normalized spatial discrepancy was therefore computed between a set of 20 consecutive ion-exchange (Mono Q 5/50 GL column, GE Healthcare) and size-exclusion (Superdex 75 16/600 HL column) chromatographies using an Äkta system (GE Healthcare). The purified fragment was subjected to N-terminal Edman sequencing. A PVDF membrane was rinsed three times with a water/ethanol mixture (10:90) and inserted in the A cartridge of a Procise 494A sequencer. After five cycles of Edman degradation, the sequence DTYSGL was identified. The correct cleavage after Arg834 generated a C-terminal fragment of theoretical mass 32,398 Da, in agreement with the 32-kDa band observed by SDS–PAGE analyses.

For production and purification of the TssM26Ct fragment (Thr869 to Glu1107), E. coli BL21 (DE3) cells cultivated in the TB medium carrying plasmid pETG20A-TssM26Ct were grown to A600 nm = 0.6 and the expression of TssM26Ct was induced by the addition of 0.5 mM IPTG for 16 h at 17 °C. Cells were collected by centrifugation at 10,000g for 15 min. The pellet was resuspended in lysis buffer and lysed by sonication. The lysate was clarified by centrifugation at 20,000g for 4 °C for 15 min, and the supernatant containing the Trx–His–TssM26Ct fusion protein was purified by consecutive Ni²⁺ affinity and size-exclusion (Superdex 75 column) chromatographies on an Äkta purifier (GE Healthcare). The fractions containing the protein of interest were pooled and the 6×His-tagged TEV protease was added (5% w/v). The cleaved protein was purified using Ni²⁺ affinity, removing the Trx–His6, followed by size-exclusion chromatography (Superdex 75 column) on an Äkta purifier (GE Healthcare). Over 100 mg of TssM26Ct fragment were obtained per litre of culture. The purified protein was verified by mass spectrometry, before being concentrated up to 8.7 mg ml⁻¹ in 20 mM Tris-HCl pH 8.0, NaCl 150 mM.

The production of nanobody nb25 and the formation of its complex with TssM26Ct have been described previously. The production of unacylated TssF was previously described33. The TssJ–TssL–TssM core complex was obtained by mixing TssM26Ct (8.7 mg ml⁻¹) with purified TssJ (30 mg ml⁻¹) in a 1:1.2 molecular ratio and the complex was then concentrated up to 15 mg ml⁻¹ using centricon (cut-off of 10,000 Da) in 20 mM Tris-HCl pH 8.0, 150 mM NaCl.

SAXS and ab initio 3D shape reconstruction. SAXS analyses were performed at the ID29 beamline (European Synchrotron Radiation Facility, Grenoble, France) at a working energy of 12.5 keV (λ = 0.931 Å). Thirty microtiter plates of protein solution at 1.6 mg ml⁻¹ in Tris-HCl 20 mM pH 8.0, NaCl 150 mM, were loaded by a robotic system into a 2-mm quartz capillary mounted in a vacuum. Ten independent 10-s exposures were collected on a Pilatus 6M F detector placed at a distance of 2.85 m for each protein concentration. Individual frames were processed automatically and independently at the beamline by the data collection software (BsxCUBE), yielding radially averaged normalized intensities as a function of the momentum transfer q, with q = 4πsin(θ)/λ, where 2θ is the total scattering angle and λ is the X-ray wavelength. Data were collected in the range q = 0.04–6 nm⁻¹. The ten frames were combined to give the average scattering curve for each measurement. Data points affected by aggregation, possibly induced by radiation damage, were excluded. Scattering from the buffer alone was also measured before and after each sample analysis and the average of these two buffer measurements was used to correct the data. Further background subtraction was accomplished using the program PRIMUS. PRIMUS was also used to perform Guinier analysis of the low q data, which provides an estimate of the radius of gyration (Rg). Regularized indirect transforms of the scattering data were performed with the program GNOM to obtain P(r) functions of interatomic distances. The P(r) function has a maximum at the most probable intermolecular distance and goes to zero at Dmax, the maximum intramolecular distance. The values of Dmax were chosen to fit with the experimental data and to have a positive P(r) function. Three-dimensional bead models that fitted with the scattering data were built with the program DAMMIF. Twenty independent DAMMIF runs were performed using the TssJ–Trx–His–TssM–TssF complexes, with data extending up to 0.35 nm⁻¹, using slow mode settings, assuming no symmetry and allowing for a maximum 500 steps to grant convergence. The models resulting from independent runs were superimposed using the DAMAVER suite. This yielded an initial alignment of structures based on their axes of inertia followed by minimization of the normalized spatial discrepancy. The normalized spatial discrepancy was therefore computed between a set of 20 independent reconstructions, with a range of normalized spatial discrepancies from 0.678 to 0.815. The aligned structures were then averaged, giving an effective occupancy to each voxel in the model, and filtered at half-maximal occupancy to produce models of the appropriate volume that were used for all subsequent analyses. All the models were similar in terms of agreement with the experimental data, as measured by DAMMIF χ parameter and the fit of the model to the experimental curve. The DAMMIF average volume was used as the final model of the TRX–His–TssJ and TRX–His–TssM complexes.
Ni-TNA-Nanogold labelling. The TssLM complex was spotted onto a glow-discharged carbon coated grid (CF-400, Electron Microscopy Sciences). After 1 min, excess liquid was blotted, and the grid was washed on a drop of cold purification buffer (50 mM Tris pH 8, 50 mM NaCl, 0.025% (w/v) DM-NPG containing 50 mM imidazole, quickly blotted and deposited on a second drop of the same buffer in the presence of 5 mM Ni-TNA-Nanogold beads (NanoProbes). After 2 min, the grid was rinsed sequentially for 20 s with one drop of purification buffer, one drop of the same buffer without detergent and three drops of 2% uranyl acetate. Images were collected on an FEI Tecnai F20 FEG microscope operating at a voltage of 200 kV, equipped with a direct electron detector (Falcon II). Particles were selected manually using EMAN2. The assay was performed at least in triplicate, from independent TssLM complex preparations, and representative particles are shown.

Anti-Strep labelling. The TssLM complex was mixed with monoclonal anti-Strep antibodies (Sigma) at a ratio of complex:antibody of 2:1. The mixture was incubated at 4 °C for 30 min and the labelled complex was isolated by gel filtration. The sample was analysed by negative-stain EM as described above for negative-stain EM of the unlabelled TssLM complex. The assay was performed at least in triplicate, from independent TssLM complex preparations, and representative particles are shown.

Crystalization and structure determination. The crystallization of the TssM<sub>32Ct</sub>–nb25 complex has been described previously<sup>25</sup>. For TssM<sub>32Ct</sub> alone, several kits were used for crystallization screening, including STURA, WIZARD, MDL, INDEXX and PEGs. A hit was observed in the PEGs kit, within a well several kits were used for crystallization screening, including STURA, WIZARD, MDL, INDEXX and PEGs. A hit was observed in the PEGs kit, within a well

4.0 A˚ at the Soleil Proxima 2 beamline (Saint Aubin, France). Further crystals were recovered by ultracentrifugation at 100,000 g for 40 min. These dimers were not biologically relevant. Molecular contacts were analysed by the PISA server<sup>33</sup> and figures were prepared with Chimera<sup>2</sup> and Pymol<sup>34</sup>.

The crystal structures of the TssM<sub>32Ct</sub>–nb25 complex, and of the TssM<sub>32Ct</sub> fragment and of the TssM<sub>32Ct</sub>–TssJ complexes, have been deposited in the Protein Data Bank under accession numbers 47Y7, 47Y1 and 4Y7O respectively.

Docking TssM<sub>26Ct</sub>–TssJ structure and TssL<sub>cyto</sub>. The crystal structures of the TssM<sub>26Ct</sub>–TssJ complex and of the Tsl complex containing the cytoplasmic domain (Protein Data Bank 3U66<sup>35</sup>) were docked automatically using Chimera<sup>2</sup> after map segmentation.

Refinement of docked TssM<sub>26Ct</sub>–TssJ penamer in the EM density map. The atomic model of the docked TssM<sub>26Ct</sub>–TssJ structure was refined in the EM density with RFree<sup>28</sup>. First, missing side-chain and polar hydrogen atoms were added with Modeller<sup>36</sup>. The structure was minimized using 2,000 steps of least-squares conjugate gradient refinement in the presence of distance restraints for hydrogen bonds and backbone dihedral angle restraints to maintain secondary structures. The minimization was performed with the real-space objective function calculated by RFree<sup>28</sup>. The Cs symmetry was enforced by strict non-crystallographic symmetry restraints. The total energy included internal parameters (bond length, bond angle, improper and dihedral angles) and non-bonded interactions with full Van der Waals and electrostatic potentials using a 7.5 Å cutoff. The final correlation coefficient between the EM reconstruction and the refined model was 0.929 (as calculated by RFree<sup>28</sup>), whereas it was 0.766 before minimization.

Modelling of TssM<sub>32Ct</sub>–J decamer. The atomic position of a TssM<sub>32Ct</sub>–J proto- mer from the outer ring of the penamer served as the starting structure to generate a TssM<sub>26Ct</sub>–J decamer model with cyclic ten-fold symmetry using CNS<sup>37</sup>. The symmetry was enforced by strict non-crystallographic symmetry restraints (rotations of 36° around the symmetry axis). First, 5,000 steps of rigid body minimization were performed including only inter-protoner energetic contributions (full Van der Waals and electrostatic potentials). After a short all-atom minimization (300 steps), 1,5 ps of molecular dynamics simulation at 1,000 K was performed, followed by 300 steps of minimization and 10 ps of molecular dynamics simulation at 200 K. Minimizations and molecular dynamics simulations were realized with both intra-protoner and inter-protoner energetic contributions activated, and the backbone conformation of the protomer was restrained with harmonic constraints.

Substituted cysteine accessibility method. Cysteine accessibility experiments were performed on whole cells, mainly as described<sup>25,38</sup> with modifications. A 20 ml culture of wild-type or AtsbC strains producing a periplasmic cysteine-less TssM (Cys527-to-Ser) or derivatives bearing cysteine substitutions were induced for tssJ gene expression with 0.05 μg/ml 3-aminohexadecanoic acid (AHA) for 1 h. Cells were harvested and resuspended in buffer A (100 mM Hepes (pH 7.5), 150 mM NaCl, 25 mM MgCl<sub>2</sub>) to a final A<sub>600</sub> of 12 in 50 μl of buffer A. Bovine serum albumin (BSA)-coupled maleimide (Sigma-Aldrich) was added to a final concentration of 100 μM (from a 20 mM stock freshly dissolved in DMSO) and the cells were incubated for 30 min at 25 °C. Mercuric ethylmalonate (20 mM final concentration) was added to quench the biotinylation reaction, then cells were washed twice in buffer A and resuspended in buffer A containing N-ethyl maleimide (final concentration 5 mM) to block all free sulfhydryl residues. After incubation for 20 min at 25 °C, cells were disrupted by sonication. Membranes were recovered by a perchloric acid treatment (60 mM final concentration) and loaded on a Laemmli buffer before SDS–PAGE analysis and immunodetection with anti-Flag antibodies (to detect the TssM proteins). Controls were performed by labelling total membranes from the same samples instead of whole cells. The assay was performed in triplicate, from three independent cultures, and a representative experiment is shown.

SDS–PAGE, protein transfer, immunostaining and antibodies. SDS–PAGE was performed on Bio-Rad Mini-PROTEAN systems using standard protocols. For immunostaining, proteins were transferred onto 0.2-μm nitrocellulose membranes (Amersham Protran). Immunoblots were probed with primary antibodies against goat secondary antibodies coupled to alkaline phosphatase and developed in alkaline buffer in presence of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. The anti-ToB was from our laboratory collection, whereas the anti-HA (3F10 clone, Roche), anti-Flag (M2 clone, Sigma Aldrich), anti-StrepII (Sigma Aldrich), anti-Shi (Sigma Aldrich) monoclonal antibodies and...
alkaline-phosphatase-conjugated goat anti-rabbit or mouse secondary antibodies (Millipore) were purchased as indicated.

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Extended Data Figure 1 | Functional and dynamic properties of fluorescently labelled Tss proteins. a, GFP–TssM and GFP–TssL fusion proteins are functional. Top: Hcp release assay. Hcp release was assessed by separating whole cells (C) and supernatant (S) fractions from the indicated strains. A total of $1 \times 10^9$ cells and the TCA-precipitated material from the supernatant of $2 \times 10^9$ cells were analysed by western blot using anti-Flag monoclonal antibody (lower panel) and anti-TolB polyclonal antibodies as a lysis control (upper panel). The molecular mass markers (in kilodaltons) are indicated on the left. Bottom: anti-bacterial assay. The anti-bacterial activity was assessed by mixing kanamycin-resistant prey *E. coli* K-12 cells with the indicated attacker cells for 16 h at 37°C in SIM. The number of recovered *E. coli* prey cells is indicated in the graph (as log of colony-forming units (c.f.u.)). The circles indicate values from three independent assays, and the average is indicated by the bar. b, TssJ–sfGFP and TssJ–mCh fusion proteins are non-functional. Hcp release was assessed by separating whole cells (C) and supernatant (S) fractions from the indicated strains. A total of $1 \times 10^9$ cells and the TCA-precipitated material from the supernatant of $2 \times 10^9$ cells were analysed by western blot using anti-Flag monoclonal antibody (lower panel) and anti-TolB polyclonal antibodies as a lysis control (upper panel). The molecular mass markers (in kilodaltons) are indicated on the left. c, sfGFP–TssM and sfGFP–TssL cluster in foci. Large fields of fluorescence microscopy recordings showing localization of the sfGFP–TssL (left) and sfGFP–TssM (right) fusion proteins. The positions of selected foci are indicated by arrowheads. Scale bars, 5 μm. d, sfGFP–TssM and sfGFP–TssL foci are stable and static. Mean square displacement (in arbitrary units (a.u.)) of sfGFP–TssM (blue line) and sfGFP–TssL (red line) clusters were measured by sub-pixel tracking of fluorescent foci and plotted over time (in minutes). e, The TssBC sheath tubular structures assemble on TssJLM membrane complexes. Statistical analyses reporting the average number of sheath per cell compared with the number of membrane complexes per cell, highlighting the observation that the number of membrane complexes is at least equal to the number of sheathes. Lower and upper boundaries of the boxes correspond to the 25% and 75% percentiles respectively. Black bold horizontal bar, median values for each strain; whiskers, 10% and 90% percentiles; n indicates the number of cells studied per strain. f, Long-term fluorescence microscopy recordings. Time-lapse fluorescence microscopy recordings showing localization and dynamics of the sfGFP–TssL and TssB–mCherry fusion proteins. Individual images were taken every 15 min. Assembly/contraction of the sheath and TssL localization events are schematized in the lowest panel. Scale bars, 1 μm. g, Statistical analysis of sfGFP–TssM and sfGFP–TssL localization in various tss backgrounds. Shown are box-and-whisker plots of the measured number of sfGFP–TssM and sfGFP–TssL foci per cell for each indicated strain with the lower and upper boundaries of the boxes corresponding to the 25% and 75% percentiles respectively (horizontal bar, the median values for each strain; whiskers, the 10% and 90% percentiles); n indicates the number of cells studied per strain.
Extended Data Figure 2 | Expression and purification of the T6SS membrane core complex. a–e, T6SS operon genomic organization and constructs used for in vitro analyses. a, Schematic representation of the T6SS sci-1 gene cluster from enteric aggregative E. coli. The numbers on top refer to the gene locus tag (EC042_XXXX). Genes encoding core components (identified by their names on bottom, for example, ‘B’ refers to the tssB gene) are coloured grey. Genes of unknown function are coloured white. The three genes used to reconstitute the core membrane complex are coloured orange (tssJ), blue (tssl) and green (tssM). b, Schematic representation of the engineered constructs: the tssJ, tssl and tssM genes were amplified with an additional Shine Dalgarno (SD) sequence and 3’ StrepII, 5’ Flag and 5’ 6×His tags respectively. These three fragments were cloned into the pRSF-Duet vector (c). This construct allows the production of the C-terminally StrepII-tagged TssJ outer membrane (OM) lipoprotein and N-terminally Flag-tagged TssL and 6×His-tagged TssM inner-membrane (IM) proteins (d, e). The proteins are schematized and their boundaries and principal characteristics (TM, transmembrane segments; SP, signal peptide; CYS, acylated cysteine) are indicated (d) and their topologies are shown (e). The additional TssM constructs (TssMp, TssM32Ct and TssM26Ct) used for SAXS or X-ray analyses are shown at the bottom. f–h, Purification and biochemical characterization of the T6SS membrane core complex. f, Analytical size-exclusion chromatography analysis of the purified TssJLM complex (continuous line) on a Superose 6 column, calibrated with 75-, 158-, 440- and 660-kDa molecular mass markers (dotted lines). The molecular mass of each marker (in kilodaltons) is indicated on the top of the corresponding peak. An arrow indicates the position of the peak fraction corresponding to the TssJLM complex. g, SDS–PAGE of the purified TssJLM complex analysed by Coomassie staining (CB) or immunoblotting using anti-His (α-His), -Flag (α-Flag) and -StrepII (α-STREP) antibodies. h, Left: cysteine labelling of the purified TssJLM complex in reducing and denaturing conditions as described in Methods. The total number of cysteine residues was nine for TssM, five for TssL and none for TssJ (the N-terminal cysteine is acylated). Right: the relative amount of TssL compared with TssM (densitometry relative to the number of free cysteine residues, fixed at 1 for TssM).
Extended Data Figure 3 | Architecture of the T6SS membrane core complex. 

a, Negative-stain EM of the EAEC TssJLM complex. Representative micrograph of the data set used for image processing. Isolated TssJLM complexes were clearly visible (white circles).
b, Plot of the rotational autocorrelation function for a representative class average of an end-view.
c, FSC curve of the TssJLM reconstruction. The ‘gold standard’ FSC curve was calculated in Relion using the masked reconstruction of the TssJLM complex. The resolution at 0.143 correlation was 11.56 Å.
d, Top: side and corresponding cut-away views of the 3D reconstruction for the whole TssJLM complex. Bottom: local resolution as calculated by Resmap. The TssJLM volume (left reconstruction, side view; right reconstruction, cut-away view) is coloured according to the local resolution from high resolution (≈12 Å) in blue to low resolution (>30 Å) in red.
e, FSC curve of the TssJLM base. The ‘gold standard’ FSC curve was calculated in Relion using the unmasked reconstruction of the TssJLM base. The resolution at 0.143 correlation was 16.6 Å.
f, Top, side and bottom views of the 3D reconstruction after specific refinement of the base.
**Extended Data Figure 4 | Structural analysis and segmentation.**

**a**, Segmentation of the TssJLM reconstruction. Left: above the base, ten equivalent densities could be defined by segmentation. They are arranged in two concentric rings. The internal ring is represented in green in the top panel and the external ring is represented in blue in the bottom panel. Right: cut-out views of the complex showing the arrangement of the two concentric rings at different levels (grey lines) along the periplasmic portion of the TssJLM complex. The cut-out views are seen from the bottom of the complex.

**b**, Requirement of TssJ lipidation for complex assembly and insertion into the outer membrane. Left: membrane fractionation by differential solubilization followed by immunoblot analysis. Total membrane extracts from cells producing the wild-type TssJLM complex or the TssJLM complex with an unacylated variant of TssJ (Cys1-to-Ser substitution, CS) were solubilized by lauroyl sarcosine to separate inner membranes and outer membranes. HisTssM, FlagTssL and TssJ Strep (indicated on the right) were revealed by anti-His, anti-Flag and anti-StrepII antibodies respectively. Controls included immunodetection of the inner membrane DglA diacylglycerol lipase and the outer membrane OmpF porin. Wild-type TssJLM complex co-fractionates with both the inner and outer membrane fractions whereas the Cys1-to-Ser substitution mutated complex co-fractionates only with the inner-membrane fraction. Top right: negative-stain EM of the mutated TssJCSLM complex. Representative micrograph of the data set used for image processing. Isolated TssJCSLM complexes were clearly visible (white circles). Bottom right: gallery of representative class averages generated after reference-free 2D classification in Relion. End to side views are shown from top left to bottom right.

**c**, Orientation of the TssJLM complex in the cell envelope. Left: schematic representation of the TssJ (J, orange), TssL (L, blue) and TssM (M, green) proteins. Their localization, main characteristics (lipidation or transmembrane segments shown in black) and the location of the 6×His and StrepII tags (red balls) are indicated. The strepII and 6×His tags were introduced at the C terminus and N terminus of TssJ and TssM respectively. Middle: immune and Nanogold labelling coupled to EM. Anti-StrepII or Nanogold-NTA were incubated with the TssJLM complex and visualized by negative-stain EM. A gallery of representative views is presented (top row, StrepII labelling; bottom row, Ni-NTA labelling). StrepII antibodies (a schematic diagram with StrepII antibodies depicted as blue circles is shown on top) and Nanogold-NTA are highlighted in red circles. Right: the positions of the StrepII antibody (targeting TssJ C terminus) and of the Ni-NTA gold particle (targeting TssM N terminus) are indicated on the TssJLM reconstruction.

**d**, SAXS data and low-resolution structure of the TssMp–TssJ complex. Top left: experimental scattering data (green crosses) and the fitting curve (continuous red line) calculated from an *ab initio* model of the TssMp–TssJ complex. Top right: Guinier plot (dots) with the linear fit (continuous line). Bottom left: distance distribution function of the TssMp–TssJ complex. Bottom right: SAXS envelope (grey surface) of the ‘best representative’ model of the TssMp–TssJ complex. Each view is rotated by 90° around the y-axis.

**e**, Location of the TssMp–TssJ complex SAXS envelope in the 3D reconstruction of TssJLM complex. Left: the volume of the TssMp–TssJ complex determined by SAXS was docked into the EM 3D reconstruction of the TssJLM complex (top). Two optimal docking positions were found, both with 82% correlation with the EM map (coloured magenta and pink). The corresponding volumes in the EM map were extracted (bottom). They correspond to the same volume displayed in Extended Data Fig. 4a. Right: direct comparison of the SAXS (magenta) and EM (blue and green) volumes corresponding to the TssMp–TssJ complex. The volumes are equivalent in size and shape.

**f**, TssL cytoplasmic domain docking into the TssJLM complex base. Fitting of the TssL cytoplasmic domain (TssL*cyto*)3 dimer in green ribbons in the hooks found in the base. Top and bottom: side and bottom views, respectively.
Extended Data Figure 5 | Crystal structure of the TssJ–TssM C-terminal domain complex. **a**, Amino-acid sequence of TssM. The different domains as well as the fragments used in this study are indicated (yellow, transmembrane helix; grey, cytoplasmic domain; green, blue and purple, periplasmic domain; blue and purple, C-terminal domain corresponding to the TssM32Ct fragment; purple, C-terminal domain corresponding to the TssM26Ct fragment). **b**, Crystal structure of the TssM32Ct–nb25 complex. The two proteins are represented as rainbow-coloured ribbons. The complementary determining regions (CDRs 1–3, coloured blue, green and red, respectively) of nb25 are indicated. The inset highlights the TssM32Ct–nb25 interface: the TssM32Ct surface is coloured beige whereas nb25 is represented as rainbow-coloured ribbons; the side chains of the amino acids in contact with TssM32Ct are indicated. The nb25 nanobody binds the TssM C-terminal domain, and covers a surface area of 580Å² by inserting its protruding CDR3 between TssM32Ct loops L5–6 and L9–10. The contacts between the two proteins are listed in Extended Data Table 2a. **c**, Crystal structure of the TssM 26Ct–TssJ complex. Left: the two proteins are represented as ribbons and coloured in rainbow mode. Middle: same view rotated by 90°. The TssJ loop 1–2, previously shown to contact TssM32Ct, is indicated. Right: TssM32Ct–TssJ interface. Top panel: the TssM32Ct surface is coloured violet whereas TssJ is represented as rainbow-coloured ribbons. The TssJ side-chains of the amino acids in contact with TssM are indicated. The loops are numbered according to the flanking β-strands. Bottom panel: the TssJ surface is coloured beige whereas TssM32Ct is represented as rainbow-coloured ribbons. The TssM side-chains of the amino acids in contact with TssJ are indicated. The contacts between the two proteins are listed in Extended Data Table 2b. **d**, Comparison of the binding sites of nb25 and TssJ on TssM. Left: the structure of the TssM32Ct–TssJ complex (rainbow coloured) has been superimposed to the structure of the TssM32Ct–nb25 complex (only nb25 is shown in grey for clarity). Right: the same partners as in the left panel in surface representation. TssM32Ct (violet), TssJ (green) and nb25 (pink). **e**, Insertion of the TssJ lipid anchor in the outer membrane. Left: TssJ structure25 with the N-terminal 24 residues (absent in the crystal structure). This N-terminal extension (in magenta), predicted to be disordered, was modelled in Chimera using Modeller. The first cysteine residue is acylated to allow anchorage to the inner leaflet of the outer membrane (orange rectangle). Right: docking of the TssM26Ct–TssJ complex in the EM 3D reconstruction of the TssJLM complex (only the uppermost (tip) part of the TssJLM complex is shown). Left panel: two TssM26Ct–TssJ were docked into the inner and outer pillars of the tip complex. Right panel: docking in each pillar of the TssJLM tip complex (C₅ symmetry). **f**, Hydrophobicity of the TssM26Ct–TssJ complex. Surface representation of the TssM26Ct–TssJ decamer (left, top view; right, side view). The hydrophobicity of the surface residues is displayed (blue to red scale from most hydrophilic to most hydrophobic). No obvious hydrophobic patch is visible at the surface of the complex.
Extended Data Figure 6 | Cell surface accessibility of TssM C-terminal domain.  

**a**, Functionality of the TssM cysteine variants. Hcp release was assessed by separating whole cells (C) and supernatant (S) fractions from the wild-type (WT) 17-2 strain and its ΔtssM derivative producing a wild-type allele of TssM or TssM cysteine substitution derivatives (as indicated). A total of $1 \times 10^9$ cells and the TCA-precipitated material from the supernatant of $2 \times 10^9$ cells were analysed by western blot using anti-HA monoclonal antibody (lower panel) and anti-TolB polyclonal antibodies as a lysis control (upper panel). The molecular mass markers (in kilodaltons) are indicated on the left.

**b**, Cysteine substitution labelling. Accessibility to cysteine residues positioned in TssM domain 4 loops was assessed by treating isolated membranes (M) or whole cells (WC) of the indicated strain (WT, wild-type 17-2; ΔtssBC; ΔtssJ) producing the indicated TssM cysteine derivative (in red letters) with the cysteine-reactive, membrane-impermeant BSA-maleimide (BSA-mal). Samples corresponding to a total of $5 \times 10^9$ cells were analysed by western blot using anti-Flag monoclonal antibody. The position of the TssM protein (~125 kDa) is indicated as well as that of a retarded band corresponding to BSA-maleimide-coupled TssM (~190 kDa; asterisk). The molecular mass markers (in kilodaltons) are indicated.

**c**, Close-up of the TssM26Ct–TssJ interface. TssM 26Ct is represented in blue ribbons. TssJ is represented in orange ribbons and orange transparent surface. TssM residues accessible from the cell exterior when the T6SS is functional are indicated by yellow spheres whereas unaccessible residues are shown by grey spheres. The accessible residues 989 and 1005 are buried at the interface between TssM and TssJ, suggesting that this interface is probably disrupted during T6SS assembly and/or function. Left and right panels are orthogonal views of the same molecule.
Extended Data Figure 7 | Comparison with other bacterial secretion systems and model for channel opening. 

a, Comparison between the T6SS TssJLM membrane core complex structure and other bacterial secretion systems. From left to right, the *E. coli* AcrAB-TolC multi-drug efflux pump (EMDB accession number emd-5915)\(^7\), the EAEC T6SS membrane core complex (this study, EMDB accession number emd-2927), the *Shigella* T3SS transmembrane complex (EMDB accession number emd-1617)\(^7\) and the *E. coli* R388 T4SS complex (EMDB accession number emd-2567)\(^7\). The positions on the inner membrane (IM) and outer membrane (OM) are indicated (C, cytoplasm; P, periplasm). Scale bar, 10 nm.

b, Docking of the Hcp tube/VgrG spike into the TssJLM 3D reconstruction. Left: before sheath contraction. The Hcp tube/VgrG spike (VgrG in yellow and Hcp in green; surface representation) was manually docked in the 3D reconstruction of the TssJLM complex (grey surface). The diameter of the channel defined by the closed tip complex is not large enough to allow the passage of the tube/spike, suggesting that large conformational changes probably occur. The cavity at the tip of VgrG could be filled by VgrG-bound PAAR modules or toxin effectors\(^2\). Right: during sheath contraction. The diameter of the C\(_{10}\)-symmetrized TssM\(_{26Ct}\) model (represented as ribbons) is compatible with the passage of the Hcp tube/VgrG spike (same colours as in the left panel).

c, Closed and open forms of the TssM\(_{26Ct}\) oligomer. Crystal structure of TssM\(_{26Ct}\) represented as ribbons and transparent surface. The TssM\(_{26Ct}\) \(\alpha\)- and \(\beta\)-domains are coloured cyan and blue, respectively. The C-terminal \(\alpha\)S-helix and the extended stretch are coloured pink. Cysteines with extracellular accessibility when the T6SS is active are coloured yellow, while the unlabelled ones are coloured red. Left: docking of the TssM\(_{26Ct}\)–TssJ crystal structure in the EM 3D reconstruction of the TssJLM tip complex. Top and bottom panels, side and top views, respectively. Right: model of a C\(_{10}\)-symmetrized oligomer of the TssM\(_{26Ct}\) domain. Top and bottom panels, side and top views, respectively.
Extended Data Table 1 | Data collection and refinement statistics

|                      | TssM\(_{32C7}\)-nb25 | TssM\(_{26C1}\) | TssM\(_{26C7}\)-TssJ |
|----------------------|-----------------------|----------------|------------------|
| **Data collection**§ |                       |                |                  |
| Space group          | P6\(_4\)              | P 4\(_{1}2_{1}2\) | P 4\(_{1}2_{1}2\) |
| Cell dimensions      |                       |                |                  |
| \(a, b, c\) (Å)      | 95.2, 95.2, 172.95    | 64.0, 64.0, 249.7 | 85.5, 85.5, 256.4 |
| \(\alpha, \beta, \gamma\) (°) | 90.0, 90.0, 120.0 | 90.0, 90.0, 90 | 90.0, 90.0, 90 |
| Resolution (Å)       | 50.0-1.92(1.97-1.92)* | 30.0-1.51(1.6-1.51)* | 50.0-2.24(2.38-2.24)* |
| \(R_{merge}\)        | 0.079 (1.08)          | 0.067 (0.59)    | 0.067 (0.73)     |
| \(I/\sigma I\)       | 18.0 (2.0)            | 19.2 (3.0)      | 21.5 (3.1)       |
| Completeness (%)     | 100.0 (100.0)         | 99.9 (89.3)     | 99.7 (98.4)      |
| Redundancy           | 11.4 (11.3)           | 9.9 (9.9)       | 10 (10)          |
| **Refinement**       |                       |                |                  |
| Resolution (Å)       | 47.6-1.92(1.97-1.92)* | 22.1-1.51 (1.55-1.51)* | 49.3-2.24(2.3-2.24)* |
| No. reflections      | 67543 (4721)          | 82127 (5359)    | 46047 (3015)     |
| \(R_{work}/R_{free}\) | 0.184/0.21(0.234/0.25) | 0.192/0.202(0.241/0.27.6) | 0.208/0.228(0.224/0.25) |
| No. atoms            | 5522                  | 3784            | 5521             |
| Protein              | 5522                  | 3784            | 5521             |
| Ligand/ion           | 15                    | 22              | 4                |
| Water                | 805                   | 536             | 379              |
| B-factors            |                       |                |                  |
| Protein              | 42.3                  | 27.4            | 51.7             |
| Ligand/ion           | 98.8                  | 55.0            | 81               |
| Water                | 49.9                  | 39.5            | 63.8             |
| R.m.s deviations     |                       |                |                  |
| Bond lengths (Å)     | 0.009                 | 0.010           | 0.010            |
| Bond angles (°)      | 1.05                  | 1.03            | 1.19             |

†Each data set has been collected on a unique crystal.
*Highest resolution shell is shown in parenthesis.
Extended Data Table 2 | Interactions and accessibility data

Extended Table 2a. Interactions between TssM and nb25

| n25/CDR3 number | n25/CDR3 type | TssMres. number | TssMres. type | distance (Å) | bond |
|-----------------|---------------|----------------|---------------|--------------|------|
| 103             | Gly           | O              | 1063          | Ala          | 2.89 | H    |
| 104             | Ile           | CA             | 1061          | Gly          | 3.08 | H    |
| 105             | Tyr           | N              | 1061          | Gly          | 2.85 | H    |
| 107             | Thr           | OG1            | 1060          | Pro          | 2.67 | H    |
| 107             | Thr           | CG2            | 1061          | Gly          | 3.56 |      |
| 109             | Tyr           | CE1            | 1067          | Ser          | 3.50 |      |
| 109             | Tyr           | OH             | 1080          | Tyr          | 3.47 | H    |
| 109             | Tyr           | OH             | 1081          | Thr          | 2.64 | H    |
| 110             | Ile           | CD1            | 1062          | Val          | 3.73 |      |
| 113             | Pro           | O              | 984           | Gly          | 3.12 |      |
| 113             | Pro           | O              | 985           | Thr          | 3.00 | H    |
| 114             | Tyr           | CE1            | 982           | Arg          | 3.55 |      |
| 114             | Tyr           | CZ             | 982           | Arg          | 3.63 |      |
| 114             | Tyr           | OH             | 1010          | Trp          | 2.99 | H    |
| 114             | Tyr           | O              | 982           | Arg          | 2.88 | H    |
| 115             | Gly           | O              | 982           | Arg          | 2.76 | H    |
| 116             | Met           | O              | 1008          | Pro          | 3.32 |      |
| 117             | Asp           | OD1            | 982           | Arg          | 2.80 | H    |

Extended Table 2b Interactions between TssM and TssJ.

| TssJ number | TssJ type | TssM number | TssM type | distance (Å) |
|-------------|-----------|-------------|-----------|--------------|
| 37          | Asn       | Nd2         | 1005      | Asn          | O           | 3.06 H |
| 39          | Ser       | Ch           | 985       | Thr          | Og1         | 3.80   |
| 43          | Ile       | CG2          | 987       | Ala          | Cb          | 3.79   |
| 45          | Leu       | Cd1          | 985       | Thr          | Cg2         | 3.63   |
| 46          | Ser       | O             | 990       | Met          | Ca          | 3.14   |
|             |           | O             | 1005      | Asn          | Nd2         | 3.27 H |
|             |           | O             | 1005      | Asn          | Nd2         | 3.19 H |
| 48          | Val       | Cg2          | 1004      | Val          | Cb          | 3.82   |
| 65          | Tyr       | Ce1          | 1007      | Met          | Cg          | 3.51   |
|             |           | Oh            | 1007      | Met          | N           | 3.30 H |
| 87          | Trp       | Ch2           | 990       | Met          | C           | 3.56   |
| 89          | Gln       | Oe1          | 1031      | Thr          | O           | 2.87 H |
|             |           | Ne2          | 1031      | Thr          | Og1         | 2.79 H |
| 112         | Met       | C            | 1006      | Gln          | Ne2         | 3.35   |
| 113         | Phe       | O             | 1007      | Met          | N           | 3.24 H |
| 114         | Leu       | Cd1          | 1003      | Tyr          | OH          | 3.16   |
|             |           | N             | 1005      | Asn          | O           | 3.33 H |
|             |           | C            | 1006      | Gln          | O           | 3.40   |
|             |           | O             | 1007      | Met          | Cg          | 3.51   |
|             |           | Cd1           | 1008      | Pro          | Cd          | 3.73   |
| 116         | Pro       | Cd            | 1007      | Met          | Cg          | 3.74   |

Extended Table 2c. TssM cysteine accessibility.

| Cysteine position | Labelled at rest | Labelled in action | Position | WAS # |
|-------------------|------------------|--------------------|----------|-------|
| 972               | -                |                    | helix α3 | 107 / 107 |
| 989               | -                | ++                 | loop β3-β4 | 14 / 10 |
| 1005              | -                | ++                 | loop β5-β6 | 76 / 0 |
| 1019              | -                |                    | loop β6-β7 | 35 / 35 |
| 1035              | -                | +                  | loop β7-β8 | 113 / 113 |
| 1062              | -                |                    | loop β11-α5 | 94 / 94 |
| 1075              | -                | ++                 | loop β10-β11 | 51 / 51 |
| 1092              | -                |                    | helix α5 | 0 / 0 |
| 1109              | ++               | ++                 | C-terminal | NA* |

The letter H in right-hand columns indicates that atoms establish a hydrogen bond.

WAS, water-accessible surface of the original amino acids (measured in the unbound TssM/in the TssM-TssJ complex).

*Not visible in the electron density map.