**In situ** and high-resolution cryo-EM structure of a bacterial type VI secretion system membrane complex

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**Abstract**

Bacteria have evolved macromolecular machineries that secrete effectors and toxins to survive and thrive in diverse environments. The type VI secretion system (T6SS) is a contractile machine that is related to *Myxococcus* phages. It is composed of a phage tail-like structure inserted in the bacterial cell envelope by a membrane complex (MC) comprising the TssJ, TssL and TssM proteins. We previously reported the low-resolution negative-stain electron microscopy structure of the enteropathogenic *Escherichia coli* MC and proposed a rotational 5-fold symmetry with a TssJ:TssL:TssM stoichiometry of 2:2:2. Here, cryo-electron tomography analyses of the T6SS MC confirm the 5-fold symmetry in situ and identify the regions of the structure that insert into the bacterial membranes. A high-resolution model obtained by single-particle cryo-electron microscopy highlights new features: five additional copies of TssJ, yielding a TssJ:TssL:TssM stoichiometry of 3:2:2, an 11-residue loop in TssM, protruding inside the lumen of the MC and constituting a functionally important periplasmic gate, and hinge regions. Based on these data, we propose an updated model on MC structure and dynamics during T6SS assembly and function.

**Keywords** cryo-electron microscopy; membrane protein complex; type VI secretion systems

**Subject Categories** Microbiology, Virology & Host Pathogen Interaction; Structural Biology

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**Introduction**

In a competitive environment, the ability to communicate and outcompete neighbours provides bacteria with key advantages to survive. The type VI secretion system (T6SS) is a macromolecular complex involved in the release of toxins that disrupt essential functions in competitor cells (Russell et al., 2014). The T6SS is associated with increased survival and pathogenicity in bacteria expressing it (Zhao et al., 2018). It is composed of 13–14 core proteins (Boyer et al., 2009), usually encoded in the same locus in the genome (Mougous et al., 2006). The T6SS assembles a molecular spring-loaded dagger, which punctures the target cell to secrete fully folded effector proteins into neighbouring bacteria (Russell et al., 2011) or eukaryotic hosts (Hachani et al., 2016) The full assembly consists of the trans-envelope TssJLM membrane complex (MC) (Durand et al., 2015) that tethers the TssKFGE-VgrG baseplate (Brunet et al., 2015; Cherrak et al., 2018), onto which the tail polymerizes. This tail comprises the inner tube made of stacks of Hcp hexamers wrapped by the TssBC sheath proteins that polymerize in a helical conformation (Robb et al., 2013; Brunet et al., 2014; Clemens et al., 2015; Kudryashev et al., 2015; Chang et al., 2017; Wang et al., 2017), and tipped by the spike VgrG, which can be sharpened by the PAAR protein (Shneider et al., 2013; Renault et al., 2018). Effectors are either associated within the Hcp lumen, or directly or indirectly bound to the VgrG or PAAR spike (Shneider et al., 2013; Silverman et al., 2013; Flaugnatti et al., 2016; Unterweger et al., 2017; Quentin et al., 2018). Upon contact with a neighbouring cell, unknown signals trigger the contraction of the sheath causing the tube and spike to pierce the membranes of the target cell, hence delivering the effectors (Basler et al., 2012).

While the baseplate, tube and sheath proteins are conserved amongst contractile injection systems, the MC is specific to the
T6SS. TssJ is an outer membrane lipoprotein (Aschtgen et al., 2008) that positions first at the site of assembly, and then recruits TssM and TssL (Durand et al., 2015). TssM and TssL are two inner membrane proteins that share homology with two accessory subunits associated with type IV secretion system (T4SS)b, lcmF and lcmH/DotU (Ma et al., 2009; Aschtgen et al., 2012; Durand et al., 2012; Logger et al., 2016). Not only does the MC anchor the baseplate to the inner membrane, but it also serves as a channel to allow the passage of the tail tube/spike, and to maintain the integrity of the attacking cell during the translocation of the inner tube (Durand et al., 2015). The different subunits and the MC have been extensively biochemically characterized and several crystal structures of the components are available from various bacterial species (Felisberto-Rodrigues et al., 2011; Rao et al., 2011; Durand et al., 2012, 2015; Robb et al., 2012, 2013; Chang & Kim, 2015). We previously reported the negative-stain electron microscopy (EM) structure of the TssJLM complex from enteropathogenic Escherichia coli (EAEC). We determined that 10 TssJ lipoproteins are bound to 10 TssM proteins, forming two concentric rings of pillars and arches that span the periplasm. The arches were shown to link to a flexible base composed of the N-terminal part of TssM and 10 copies of TssL that span the periplasm. The arches were shown to link to a flexible base composed of the N-terminal part of TssM and 10 copies of TssL that span the periplasm (Nazarov et al., 2018) raised questions on whether the purified TssJLM MC reflects the in vivo situation. In addition, although most of the available crystal structures can be readily fitted into this EM structure, we currently lack molecular details on the whole complex, such as the precise location of the membranes, of the transmembrane helices, and the potential presence of a periplasmic channel.

Here, we first present the in situ cryo-electron tomography (cryo-ET) structure of the EAEC TssJLM MC. These data confirm the 5-fold symmetry of the complex in vivo and provide information on the location of the inner and outer membranes. We then present the single-particle (SPA) cryo-electron microscopy (cryo-EM) structure of the MC that describes the molecular architecture of the periplasmic portion of the complex. This high-resolution cryo-EM structure reveals the presence of five additional copies of TssJ at the tip of the complex and provides detailed structural information on the periplasmic gate that narrows the MC channel. Finally, we demonstrate that this periplasmic gate and the additional TssJ are required for T6SS-dependent activity in vivo.

Results

Structure of the T6SS MC within the cell envelope

To observe the T6SS MC in its native cellular environment, we performed cryo-ET on bacterial cells (Weiss et al., 2017). With the aim to have a sufficient number of particles to obtain an in situ structure by subtomogram averaging, we imaged E. coli BL21(DE3) cells in which TssJLM was heterologously overproduced. This strain does not possess T6SS genes, thereby preventing any crosstalk or protein–protein interactions between TssJLM and other natively present T6SS proteins. Since E. coli is too thick to be directly imaged by cryo-ET, we pursued three different approaches to tackle sample thickness. The first approach consisted of engineering a minicell-producing skinny strain (Farley et al., 2016) of E. coli BL21(DE3), and thereby generating a minicell strain that is compatible with the T7-based expression system. Although this strain produced minicells as small as 450 nm in diameter (Fig EV1A), their size still affected the contrast to an extent that did not allow for subtomogram averaging. Nevertheless, characteristic inverted Y-shaped particles (side views, Fig EV1B) and star-shaped particles (top views, Fig EV1C) could occasionally be observed in the periplasm of these minicells. In a second approach, tomograms were recorded of cells that were partially lysed and exhibited a high contrast (Fig EV1D and E). These cells, which have previously been described as “ghost cells” due to their transparent appearance, still had mostly intact membranes and showed their cytoplasmic macromolecular complexes such as ribosomes (Fu et al., 2014). In a third approach, we used a state-of-the-art cryo-sample thinning method called focused ion beam (FIB) milling to thin E. coli BL21(DE3) cells in which TssJLM was heterologously overproduced (Fig EV1F and G). FIB milling allows to etch through a lawn of bacterial cells and to thin them down to under 200 nm (Marko et al., 2007; Medeiros et al., 2018a,b). This approach was more native, as it was performed on intact rod-shaped wild-type BL21(DE3) cells. These tomograms had a high contrast and provided great detail. To confirm the relevance of these observations, we carried out control experiments in the native T6SS+ EAEC 17-2 strain. TssJLM particles were frequently observed by cryo-ET, both when heterologously overproduced (Fig EV1H, note that the particles could occasionally be found detached from the outer membrane: Fig EV1I) as well as under native conditions, i.e. in wild-type EAEC 17-2 cells (Fig EV1I).

Subvolumes of star-shaped (top views) and inverted Y-shaped (side views) particles were manually picked, computationally extracted, and subsequently aligned and averaged. The resulting average was similar to the in vitro T6SS MC structure published previously (Durand et al., 2015), with a tip and a core, made of 5 pairs of pillars forming a narrow central channel, that splits into 10 arches (Fig 1A–E). Importantly, the 5-fold symmetry was evident without applying symmetry (Fig 1A’). As it is the case for the TssJLM MC solved by EM (Durand et al., 2015), the periplasmic core and the arches were well resolved, whereas the inner membrane-embedded base and the outer membrane-embedded cap were poorly resolved. The Fourier shell correlation (FSC) curve indicated a resolution of 25 Å at a coefficient of 0.5 (Appendix Fig S1A). The prevalence of top views indicated that the average might be affected by a missing wedge (Appendix Fig S1B).

After aligning and averaging a set of subvolumes, it can be useful to place the isosurface of the average back in the original tomographic volume to analyse the positions and orientations of the individual aligned particles. In this way, we obtained a clear view of the location of the MC within the cell envelope (Fig 2A, Movie EV1 and Appendix Fig S1C–E, Movie EV2). The position of the membranes with respect to the TssJLM highlighted that the tip was embedded in the outer membrane without crossing it, while the MC was anchored in the inner membrane at the lower part of the arches (Fig 2B). In some cases, densities could be seen spanning the inner membrane and extending into the cytoplasm (Fig 2C). In what could be an overproduction artefact, TssJLM particles were also found in cytoplasmic membrane invaginations (Fig 2A). Altogether, these
data confirmed the 5-fold symmetry of the T6SS TssJLM MC in situ and provided further insights into the position of the inner and outer membranes.

**Structural analysis of the TssJLM complex by cryo-EM**

We previously reported the negative-stain EM structure of the EAEC TssJLM MC (Durand et al., 2015). Here, we used the same purification procedure and performed SPA cryo-EM to obtain the cryo-EM structure of the 1.7-MDa T6SS MC at 4.9 Å overall resolution. With a 5-fold symmetry imposed, the local resolution ranged between 4.1 and 21.7 Å (Fig 3A and Appendix Fig S2A–F).

The 5-fold symmetry is clearly visible in the 2D classes top views and the volume slices of the reconstruction, which retain a 5-pointed star shape (Appendix Fig S2C and G). When no symmetry was applied during the reconstruction, the overall resolution decreased to 7.5 Å but the pentameric nature of the complex was maintained, with only one of the 10 arches displaying weaker density than the others causing the drop in resolution (Appendix Fig S2H and I). This could indicate partial assembly of the complex in vivo or disassembly during its purification.

The overall structure resembles that obtained by negative stain (Appendix Fig S2J; Durand et al., 2015). The architecture of the complex comprises a tip connected to a core region that extends to a base, through a double ring of pillars with arches in proximity to the inner membrane (Fig 3A and B). Several notable features are already evident from the cryo-EM density map of the full complex (Fig 3A). First, the tip region and the base are disordered and appear to be filled by random densities. Second, cross sections of the full complex show that the channel, across which

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**Figure 1. Subtomogram average of the TssJLM complex in situ.**

A–E Isosurface of the subtomogram average (in pink) with an applied C5 symmetry and 0.69 nm tomographic slices (A–E) at the indicated heights. Scale bars 10 nm.

The average is shown in side view, whereas the slices represent perpendicular slices. The 5 pairs of pillars formed a narrow central channel and separated into 10 arches towards the base (base not visible at the used threshold). The division of the structure into subparts was adapted from Durand et al. (2015). Subvolumes were extracted from cryotomograms of ghost cells and FIB-milled intact Escherichia coli BL21 cells heterologously expressing TssJLM.

A′ Slice (0.69 nm) through the non-symmetrized average. Note that the C5 symmetry was visible.
tube/spike transport might occur, is closed by a gate at the intersection between arches and pillars, above the inner membrane (Fig 3B–E and Appendix Fig S3A-D, respectively). This gate is also visible in the reconstruction without symmetry applied (Appendix Fig S2H).

To better characterize the flexibility of the base of the complex, we collected tomograms on the same frozen EM grids that were used to collect the SPA dataset (Appendix Fig S4A–C). The base of individual complexes appeared as very heterogeneous, with single arches often pointing in opposite directions, or on the contrary several arches clumping together (Appendix Fig S4A). On the other hand, the core was rigid and resembled a 5-branched star. Moreover, about 20% of the particles possessed only 3 or 4 out of 5 branches (Appendix Fig S4B), indicating that partial assemblies could be stable. The particles were lying in a thin layer of ice (25 nm) and were found in different orientations (Appendix Fig S4C1 and C2).

The structure of the base

To try and overcome the inherent flexibility of the complex and better discern different features of the base, we performed a density subtraction of the tip, core and arches followed by a focused refinement of the base with and without symmetry applied. We thus obtained 2D classes and a 3D structure of the base at 17 Å resolution when a C5 symmetry was applied (Fig 3F and Appendix Fig S4E and F). When observing the cross section of the base in the full complex, as indicated by arrows in Fig 3B, and in the subtracted structure (Appendix Fig S4E), two 110 Å-wide linear densities are clearly visible, separated by 40 Å. This double layer of density is consistent with the density diagram of a lipid bilayer with the head groups being the most dense at a distance of ~4 nm from each other and fits well a lipid bilayer composed of PE, obtained using the CHARMM-GUI (Jo et al., 2007; Appendix Fig S4E). We propose that the inner membrane subdomain of the T6SS MC could be filled by a
Figure 3.
l lipid bilayer. However, such hypothesis will have to be further explored in the future.

**An additional TssJ is present in the full complex**

To focus on the best-resolved region of the cryo-EM map, the base was subtracted from the tip, and a 2D classification and a masked 3D refinement were performed to obtain the structure of the core at 4.5 Å (Figs 3G and EV2A and B), with a local resolution ranging from 4 Å to > 10 Å (Fig EV2C). The known crystallographic structure of the C-terminus of TssM (aa. 869–1129) bound to TssJ (PDB 4Y7O) could be easily fitted in the outer and inner pillars, with a correlation of 0.8505 and 0.565, respectively, leaving an extra density (Fig EV2D and E). Interestingly, an extra TssJ subunit, TssJ$, which was not observed in the low-resolution complex (Durand et al, 2015) fits in this extra density with a correlation of 0.879 (Fig EV2E and F). We thus conclude that the T6SS MC comprises 15 TssJ proteins, and 3 TssJs for 2 TssM (Fig 4A). No additional residues were visible for the N- and C-termini of TssJ (1–21 and 151–155), disordered in both the crystal and the cryo-EM structures. After placing the extra TssJ, we refined the TssM-TssJ crystal structure and the newly fitted TssJ copy against the cryo-EM map to obtain the structure of each TssM and TssJ monomer within the whole MC.

TssJ$ binds to the MC through previously unknown interfaces. If we consider TssJ and the TssJ subunits from the inner and outer pillars (TssJ.i and TssJ.o, respectively; Figs 4A–C and EV3A and B) with TssJ.i and TssJ.o being in contact with TssM.i and TssM.o, respectively, they sit in the same position as in the crystal structure and in the outer and inner pillars (Fig 4B). By contrast, TssJ binds to TssM.o and TssJ.i strongly (Figs 4C and EV3C and Table 1). In particular, the interaction of TssJ$ with TssJ.i is specifically strong, as their contact is mediated not only by hydrogen bonds but also by salt bridges (R31 with E34, and D97 with R33; see Fig EV3C and Table 1). TssJ binds to TssM.i via hydrogen bonds only and this interaction is comparable to that between TssJ.i and TssJ.o with TssM.i and TssM.o, respectively (surface of interaction 573 Å$^2$ and −2.6 kcal/mol ΔG; Fig 4B and C, Table 1).

**TssJ$ is required for MC assembly and T6SS activity**

To gain further information on the function and in vivo relevance of TssJ$, mutations that specifically impact the TssJ$-TssJ.i interface were engineered onto the native locus. Two residues, R31 and D97 (Fig EV3C), were targeted as they form a salt bridge with E34 and R33 in TssJ.i, respectively (Table 1 and Fig EV3C). The R31E, D97A and D97K substitutions were then tested for their ability to outcompete a fluorescent E. coli competitor strain. Although the R31E and D97A did not significantly impact T6SS antibacterial activity, the D97K mutation abolished proper function of the T6SS (Fig 4D). The assembly and stability of the T6SS MC were then assessed by fluorescence microscopy using a chromosomally encoded and functional fusion protein between TssM and the super-folder GFP (sfGFPTssM, Durand et al, 2015; Appendix Fig S5A). As previously observed, sfGFPTssM formed stable foci (Durand et al, 2015). By contrast, cells producing the TssJ D97K variant presented small and unstable fluorescent sfGFPTssM foci (Fig 4E). Time-lapse fluorescence microscopy recordings showed that about 90% of the foci observed in the sfGFPTssM strain (n = 50) are stable over the 600-s recording time, in agreement with the previous observation that the EAEC T6SS MC is stable and serves for several contraction/elongation cycles (Durand et al, 2015). By contrast, with a mean lifetime of ~107 s (n = 50) the sfGFPTssM fluorescent foci observed in cells producing the TssJ D97K variant are drastically less stable (Fig 4E). We then tested whether the TssJ D97K variant promotes sheath assembly, using chromosomally encoded functional sfGFPTssB fusion. Contrarily to the wild-type parental cells in which dynamic sheaths can be observed, no sheath polymerization occurs in presence of TssJ$-D97K (Appendix Figs S5A and B). Altogether, these results demonstrate that the TssJ$-TssJ.i interface is required for the stability of the T6SS MC, sheath formation and T6SS antibacterial activity.

**A flexible hinge within the TssM periplasmic domain**

We were able to confidently build de novo the periplasmic domain of TssM including its N-terminal fragment (residues 579–869) that was missing in the crystal structure (Durand et al, 2015). This was done in Coot (Emsley et al, 2010) by using Phyre2 secondary structure predictions (Kelley et al, 2015) and RaptorX residue contact predictions (Källberg et al, 2012) as validation tools (Fig 5A and Appendix Fig S6A–C). The cryo-EM structure of the TssM periplasmic domain slightly differs from the X-ray structure (Fig EV4A). From the C-terminus, helix 869–891 extends to amino acid 841 with a slight kink at residue Pro-870. The remaining N-terminal fragment forms an $\alpha$-helical domain comprising 8 helices that snake back and forth to the inner membrane (Fig 5B). The region closest to the membrane was too flexible to be resolved and for an atomic model to be built (Appendix Fig S6B). The cryo-EM pseudoatomic model of the fully assembled TssM-TssJ complex shows it forms a bell shape composed of two rings of pillars that twist around each other (Fig 5A). Within each asymmetric unit, two copies of TssM are present, named TssM.o in the outer pillar and TssM.i in the inner pillar. The inner and the outer pillar TssM proteins are superimpos-
Figure 4.

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an area of 1.529 Å², a binding energy of -12 kcal/mol and a ΔG of -5.32 kcal/mol (Figs EV3A and EV4D). Finally, TssM.o-1 also interacts with TssM.i and the two are oriented at 68° from one another (Figs EV3A and EV4E).

A poorly defined density that sits in the core region between TssM.i and TssM.o-1 was attributed to the C-terminus of TssM. If we were to build a small loop that terminates into a helix at the C-terminus, we would not be able to reach the membrane region as previously proposed (Durand et al., 2015; Fig EV4F). This same loop is disordered in the outer pillar monomer (TssM.o). Additionally, the resolution of the pillars gets worse towards the basal side, and no secondary structure could be identified when we tried to build de novo the atomic model of TssM. Despite this high degree of flexibility, we produced a model of the periplasmic region between amino acids 390 and 550 based on RaptorX contact predictions. This model fits well (correlations of 0.819 and 0.827) into the remaining densities (Fig EV4G). The EM density shows that while they are separated at the level of the arches, the inner and the outer pillar rejoin at the level of the inner membrane (Fig EV4G).

The periplasmic TssM gate

The inner pillars of TssM form a channel with a diameter that varies between 2.6 Å and >20 Å (Fig 5C). The site of constriction observed in the cryo-EM density (Fig 3B and E) corresponds to loop 776–786 in the atomic model of TssM (Fig 5D). Specifically, residues Gln-779 and Asn-780–781 maintain the constriction via polar interactions (Fig 5D). In the outer pillar, the same loop interacts with loop 600–625 on the neighbouring pillar, providing further stabilization of the structure (Appendix Fig S7A). Conservation analysis of the sequence with related proteins, predicted by the ConSurf server (Celniker et al., 2013), indicated that the sequence of the loop 776–786 is poorly conserved amongst species (Appendix Fig S7B) although the presence of a loop at this position is a conserved feature. As previously proposed (Durand et al., 2015), these data suggest that the purified TssULM MC is in a closed state. Large conformational changes, including modification of the constriction and movement of the inner pillars, are therefore required to allow the passage of the tube/spike during sheath contraction.

The TssM periplasmic gate is required for MC assembly

To test the function of the periplasmic gate, several mutations were engineered at the tssM locus in the wild-type EAEC 17-2 strain and the function of the T6SS was assessed as previously described (Fig 5E–G). To covalently stabilize the contacts between the inner pillars and thus prevent MC opening, Q779 and N780 were substituted with cysteines (Q779C-N780C). Conversely, a constitutively open gate was created by deleting a large portion of the constriction loop (Δ777–783). Antibacterial competition assays showed that the Q779C-N780C variant loses the ability to outcompete competitor cells, whereas the single control mutant Q779C did not (Fig 5E).

Table 1. Bonds between the Tssj and TssM subunits.

| Monomer1    | Monomer2    | Area (Å) | ΔG   | Nhβ | Nsb |
|-------------|-------------|----------|------|-----|-----|
| TssM.i      | TssM.i-1    | 1,528.78 | -5.32 | 9.2 | 4.8 |
| TssM.i      | TssM.o-1    | 1,168.6  | -7.54 | 4.8 | 6.2 |
| TssM.i      | TssM.o      | 1,126.48 | -5.2  | 5.8 | 5.4 |
| TssM.o      | Tssj       | 561.7    | -2.38 | 6.6 | 0  |
| TssM.i      | Tssj.i      | 546.44   | -9    | 1   | 0  |
| Tssj.o      | TssM.o      | 528.58   | -10.14| 5   | 0  |
| Tssj‘       | Tssj.i      | 480.62   | -0.82 | 4.6 | 5.4 |
| TssM.i      | Tssj.o-1    | 190.26   | 1.7   | 4   | 0  |
| Tssj.i      | TssM.o      | 109.72   | -1.14 | 0.2 | 0  |
Figure 5.
assembled when the integrity of the periplasmic gate is impacted. As expected, these mutant cells did not assemble T6SS sheaths (Fig 5G and Appendix Fig 8).

Discussion

In this study, we report the in situ and in vitro structures of the T6SS TssJLM MC from EAEC. The cryo-ET structure confirmed the fivefold symmetry and general architecture in vivo, while the high-resolution cryo-EM structure provided molecular details about the periplasmic portion of the complex (Figs 1 and 3 and Movie EV3). As previously defined, the pentameric propeller-like structure composed of 10 pillars intertwined with each other was observed both in situ and from purified material (Figs 1 and 3C–E). Cryo-ET analyses allowed to position both the inner and outer membranes. As anticipated based on biochemical experiments showing that TssJ is a periplasmic lipoprotein attached to the outer membrane by an acyl anchor (Aschghten et al, 2008, 2008), the tip of the complex is embedded in the outer membrane (Fig 2B). However, neither the predicted detergent cap in the SPA cryo-EM structure nor the cryo-ET data indicate that TssJLM breaches or crosses the outer membrane (Figs 2 and 3A and B). Nevertheless, it has been shown that this region is extracellularly exposed in wild-type EAEC cells (Durand et al, 2015). Although the cryo-EM structure demonstrates that the C-terminal region of TssM locates in the periplasm, we propose that the recruitment of specific T6SS components induces MC conformational changes and cell surface exposition of the TssM C-terminus. While our previous study suggested that the inner membrane locates at the level of the arches (Durand et al, 2015), the cryo-ET analyses revealed that the inner membrane surrounds the base (Fig 2B). Moreover, some tomograms also revealed the most basal parts of the MC, which cross the inner membrane into the cytoplasm (Fig 2A and C). These cytoplasmic densities, which we can assume would be connected to the baseplate in a fully assembled T6SS, had a heterogeneous appearance that highlighted the flexibility of the base, as discussed below. The in situ cryo-ET structure presents an apparent elongation of the tip region compared to the cryo-EM structure (Appendix Fig S9A and B and Movie EV3), which could correspond to an additional density associated to the outer membrane or which could alternatively be explained by the missing wedge. Nevertheless, the similarities between both structures allowed the atomic model of TssJ-TssM to be docked into the in situ average (Appendix Fig S9C and D), whereas the cryo-EM structure could be placed in a cellular context (Appendix Fig S9E and Movie EV3).

The base of the complex in this higher resolution structure was not better resolved than in the negative-stain structure (Fig 3A and F and Appendix Fig S2D). This base should comprise 10 copies of the TssM and TssL cytoplasmic domains (Durand et al, 2015). TssL forms dimers (Durand et al, 2012; Zoued et al, 2016, 2018), and the cryo-EM structure of its cytoplasmic hook-like domain has been reported from various species including EAEC, P. aeruginosa and V. cholerae (Durand et al, 2012; Robb et al, 2012; Chang & Kim, 2015; Wang et al, 2018). The TssM cytoplasmic domain is comprised between the N-terminal transmembrane hairpin and a third transmembrane helix (Ma et al, 2009; Logger et al, 2016). No high-resolution structure of the TssM cytoplasmic domain is available, although a model has been built based on homology with DPY-30 and NTPases (Logger et al, 2016). Unfortunately, due to the poor resolution of the base, we did not succeed to confidently fit the TssL and TssM cytoplasmic domains in this density. Additional assays to improve the resolution such as the use of nanodiscs or amphipols proved to be unsuccessful (Appendix Fig S10A). The flexibility of the TssJLM base, which did not allow for it to be resolved, might be due to the absence of other T6SS cytoplasmic components, such as the baseplate. A similar observation was made for the type III secretion system, in which the presence of the cytoplasmic sorting platform orders the IM components (Hu et al, 2017). One may hypothesize that this flexibility is essential for the docking of the hexameric baseplate and to accommodate the fivefold to sixfold symmetry mismatch. One alternative hypothesis is that the disorder at the centre of the base structure is caused by the presence of a lipid bilayer encircled by the TssM and TssL proteins (Appendix Fig S4E). In the in vitro situation, the MC assembles first, before the recruitment of the baseplate (Brunet et al, 2015; Durand et al, 2015), and hence, one can expect that a lipid bilayer at the entrance of the TssJLM lumen would be present before baseplate docking to prevent the leakage of solutes and proton-motive force.

The high-resolution structure of the EAEC TssJLM MC also revealed new interesting and functional features. First, five
additional TssJ subunits, called TssJ', were identified in the tip complex. These TssJ' proteins interact with the TssJ proteins of the inner pillars (TssJ.i). Mutations that interfere with TssJ'-TssJ.i interaction impaired the functional integrity of the MC and hence inactivated the T6SS (Fig 4D and E and Appendix Fig S5). Second, we identified an 11-amino acid loop in TssM that protrudes from each inner pillar to the centre of the channel, thus creating a constriction that is observed in the density map (Fig 3B and E and Appendix Fig S5).
Appendix Fig S3). Each loop is stabilized by the adjacent loop via Asn/Gln pairings (Fig 5D). Such weak interactions could be easily displaced by the VgrG/PAAR spike upon baseplate docking or during firing. Our mutational analyses demonstrate that this constriction is important for TssULM MC formation and T6SS activity. Periplasmic constrictions are usual features of trans-envelope complexes. The best characterized examples include the OM T2SS and T3SS secretins, and the CsgG curli secretion channel, where one or two periplasmic gates are present to prevent leakage (Appendix Fig S11A and B; Spagnuolo et al, 2010; Goyal et al, 2014; Yan et al, 2017). While we do not know the role of this constriction in the T6SS, we propose that these loops may stabilize the MC in its closed conformation during the resting state. The cryo-EM structure defined two hinge regions that exhibit a certain degree of flexibility (Fig EV4B and G). These two hinges result in the formation of the two layers of pillars, with the inner layer obstructing the channel. A large conformational change of these pillars is therefore necessary to open the channel for the passage of the tube/spike complex. Interestingly, with an interaction surface of 1,540 Å² (AG of 6.1 kcal/mol) the TssM inner pillars contacts are considerably less stable than the contacts within the T2SS secretin (interaction surface of 5,353.7 Å² and AG of −52.4 kcal/mol). The displacement of the pillars could be controlled by the flexibility of the hinge regions.

Based on these data, we propose a model in which the T6SS TssULM MC is assembled in a closed state. In this conformation, five pillars are oriented towards the centre of the complex to close the complex at the outer membrane and hence to protect the cell from periplasmic leakage or from the entry of toxic compounds. This conformation is further stabilized by the interactions of the TssM protruding loops. The flexibility of the MC cytoplasmic base allows the proper positioning of the TssKFGE-VgrG baseplate and accommodates the five-to-six symmetry. The docking of the baseplate positions the VgrG/PAAR spike in proximity to the inner membrane. Once in contact with the target cell, a signal transmitted to the baseplate triggers the contraction of the sheath, allowing the passage of the tube/spike complex through the MC. The hinge regions undergo a tectonic conformational change that opens the channel and the tip complex. The MC then returns to the resting, closing state allowing a new cycle to start (Fig 6). This model is still incomplete and rather speculative. Many aspects of the secretion mechanism by the T6SS remain elusive. Further investigations are therefore needed to provide a complete molecular understanding of this mechanism.

Materials and Methods

Strains and media

Strains used in this study are listed in Appendix Table S1. The E. coli K-12 W3110 bearing the pUA66-rnmB vector (KanR and GFP+) (Zaslaver et al, 2006) was used as recipient for antibacterial competition assays. Strains were routinely grown in lysogeny broth (LB)-rich medium or in Sci-1-inducing medium (SIM; M9 minimal medium, glycerol 0.2%, vitamin B1 1 µg/ml, casamino acids 100 mg/ml, LB 10%, supplemented or not with bacto agar 1.5%; Brunet et al, 2011) with shaking at 37°C.

Strain construction

In this study, tssM and tssJ point mutations were engineered at the native locus on the chromosome by allelic replacement using the pKO3 suicide vector (Link et al, 1997) into the enteroaggregative E. coli 17-2 strain. Briefly, 17-2 WT strain was transformed with a pKO3 plasmid in which a fragment of the tssM or tssJ gene carrying the point mutations has been cloned (see below). Insertion of the plasmid into the chromosome was selected on chloramphenicol plates at 42°C. Plasmid sequences removal was then selected on 5% sucrose plates without antibiotic, and tssM point mutation recombinant strains were screened by PCR and confirmed by DNA sequencing (Eurofins, MWG). Chromosomal fluorescent reporter insertions into the enteroaggregative E. coli 17-2 strain mutated in tssM or tssJ were achieved by using a modified one-step inactivation procedure (Dat-senko & Wanner, 2000) as previously described (Aschtgen et al, 2008) using plasmid pKOBEG (Chaveroche et al, 2000). Briefly, a kanamycin cassette was amplified from plasmid pKD4 using oligonucleotide pairs carrying 5’ 50-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, inserted at the gene locus on the bacterial chromosome, was then excised using plasmid pCP20, leaving an FRT scars. Gene deletions were confirmed by colony PCR and sequencing.

Fluorescence microscopy, image treatment and analyses

Fluorescence microscopy experiments were performed as described (Brunet et al, 2013; Zoued et al, 2013). 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 1.5% agarose pad and covered with a cover slip. Fluorescence micrographs were captured using AxioImager M2 microscope (Zeiss) equipped with an OrcaR2 digital camera (Hamamatsu). For time-lapse fluorescence microscopy, images were recorded with a Nikon Eclipse Ti microscope equipped with an Orca-Flash 4.0 LT digital camera (Hamamatsu) and a perfect focus system (PFS) to automatically maintain focus so that the point of interest within a specimen is always kept in sharp focus at all times despite mechanical or thermal perturbations. Fluorescence images were acquired with a minimal exposure time to reduce bleaching and phototoxicity effects, typically 200 ms for TssBsgFP and 300 ms for sGFFTssM. For image treatment, noise and background were reduced using the “Subtract Background” (20 pixels Rolling Ball) and Band plugins of imageJ (Schneider et al, 2012). The sGFP foci were automatically detected using the microbeJ plugin (Ducret et al, 2016). Floating bars representing the number of detected foci for each strain were made using GraphPad (https://www.graphpad.com). Microscopy analyses were performed at least three times, each in technical triplicate, and a representative experiment is shown.

Interbacterial competition assay

The antibacterial growth competition assay was performed as previously described (Flaugnatti et al, 2016). Wild-type E. coli K-12 strain
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Chiara Rapisarda et al Architecture of the T6S membrane complex

The 16,000 movies collected were aligned using MotionCor2, with Cryo-EM image processing were collected at 1.38 Å pixel size with a 5 s exposure time and composed of 25 frames at a defocus range between 0.7 and 2 Å. The defocus range chosen for the automatic collect was 20/micrograph were acquired for 1 s with a total electron flux of with a pixel size of 1.24 Å. Dose-fractionated movie frames camera (Thermo Fisher, Waltham, MA, USA) in linear mode and Talos Arctica electron microscope equipped with a Falcon 3EC et al plunge-frozen in liquid ethane (Zhang, 2016) for 2 s in a Leica EM GP at 80% humidity and 4°C. The bacterial spots were resuspended in LB and bacterial suspensions were normalized to A600 nm = 0.5. For the enumeration of viable prey cells, bacterial suspensions were serially diluted and spotted onto kanamycin LB plates. The assays were performed from at least three independent cultures, with technical triplicates, and a representative technical triplicate is shown.

Protein preparation

The expression and purification of the TssJLM complex were carried out as previously described (Durand et al, 2015), with the exception that the cryo-EM grids were prepared immediately after the HisTrap Elution. For the amphiplo- containing sample, the Strep-Trap elution was incubated with amphiploes A8-35 (Anatrace, USA) and subjected to gel filtration on a superfuse 6 (GE Healthcare, UK) to remove residual detergent.

Cryo-EM grids preparation and data acquisition

C-flat™ (CF-2/1-2C) grids were coated with graphene oxide as previously described (Martin et al, 2016). 3.5 µl of the sample at 0.2 mg/ml was loaded on the copper side and then blotted on the same side for 2 s in a Leica EM GP at 80% humidity and 4°C, before being plunge-frozen in liquid ethane (−184°C). Micrographs (Appendix Fig S2B) at a nominal magnification of 120,000× were collected in a Talos Arctica electron microscope equipped with a Falcon 3EC camera (Thermo Fisher, Waltham, MA, USA) in linear mode and with a pixel size of 1.24 Å. Dose-fractionated movie frames 20/micrograph were acquired for 1 s with a total electron flux of 120 e/Å²/s. The defocus range chosen for the automatic collect was 0.7 to 2 µm, which resulted in an actual range between 0.4 and 5 µm.

For the amphiplo- containing MC collection, 3019 movies composed of 25 frames at a defocus range between 0.7 and 2 µm were collected at 1.38 Å pixel size with a 5 s exposure time and 15 e/pix/s exposure rate at the Krios 2 at the Diamond eBIC facility.

Cryo-EM image processing

The 16,000 movies collected were aligned using MotionCor2, with dose weighting (6 e-/Å²/frame) and with 5 × 5 patches applied (Zheng et al, 2017). gCTF was used to estimate the CTF parameters (Zhang, 2016) and low-quality images were discarded. Relion 2.1 (Scheres, 2012) autopicked 227,527 particles and after several rounds of 2D classification in cryosparc (Punjani et al, 2017) and a heterogeneous ab initio reconstruction (two classes), 37,435 particles were converted using the script csparc2star.py (Asarnow, 2016) and selected for a final 2D classification in relion 2.1 (Appendix Fig S2C), of which 36,828 particles were selected. An initial unmasked refinement using the ab initio model from cryosparc gave us a resolution of 7.6 Å with 5-fold applied symmetry and a soft mask of 450 Å. This refined structure was used to do a movie refinement with all the frames and a polishing step with RELION2.1. The final masked refinement of the full structure gave a final resolution of 4.9 Å with a C5 symmetry applied, and 7.9 Å with no symmetry applied (Appendix Fig S2E and I). The disordered tip and base were subtracted and a masked refinement around the core structure yielded a final resolution of 4.6 Å (Fig EV2B). The base focused refinement was also performed on subtracted particles, without the tip and the core regions, to a resolution of 17 Å (Appendix Fig S4F).

The resolution for all densities except the base was calculated by masked post-processing according to the “gold standard” method using 0.143 as the FSC value cut-off, or 0.5 for the low-resolution reconstruction (Rosenthal & Henderson, 2003), and the local resolution of the core was calculated by relion 2.1 (Fig EV2C).

Model building

Model building proceeded by fitting the PDB 4Y7O (Durand et al, 2015) into the density 2 times for each pillar, with the cross-correlation being calculated using Chimera (Pettersen et al, 2004; Fig EV2D). To complete the structure of TssM beyond the known region, which spanned aa. 869–1129, we used the de novo tracing strategy that we introduced in an earlier work (Cherrak et al, 2018).

Briefly, we iterated between manual model building and structural refinement on Coot (Emsley et al, 2010) using bulky sidechains and secondary structure predictions obtained by Phyre2 (Kelley et al, 2015) as guides, and sequence-structure registration based on contact prediction obtained by RaptorX (Källberg et al, 2012). The map of the predicted contacts was aligned with those of the built PDB, the algorithm introduced by the MapAlign software (Ovchinnikov et al, 2017). Where discrepancies were observed, the register was modified to fit the predicted contact maps (Appendix Fig S6). The model was eventually refined using one round of rosetta.refine (Wang et al, 2016) and phenix.real_space_refine (Afonine et al, 2018). This procedure allowed us to extend the structure of TssM to the fragment spanning residues 579–869, and to produce a model of the fragment between amino acids 390 and 550.

Validation of the data

The model was validated as in the protocol in Refmac5 (Murshudov et al, 2011). The FSC map to model was calculated with the sharpened map (FSCshap). The model was shaken by 0.5 Å and the FSC map to model was calculated with one Half map (FSCwork). This refined model was then used to calculate the FSC map to model with the other Half map (FSCfree: Appendix Fig S12A).

The cross-correlation between each amino acid in the model and map was also calculated with phenix.real_space_refine (Afonine...
**Table 2. Cryo-EM data collection, refinement and validation statistics.**

|                           | TssJLM core complex (EMD-0264) (PDB 6HS7) | TssJLM complex (EMD-0265) | TssJLM C1 complex (EMD-0266) | TssJLM base complex (EMD-0267) |
|---------------------------|------------------------------------------|---------------------------|-------------------------------|--------------------------------|
| Data collection and processing |                                           |                           |                               |                                |
| Magnification (×)          | 120,000×                                 | 120,000×                  | 120,000×                      | 120,000×                       |
| Voltage (kV)              | 200                                      | 200                       | 200                           | 200                            |
| Electron exposure (e⁻/Å²) | 120                                      | 120                       | 120                           | 120                            |
| Defocus range (µm)        | 0.4–5                                    | 0.4–5                     | 0.4–5                         | 0.4–5                          |
| Pixel size (Å)            | 1.24                                     | 1.24                      | 1.24                          | 1.24                           |
| Symmetry imposed          | C5                                       | C5                        | C1                            | C5                             |
| Initial particle images (no.) | 167,825                                | 167,825                   | 167,825                       | 167,825                        |
| Final particle images (no.) | 36,828                                  | 36,828                    | 36,828                        | 36,828                         |
| Map resolution (Å)        | 4.6                                      | 4.9                       | 7.9                           | 17                             |
| FSC threshold             | 0.143                                    | 0.143                     | 0.143                         | 0.143                          |
| Map resolution range (Å)  | 3.9 and 18 Å                            | 3.8–33 Å                  | –                             | –                              |
| Tomography                |                                          |                           |                               |                                |
| Number of grid points     |                                          |                           |                               |                                |
| Voxel size                |                                          |                           |                               |                                |
| Projections               |                                          |                           |                               |                                |
| Refinement                |                                          |                           |                               |                                |
| Initial model used (PDB code) | 4Y7O                                    |                           |                               |                                |
| Model resolution (Å)      |                                          |                           |                               |                                |
| FSC threshold             | 4.6 Å                                    |                           |                               |                                |
| Model resolution range (Å) |                                         |                           |                               |                                |
| Map sharpening B factor (Å²) |                                         |                           |                               |                                |
| Model composition         |                                          |                           |                               |                                |
| Non-hydrogen atoms        | 52,890                                   |                           |                               |                                |
| Protein residues          | 6,905                                    |                           |                               |                                |
| Ligands                   |                                         | N/A                       |                               |                                |
| B factors (Å²)            |                                         |                           |                               |                                |
| Protein                   |                                         |                           |                               |                                |
| Ligand                    |                                         | N/A                       |                               |                                |
| R.m.s. deviations         |                                          |                           |                               |                                |
| Bond lengths (Å)          | 0.006                                    |                           |                               |                                |
| Bond angles (°)           | 0.989                                    |                           |                               |                                |
| Validation                |                                          |                           |                               |                                |
| MolProbity score          | 1.92                                     |                           |                               |                                |
| Clashscore                | 6.39                                     |                           |                               |                                |
| Poor rotamers (%)         | 0.04                                     |                           |                               |                                |
| Ramachandran plot         |                                          |                           |                               |                                |
| Favoured (%)              | 89.46                                    |                           |                               |                                |
| Allowed (%)               | 10.54                                    |                           |                               |                                |
| Disallowed (%)            | 0                                        |                           |                               |                                |

Strains, media and chemicals

The strains, plasmids and nucleotides used in this study are listed in Appendix Tables S1 and S2. For the cryo-ET studies, *E. coli* K-12 BL21(DE3) and enteroaggregative *E. coli* EAEC strain 17-2 were used.
for protein overexpression before plunge freezing. Strains were routinely grown in LB-Miller or in Sci-1-inducing medium (SIM; M9 minimal medium, glycerol 0.2%, vitamin B1 1 mg/ml, casamino acids 100 mg/ml, LB 10%, supplemented or not with bacto agar 1.5%; Brunet et al., 2011) with shaking at 37°C. Plasmids were maintained by the addition of ampicillin (100 mg/ml for E. coli K-12, 200 mg/ml for EAEC), kanamycin (50 mg/ml) or chloramphenicol (30 mg/ml). Expression of genes from pRSF (in BL21) and pBAD33 (in EAEC) vectors was induced for 2–3 h with 1 mM of isopropyl-b-D-thio-galactopyranoside (IPTG) or 0.3% l-arabinose, respectively.

Preparation of frozen-hydrated specimens

Plunge freezing was performed according to Weiss et al. (2017). Escherichia coli BL21 or EAEC cells were concentrated by centrifugation to an OD~100 of 3–20 and then mixed with protein A–10 nm gold conjugate (CytoDiagnostics Inc.). The higher concentrations of cells were used when preparing grids for cryo-focused ion beam (cryo-FIB) milling to form “bacterial lawns” of several layers of bacteria on top of each other. Bacterial lawns were found to be more amenable to cryo-FIB milling than individual cells. A 3 µl droplet of the sample was applied to a carbon-coated EM copper grid (R2/1, Quantifoil) that had been previously glow-discharged for 90 s at −25 mA using a Pelco easiGlow™ (Ted Pella, Inc.). The grid was plunge-frozen in liquid ethane–propane (37%/63%) using a Mark IV Vitrobot (Thermo Fisher Scientific). The forceps were mounted in the Vitrobot (27°C, humidity 95%), and the grid was blotted from both sides or only from the backside by installing a Teflon sheet (instead of a filter paper) on the front blotting pad. Grids were stored in liquid nitrogen.

Cryo-focused ion beam milling

Cryo-focused ion beam (cryo-FIB) milling was used to prepare samples of plunge-frozen cells that could then be imaged by electron cryotomography (Marko et al., 2007). Our cryo-FIB milling workflow has been detailed in Medeiros et al. (2018b). Frozen grids with lawns of E. coli BL21 cells overexpressing TssJLM were clipped into modified Autogrids provided by J. Plitzko or a commercial prototype provided by Thermo Fisher. We then transferred the grids into the liquid nitrogen bath of a loading station (Leica Microsystems) and allowed the definition of each structure’s longitudinal axis as the particle rotation axes were generated using the stalkInit program from the PEET package (Nicastro, 2006). This approach summarized the data collection, refinement and validation statistics.
Table 3. Cryo-ET data collection, refinement and validation statistics.

|                      | STA_AvgVol_15000 (EMD-4561) | STA_AvgVol_23500 (EMD-4562) |
|----------------------|-------------------------------|-------------------------------|
| **Data collection and processing** |                               |                               |
| Magnification        | 42,000                        | 42,000                        |
| Voltage (kV)         | 300                           | 300                           |
| Electron exposure (e-/Å²) | 90                            | 90                            |
| Defocus range (µm)   | 0 to −8                       | 0 to −8                       |
| Pixel size (Å)       | 6.898 (binned once), or 3.45 un binned | 6.898 (binned once), or 3.45 un binned |
| Symmetry imposed     | C5                            | C5                            |
| **Initial particle images (no.)** | 25,276                        | 28,463                        |
| **Final particle images (no.)** | 15,000                        | 23,500                        |
| **Map resolution (Å)** | 20                            | NA                            |
| **FSC threshold**    | 0.143                         |                               |
| **Map resolution range (Å)** | NA                           |                               |
| **Tomography**       |                               |                               |
| Number of grid points | 928 × 928 × 400              | 928 × 928 × 400              |
| Voxel size           | 13.8 × 13.8 × 13.8            | 13.8 × 13.8 × 13.8           |
| Projections          | 61                            | 61                            |

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Data availability

The cryo-EM structure of the TssJLM core, full complex (C5 and C1 symmetry) and base were deposited in the EMDB under ID codes EMD-0264, EMD-0265, EMD-0266 and EMD-0267, respectively. The TssJLM core atomic model was deposited in the PDB under ID code 6HS7. The cryotomography maps were deposited under ID codes EMD-4561 and EMD-4562. Raw cryo-EM data are available on request.

Expanded View for this article is available online.

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Author contributions

RF, ED, MP and EC conceived the project. CR, RF, ED and MP designed the experiments. YC performed the in vivo studies with the help of VS. RK performed all the cryotomography work with help from MP and ED. CR carried out the single-particle cryo-EM study with help from RF. RP performed the amino acids interaction analysis and validated the model. LL engineered the E. coli mini cells. CR, RK, EC and RF wrote the manuscript with contribution from all of the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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