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1 ***In situ* and high-resolution Cryo-EM structure of the Type VI secretion membrane complex**

2
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23 **Abstract**

24
25 Bacteria have evolved macromolecular machineries that secrete effectors and toxins to survive and
26 thrive in diverse environments. The type VI secretion system (T6SS) is a contractile machine that is
27 related to *Myoviridae* phages. It is composed of a phage tail-like structure inserted in the bacterial
28 cell envelope by a membrane complex (MC) comprising the TssJ, TssL and TssM proteins. We
29 previously reported the low-resolution negative-stain electron microscopy structure of the
30 enteroaggregative *Escherichia coli* MC, and proposed a rotational 5-fold symmetry with a
31 TssJ:TssL:TssM stoichiometry of 2:2:2. Here, cryo-electron tomography analyses of the T6SS MC
32 confirm the 5-fold symmetry *in situ* and identify the regions of the structure that insert into the
33 bacterial membranes. A high-resolution model obtained by single particle cryo-electron microscopy
34 highlights new features: five additional copies of TssJ, yielding a TssJ:TssL:TssM stoichiometry of
35 3:2:2, an 11-residue loop in TssM, protruding inside the lumen of the MC and constituting a
36 functionally important periplasmic gate, and hinge regions. Based on these data, we propose an
37 updated model on T6SS structure, assembly and function.

38 Introduction:

39 In a competitive environment, the ability to communicate and outcompete neighbours
40 provides bacteria with key advantages to survive. The type VI secretion system (T6SS) is a
41 macromolecular complex involved in the release of toxins that disrupt essential functions in
42 competitor cells (Russell *et al*, 2014). The T6SS is associated with increased survival and
43 pathogenicity in bacteria expressing it (Zhao *et al*, 2018). It is composed of 13-14 core proteins
44 (Boyer *et al*, 2009), usually encoded in the same locus in the genome (Mougous *et al*, 2006). The
45 T6SS assembles a molecular spring-loaded dagger, which punctures the target cell to secrete fully
46 folded effector proteins into neighbouring bacteria (Russell *et al*, 2011) or eukaryotic hosts (Hachani
47 *et al*, 2016) The full assembly consists of the trans-envelope TssJLM membrane complex (MC)
48 (Durand *et al*, 2015) that tethers the TssKFGV-VgrG baseplate (Brunet *et al*, 2015)(Cherrak *et al*,
49 2018), onto which the tail polymerizes. This tail comprises the inner tube made of stacks of Hcp
50 hexamers wrapped by the TssBC sheath proteins that polymerize in a helical conformation (Robb *et*
51 *al*, 2013; Brunet *et al*, 2014; Clemens *et al*, 2015; Kudryashev *et al*, 2015; Chang *et al*, 2017; Wang *et*
52 *al*, 2017), and tipped by the spike VgrG, which can be sharpened by the PAAR protein (Renault *et al*,
53 2018; Shneider *et al*, 2013). Effectors are either associated within the Hcp lumen, or directly or
54 indirectly bound to the VgrG or PAAR spike (Unterweger *et al*, 2017; Silverman *et al*, 2013; Shneider
55 *et al*, 2013; Flaugnatti *et al*, 2016; Quentin *et al*, 2018). Upon contact with a neighbouring cell,
56 unknown signals trigger the contraction of the sheath causing the tube and spike to pierce the
57 membranes of the target cell, hence delivering the effectors (Basler *et al*, 2012).

58 While the baseplate, tube and sheath proteins are conserved among contractile injection
59 systems, the MC is specific to the T6SS. TssJ is an outer membrane lipoprotein (Aschtgen *et al*,
60 2008a) that positions first at the site of assembly, and then recruits TssM and TssL (Durand *et al*,
61 2015). TssM and TssL are two inner membrane proteins that share homology with two accessory
62 subunits associated with Type IV secretion system (T4SS)b, IcmF and IcmH/DotU (Aschtgen *et al*,
63 2012; Ma *et al*, 2009; Durand *et al*, 2012; Logger *et al*, 2016). Not only does the MC anchor the
64 baseplate to the inner membrane, but it also serves as a channel to allow the passage of the tail
65 tube/spike, and to maintain the integrity of the attacking cell during the translocation of the inner
66 tube (Durand *et al*, 2015). The different subunits and the MC have been extensively biochemically
67 characterised and several crystal structures of the components are available from various bacterial
68 species (Robb *et al*, 2013; Felisberto-Rodrigues *et al*, 2011; Durand *et al*, 2012; Rao *et al*, 2011; Robb
69 *et al*, 2012; Chang & Kim, 2015; Durand *et al*, 2015). We previously reported the negative-stain
70 electron microscopy (EM) structure of the TssJLM complex from enteroaggregative *Escherichia coli*
71 (EAEC). We determined that 10 TssJ lipoproteins are bound to 10 TssM proteins, forming two

72 concentric rings of pillars and arches that span the periplasm. The arches were shown to link to a
73 flexible base composed of the N-terminal part of TssM and 10 copies of TssL (Durand *et al*, 2015).
74 This study also revealed that the EAEC TssJLM complex assembles into a 5-fold rotationally
75 symmetric trans-envelope structure. However, the symmetry mismatch between the 5-fold
76 symmetry of the MC and 6-fold symmetry of the baseplate (Nazarov *et al*, 2018) raised questions on
77 whether the purified TssJLM MC reflects the *in vivo* situation. In addition, although most of the
78 available crystal structures can be readily fitted into this EM structure, we currently lack molecular
79 details on the whole complex, such as the precise location of the membranes, of the trans-
80 membrane helices, and the potential presence of a periplasmic channel.

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

89 Results

90 *Structure of the T6SS MC within the cell envelope*

91 To observe the T6SS MC in its native cellular environment, we performed cryo-ET on bacterial
92 cells (Weiss *et al*, 2017). With the aim to have a sufficient number of particles to obtain an *in situ*
93 structure by subtomogram averaging, we imaged *E. coli* BL21(DE3) cells in which TssJLM were
94 heterologously overproduced. This strain does not possess T6SS genes, thereby preventing any
95 crosstalk or protein-protein interactions between TssJLM and other natively present T6SS proteins.
96 Since *E. coli* is too thick to be directly imaged by cryo-ET, we pursued three different approaches to
97 tackle sample thickness. The first approach consisted of engineering a minicell-producing skinny
98 strain (Farley *et al*, 2016) of *E. coli* BL21(DE3), and thereby generating a minicell strain that is
99 compatible with the T7-based expression system. Although this strain produced minicells as small as
100 450 nm in diameter (Fig EV1A), their size still affected the contrast to an extent that did not allow for
101 sub-tomogram averaging. Nevertheless, characteristic inverted Y-shaped particles (side views, Fig
102 EV1B) and star-shaped particles (top views, Fig EV1C) could occasionally be observed in the
103 periplasm of these minicells. In a second approach, tomograms were recorded of cells that were
104 partially lysed and exhibited a high contrast (Fig EV1D, E). These cells, which have previously been

105 described as “ghost cells” due to their transparent appearance, still had mostly intact membranes
106 and showed their cytoplasmic macromolecular complexes such as ribosomes (Fu *et al*, 2014). In a
107 third approach, we used a state-of-the-art cryo-sample thinning method called focused ion beam
108 (FIB) milling to thin *E. coli* BL21(DE3) cells in which TssJLM were heterologously overproduced (Fig
109 EV1F, G). FIB milling allows to etch through a lawn of bacterial cells and to thin them down to under
110 200 nm (Medeiros *et al*, 2018a, 2018b; Marko *et al*, 2007). This approach was more native, as it was
111 performed on intact rod-shaped wild-type BL21(DE3) cells. These tomograms had a high contrast and
112 provided great detail. To confirm the relevance of these observations, we carried out control
113 experiments in the native T6SS⁺ EAEC 17-2 strain. TssJLM particles were frequently observed by cryo-
114 ET, both when heterologously overproduced (Fig EV1H, note that the particles could occasionally be
115 found detached from the outer membrane: Fig EV1J) as well as under native conditions, *i.e.* in wild-
116 type EAEC 17-2 cells (Fig EV1I).

117 Sub-volumes of star-shaped (top views) and inverted Y-shaped (side views) particles were
118 manually picked, computationally extracted, and subsequently aligned and averaged. The resulting
119 average was similar to the *in vitro* T6SS MC structure published previously (Durand *et al*, 2015), with
120 a tip and a core, made of 5 pairs of pillars forming a narrow central channel, that splits into 10 arches
121 (Fig 1A-E). Importantly, the 5-fold symmetry was evident without applying symmetry (Fig 1A'). As it is
122 the case for the TssJLM MC solved by EM (Durand *et al*, 2015), the periplasmic core and the arches
123 were well resolved, whereas the inner membrane-embedded base and the outer membrane-
124 embedded cap were poorly resolved. The Fourier shell correlation (FSC) curve indicated a resolution
125 of 25 Å at a coefficient of 0.5 (Appendix Fig S1 A). The prevalence of top views indicated that the
126 average might be affected by a missing wedge (Appendix Fig S1B).

127 After aligning and averaging a set of sub-volumes, it can be useful to place the isosurface of
128 the average back in the original tomographic volume to analyze the positions and orientations of the
129 individual aligned particles. In this way, we obtained a clear view of the location of the MC within the
130 cell envelope (Fig 2A, Movie 1 and Appendix Fig S1C-E, Movie 2). The position of the membranes with
131 respect to the TssJLM highlighted that the tip was embedded in the outer membrane without
132 crossing it, while the MC was anchored in the inner membrane at the lower part of the arches (Fig
133 2B). In some cases, densities could be seen spanning the inner membrane and extending into the
134 cytoplasm (Fig 2C). In what could be an overproduction artefact, TssJLM particles were also found in
135 cytoplasmic membrane invaginations (Fig 2A). Altogether, these data confirmed the 5-fold symmetry
136 of the T6SS TssJLM MC *in situ* and provided further insights into the position of the inner and outer
137 membranes.

138

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

140 We previously reported the negative stain EM structure of the EAEC TssJLM MC (Durand *et al*,
141 2015). Here, we used the same purification procedure and performed SPA cryo-EM to obtain the
142 cryo-EM structure of the 1.7-MDa T6SS MC at 4.9 Å overall resolution. With a 5-fold symmetry
143 imposed, the local resolution ranged between 4.1 Å and 21.7 Å (Fig 3A and Appendix Fig S2A-F).

144 The 5-fold symmetry is clearly visible in the 2D classes top views and the volume slices of the
145 reconstruction, which retain a 5-pointed star shape (Appendix Fig S2C, G). When no symmetry was
146 applied during the reconstruction, the overall resolution decreased to 7.5 Å but the pentameric
147 nature of the complex was maintained, with only one of the 10 arches displaying weaker density
148 than the others causing the drop in resolution (Appendix Fig S2H, I and S3). This could indicate partial
149 assembly of the complex *in vivo* or disassembly during its purification.

150 The overall structure resembles that obtained by negative stain (Appendix Fig S2J)(Durand *et*
151 *al*, 2015). The architecture of the complex comprises a tip connected to a core region that extends to
152 a base, through a double ring of pillars with arches in proximity to the inner membrane (Fig 3A-B).
153 Several notable features are already evident from the cryo-EM density map of the full complex (Fig
154 3A). First, the tip region and the base are disordered, and appear to be filled by random densities.
155 Second, cross-sections of the full complex, show that the channel, across which tube/spike transport
156 might occur, is closed by a gate at the intersection between arches and pillars, above the inner
157 membrane (Fig 3B-E and Appendix Fig S3A-D respectively). This gate is also visible in the
158 reconstruction without symmetry applied (Appendix Fig S2H and S3).

159 To better characterise the flexibility of the base of the complex, we collected tomograms on
160 the same frozen EM grids that were used to collect the SPA dataset (Appendix Fig S4A-C). The base of
161 individual complexes appeared as very heterogeneous, with single arches often pointing in opposite
162 directions, or on the contrary several arches clumping together (Appendix Fig S4A). On the other
163 hand, the core was rigid and resembled a 5-branched star. Moreover, about 20% of the particles
164 possessed only 3 or 4 out of 5 branches (Appendix Fig S4B), indicating that partial assemblies could
165 be stable. The particles were lying in a thin layer of ice (25 nm) and were found in different
166 orientations (Appendix Fig S4C).

167

168 *The structure of the base*

169 To try and overcome the inherent flexibility of the complex and better discern different
170 features of the base, we performed a density subtraction of the tip, core and arches followed by a
171 focused refinement of the base with and without symmetry applied. We thus obtained 2D classes
172 and a 3D structure of the base at 17-Å resolution when a C5 symmetry was applied (Fig 3F and

173 [Appendix Fig S4E-F](#)). When observing the cross section of the base in the full complex, as indicated by
174 arrows in [Fig 3B](#), and in the subtracted structure ([Appendix Fig S4E](#)), two 110 Å-wide linear densities
175 are clearly visible, separated by 40 Å. This double layer of density is consistent with the density
176 diagram of a lipid bilayer with the head groups being the most dense at a distance of ~4 nm from
177 each other and fits well a lipid bilayer composed of PE, obtained using the CHARMM-GUI (Jo *et al*,
178 2007) ([Appendix Fig S4E](#)). We propose that the inner membrane sub-domain of the T6SS MC could be
179 filled by a lipid bilayer. However, such hypothesis will have to be further explored in the future.

180

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

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

194 TssJ' binds to the MC through previously unknown interfaces. If we consider TssJ' and the TssJ
195 subunits from the inner and outer pillars (TssJ.i and TssJ.o, respectively) ([Figs 4A-C and EV3A-B](#)) with
196 TssJ.i and TssJ.o being in contact with TssM.i and TssM.o respectively, they sit in the same position as
197 in the crystal structure and in the outer and inner pillars ([Fig 4B](#)). By contrast, TssJ' binds to TssM.o
198 and TssJ.i strongly ([Figs 4C, EV3C, and Table 1](#)). In particular, the interaction of TssJ' with TssJ.i is
199 specifically strong, as their contact is mediated not only by hydrogen bonds but also by salt bridges
200 (R31 with E34, and D97 with R33; see [Fig EV3C and Table 1](#)). TssJ' binds to TssM.i via hydrogen bonds
201 only and this interaction is comparable to that between TssJ.i and TssJ.o with TssM.i and TssM.o
202 respectively (surface of interaction 573 Å² and -2.6 kcal/mol ΔG) ([Fig 4B-C, Table 1](#)).

203 *TssJ' is required for MC assembly and T6SS activity*

204 To gain further information on the function and *in vivo* relevance of TssJ', mutations that
205 specifically impact the TssJ'-TssJ.i interface were engineered onto the chromosome, at the native

206 locus. Two residues, R31 and D97 (Fig EV3C), were targeted as they form a salt bridge with E34 and
207 R33 in TssJ.i, respectively (Table 1 and Fig EV3C). The R31E, D97A and D97K substitutions were then
208 tested for their ability to outcompete a fluorescent *E. coli* competitor strain. Although the R31E and
209 D97A did not significantly impact T6SS antibacterial activity, the D97K mutation abolished proper
210 function of the T6SS (Fig 4D). The assembly and stability of the T6SS MC was then assessed by
211 fluorescence microscopy using a chromosomally-encoded and functional fusion protein between
212 TssM and the super-folder GFP (_{sfGFP}TssM, Durand *et al*, 2015) (Appendix Fig S5A). As previously
213 observed, _{sfGFP}TssM formed stable foci (Durand *et al*, 2015). By contrast, cells producing the TssJ
214 D97K variant presented small and unstable fluorescent _{sfGFP}TssM foci (Fig 4E). Time-lapse
215 fluorescence microscopy recordings showed that about 90% of the foci observed in the _{sfGFP}TssM
216 strain ($n = 50$) are stable over the 600-sec recording time, in agreement with the previous
217 observation that the EAEC T6SS MC is stable and serves for several contraction/elongation cycles
218 (Durand *et al.*, 2015). By contrast, with a mean lifetime of ~ 107 sec ($n = 50$) the _{sfGFP}TssM fluorescent
219 foci observed in cells producing the TssJ D97K variant are drastically less stable (Fig 4E). We then
220 tested whether the TssJ D97K variant promotes sheath assembly, using chromosomally-encoded
221 functional _{sfGFP}TssB fusion. Contrarily to the wild-type parental cells in which dynamic sheaths can be
222 observed, no sheath polymerization occurs in presence of TssJ-D97K (Appendix Figs S5A-B).
223 Altogether, these results demonstrate that the TssJ'-TssJ.i interface is required for the stability of the
224 T6SS MC, sheath formation and T6SS antibacterial activity.

225

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

227 We were able to confidently build *de novo* the periplasmic domain of TssM including its N-
228 terminal fragment (residues 579 to 869) that was missing in the crystal structure (Durand *et al*,
229 2015). This was done in Coot (Emsley *et al*, 2010) by using Phyre2 secondary structure predictions
230 (Kelley *et al*, 2015) and RaptorX residue contact predictions (Källberg *et al*, 2012) as validation tools
231 (Fig 5A and Appendix Fig S6A-C). The cryo-EM structure of the TssM periplasmic domain slightly
232 differs from the X-ray structure (Fig EV4A). From the C-terminus, helix 869-891 extends to amino-acid
233 841 with a slight kink at residue Pro-870. The remaining N-terminal fragment forms an α -helical
234 domain comprising 8 helices that snake back and forth to the inner membrane (Fig 5B). The region
235 closest to the membrane was too flexible to be resolved and for an atomic model to be built
236 (Appendix Fig S6B). The cryo-EM pseudoatomic model of the fully assembled TssM-TssJ complex
237 shows it forms a bell shape composed of two rings of pillars that twist around each other (Fig 5A).
238 Within each asymmetric unit, two copies of TssM are present, named TssM.o in the outer pillar and
239 TssM.i in the inner pillar. The inner and the outer pillar TssM proteins are superimposable with the

240 exception of a 23° kink located at residue 867 (Fig EV4B). These two TssM subunits interact front-to-
241 back (Fig EV4C) with an area of 1168 Å², a binding energy of -9.9 kcal/mol, and a ΔG of -5.2 kcal/mol.
242 Each TssM.i also interacts with two adjacent TssM.i within the inner TssM ring at an angle of 76°,
243 with an area of 1529 Å², a binding energy of -12 kcal/mol and a ΔG of -5.32 kcal/mol (Figs EV3A and
244 EV4D). Finally, TssM.o⁺¹ also interacts with TssM.i and the two are oriented at 68° from one another
245 (Figs EV3A and EV4E).

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

257 *The periplasmic TssM gate*

258 The inner pillars of TssM form a channel with a diameter that varies between 2.6 Å and > 20 Å
259 (Fig 5C). The site of constriction observed in the cryo-EM density (Figs 3B, E) corresponds to loop
260 776-786 in the atomic model of TssM (Fig 5D). Specifically, residues Gln-779 and Asn-780-781
261 maintain the constriction via polar interactions (Fig 5D). In the outer pillar, the same loop interacts
262 with loop 600-625 on the neighbouring pillar, providing further stabilisation of the structure
263 (Appendix Fig S7A). Conservation analysis of the sequence with related proteins, predicted by the
264 ConSurf server (Celniker *et al*, 2013), indicated that the sequence of the loop 776-786 is poorly
265 conserved amongst species (Appendix Fig S7B) although the presence of a loop at this position is a
266 conserved feature. As previously proposed (Durand *et al*, 2015), these data suggest that the purified
267 TssJLM MC is in a closed state. Large conformational changes, including modification of the
268 constriction and movement of the inner pillars are therefore required to allow the passage of the
269 tube/spike during sheath contraction.

270

271 ***The TssM periplasmic gate is required for MC assembly.***

272 To test the function of the periplasmic gate, several mutations were engineered at the *tssM*
273 locus in the wild-type EAEC 17-2 strain and the function of the T6SS was assessed as previously
274 described (Fig 5E-G). To covalently stabilize the contacts between the inner pillars and thus prevent
275 MC opening, Q779 and N780 were substituted with cysteines (Q779C-N780C). Conversely, a
276 constitutively open gate was created by deleting a large portion of the constriction loop (Δ 777-783).
277 Antibacterial competition assays showed that the Q779C-N780C variant loses the ability to
278 outcompete competitor cells, whereas the single control mutant Q779C did not (Figure 5E). Deletion
279 of the Δ 777-783 loop also impaired the T6SS antibacterial activity (Figure 5E). Fluorescence
280 microscopy recordings further showed that $_{sfGFP}$ TssM Q779C-N780C and $_{sfGFP}$ TssM Δ 777-783 do not
281 assemble TssM foci since, in contrast to the parental strain, diffuse fluorescent patterns were
282 observed (Fig 5F and Appendix Fig S8). These results demonstrate that the MC is not properly
283 assembled when the integrity of the periplasmic gate is impacted. As expected, these mutant cells
284 did not assemble T6SS sheaths (Fig 5G and Appendix Fig S8).

285 ***Discussion***

286 In this study, we report the *in situ* and *in vitro* structures of the T6SS TssJLM MC from EAEC.
287 The cryo-ET structure confirmed the 5-fold symmetry and general architecture *in vivo*, while the
288 high-resolution cryo-EM structure provided molecular details about the periplasmic portion of the
289 complex (Fig 1, Fig 3 and Movie 3). As previously defined, the pentameric propeller-like structure
290 composed of 10 pillars intertwined with each other was observed both *in situ* and from purified
291 material (Figs 1 and 3C-E). Cryo-ET analyses allowed to position both the inner and outer
292 membranes. As anticipated based on biochemical experiments showing that TssJ is a periplasmic
293 lipoprotein attached to the outer membrane by an acyl anchor (Aschtgen *et al*, 2008), the tip of the
294 complex is embedded in the outer membrane (Fig 2B). However, neither the predicted detergent cap
295 in the SPA cryo-EM structure nor the cryo-ET data, indicate that TssJLM breaches or crosses the outer
296 membrane (Fig 2, Fig 3A-B). Nevertheless, it has been shown that this region is extracellularly
297 exposed in wild-type EAEC cells (Durand *et al*, 2015). Although the cryo-EM structure demonstrates
298 that the C-terminal region of TssM locates in the periplasm, we propose that the recruitment of
299 specific T6SS components induces MC conformational changes and cell surface exposition of the
300 TssM C-terminus. While our previous study suggested that the inner membrane locates at the level
301 of the arches (Durand *et al*, 2015), the cryo-ET analyses revealed that the inner membrane surrounds
302 the base (Fig 2B). Moreover, some tomograms also revealed the most basal parts of the MC, which
303 cross the inner membrane into the cytoplasm (Fig 2A, C). These cytoplasmic densities, which we can
304 assume would be connected to the baseplate in a fully assembled T6SS, had a heterogeneous

305 appearance that highlighted the flexibility of the base, as discussed below. The *in situ* cryo-ET
306 structure presents an apparent elongation of the tip region compared to the cryo-EM structure
307 (Appendix Fig S9A, B and Movie 3), which could correspond to an additional density associated to the
308 outer membrane, or which could alternatively be explained by the missing wedge. Nevertheless, the
309 similarities between both structures allowed the atomic model of TssJ - TssM to be docked into the *in*
310 *situ* average (Appendix Fig S9C, D), whereas the cryo-EM structure could be placed in a cellular
311 context (Appendix Fig S9E and Movie 3).

312 The base of the complex in this higher resolution structure was not better resolved than in the
313 negative stain structure (Fig 3A, F, and Appendix Fig S2D). This base should comprise 10 copies of the
314 TssM and TssL cytoplasmic domains (Durand *et al*, 2015). TssL forms dimers (Zoued *et al*, 2018;
315 Durand *et al*, 2012; Zoued *et al*, 2016), and the crystal structure of its cytoplasmic hook-like domain
316 has been reported from various species including EAEC, *P. aeruginosa* and *V. cholerae* (Durand *et al*,
317 2012; Robb *et al*, 2012; Chang & Kim, 2015; Wang *et al*, 2018). The TssM cytoplasmic domain is
318 comprised between the N-terminal transmembrane hairpin and a third transmembrane helix (Ma *et*
319 *al*, 2009; Logger *et al*, 2016). No high-resolution structure of the TssM cytoplasmic domain is
320 available, although a model has been built based on homology with DPY-30 and NTPases (Logger *et*
321 *al*, 2016). Unfortunately, due to the poor resolution of the base, we did not succeed to confidently fit
322 the TssL and TssM cytoplasmic domains in this density. Additional assays to improve the resolution
323 such as the use of nanodiscs or amphipols proved to be unsuccessful (Appendix Fig S10A). The
324 flexibility of the TssJLM base, which did not allow for it to be resolved, might be due to the absence
325 of other T6SS cytoplasmic components, such as the baseplate. A similar observation was made for
326 the type III secretion system, in which the presence of the cytoplasmic sorting platform orders the IM
327 components (Hu *et al*, 2017). One may hypothesize that this flexibility is essential for the docking of
328 the hexameric baseplate, and to accommodate the five-fold to six-fold symmetry mismatch. One
329 alternative hypothesis is that the disorder at the centre of the base structure is caused by the
330 presence of a lipid bilayer encircled by the TssM and TssL proteins. In the *in vivo* situation, the MC
331 assembles first, before the recruitment of the baseplate (Durand *et al*, 2015; Brunet *et al*, 2015), and
332 hence, one can expect that a lipid bilayer at the entrance of the TssJLM lumen would be present
333 before baseplate docking to prevent the leakage of solutes and proton-motive force.

334 The high-resolution structure of the EAEC TssJLM MC also revealed new interesting and
335 functional features. First, five additional TssJ subunits, called TssJ', were identified in the tip complex.
336 These TssJ' proteins interact with the TssJ proteins of the inner pillars (TssJ.i). Mutations that
337 interfere with TssJ'-TssJ.i interaction impaired the functional integrity of the MC and hence
338 inactivated the T6SS (Fig 4D-E and Appendix Fig S5). Second, we identified an 11-amino-acid loop in
339 TssM that protrudes from each inner pillar to the centre of the channel, thus creating a constriction

340 that is observed in the density map (Fig 3B, E and Appendix Fig S3). Each loop is stabilized by the
341 adjacent loop via Asn/Gln pairings (Fig 5D). Such weak interactions could be easily displaced by the
342 VgrG/PAAR spike upon baseplate docking or during firing. Our mutational analyses demonstrate that
343 this constriction is important for TssJLM MC formation and T6SS activity. Periplasmic constrictions
344 are usual features of trans-envelope complexes. The best characterized examples include the OM
345 T2SS and T3SS secretins, and the CsgG curli secretion channel, where one or two periplasmic gates
346 are present to prevent leakage (Appendix Fig S11A-B) (Yan *et al*, 2017; Spagnuolo *et al*, 2010; Goyal
347 *et al*, 2014). While we do not know the role of this constriction in the T6SS, we propose that these
348 loops may stabilize the MC in its closed conformation during the resting state. The cryo-EM structure
349 defined two hinge regions that exhibit a certain degree of flexibility (Fig EV4B,G). These two hinges
350 result in the formation of the two layers of pillars, with the inner layer obstructing the channel. A
351 large conformational change of these pillars is therefore necessary to open the channel for the
352 passage of the tube/spike complex. Interestingly, with an interaction surface of 1540 Å² (ΔG of 6.1
353 kcal/mol) the TssM inner pillars contacts are considerably less stable than the contacts within the
354 T2SS secretin (interaction surface of 5353.7 Å² and ΔG of -52.4 kcal/mol). The displacement of the
355 pillars could be controlled by the flexibility of the hinge regions.

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

369

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391

392 **Author Contributions**

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

398

399 **Conflict of interest**

400 The authors declare no competing interests.

401

402 **Figure legends**

403

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

405 **A-E** Isosurface of the subtomogram average (in pink) with an applied C5 symmetry and 0.69 nm
406 tomographic slices (A-E) at the indicated heights. The average is shown in side view, whereas the
407 slices represent perpendicular slices. The 5 pairs of pillars formed a narrow central channel and
408 separated into 10 arches towards the base (base not visible at the used threshold). The division of
409 the structure into subparts was adapted from (Durand *et al.*, 2015). Subvolumes were extracted from

410 cryotomograms of ghost cells and FIB-milled intact *E. coli* BL21 cells heterologously expressing
411 TssJLM

412 **A'** Slice (0.69 nm) through the non-symmetrized average. Note that the C5 symmetry was visible
413

414 **Figure 2. Position of TssJLM in the cell envelope**

415 **A** Slice (9.7 nm) through a cryotomogram of a FIB-milled *E. coli* BL21 cell expressing TssJLM. The
416 average shown in Figure 1 was placed back at the positions and orientations of the individual
417 subvolumes that were used to generate the final average. The zoomed-in area highlights the position
418 of the TssJLM particles within the inner and the outer membrane. Some particles were found in
419 cytoplasmic membrane invaginations, as indicated by white arrows. Scale bar 100 nm

420 **B** Isosurface of the final average (orange) merged with the isosurface of a second average (with
421 higher threshold; grey mesh). The panel shows the positioning of TssJLM with respect to both inner
422 (IM) and outer membranes (OM). The distances corresponded to the widest and longest dimensions
423 of the TssJLM complex

424 **C** Cryotomographic slices (9.7 nm) showing side views of TssJLM. In these examples, the basal parts
425 of TssJLM (red arrows) could be seen extending into the cytoplasm. Scale bar 10 nm

426

427 **Figure 3. 3D structure of the TE complex of the T6SS**

428 **A** Autosharpened Cryo-EM density of the full TE complex composed of TssJ, TssL and TssM. The inner
429 pillars are coloured in green and the outer pillars in blue. The unstructured tip and base are in grey,
430 while the top of the core is in orange

431 **B** Vertical cross section of the cryo-EM density. Colouring is according to A. The position of the slices
432 for C-E are indicated. The density of the base appears to be composed of two leaflets, indicated by 2
433 arrows

434 **C-E** Cross sections of A, sliced at positions indicated in B

435 **F** Subtracted, masked and unsharpened cryo-EM density of the, mostly unstructured, base

436 **G** Locally sharpened cryo-EM density of the subtracted and masked core. Three different views are
437 shown. The colouring is according to A

438

439 **Figure 4. The TssJ' monomer and its function**

440 **A** Ribbon diagram and locally sharpened surface representation (transparent= of the three TssJ
441 protomers in orange, labelled TssJ.i, TssJ.o and TssJ' for the inner, outer and additional monomer
442 respectively. In light green and blue are the TssM.i and TssM.o protomers respectively

443 **B** Superimposition of the ribbon diagrams of TssM-TssJ heterodimer within the inner (green-orange)
444 and the outer (blue-orange) pillars

445 **C** Ribbon diagram of the TssM.o (outer pillar) with the TssJ.o and the TssJ' (orange)
446 **D** Fluorescence of prey cells (green) indicating their survival against attacker cells after 4 h. The
447 image of a representative bacterial competition spot is shown on the upper part. The relative
448 fluorescent level (in AU) and the number of recovered *E. coli* recipient cells are indicated in the lower
449 graph (in log₁₀ of colony forming units (cfu)). The assays were performed from at least three
450 independent cultures, with technical triplicates and a representative technical triplicate is shown.
451 The circles indicate values from technical triplicate, and the average is indicated by the bar
452 **E** Time-lapse fluorescence microscopy recordings showing localization and dynamics of the *sfGFP*TssM
453 fusion protein in the parental (WT) and TssJ mutated strains (TssJ D97K). Individual images were
454 taken every 60 seconds. The corresponding time of each micrograph is indicated in the lower right
455 part of the image. Stable foci in the WT strain and less stable foci in the TssJ D97K strain are
456 respectively indicated by white and blue arrowheads. Scale bars, 1 μm

457

458 **Figure 5. The TssM protein and the periplasmic gate**

459 **A** The high-resolution structure of MC complex in different orientations. TssJ is in orange, TssM is in
460 green and blue according to their position as an inner or outer pillar respectively.

461 **B** Pseudoatomic model of the N-terminus of TssM.i (green), showing it is composed of 8 helices,
462 coloured in different shades of red and numbered from the C-terminus (1) to the N-terminus (8).

463 **C** The pore radius formed by the inner pillar of TssM protomers is highlighted as dots and mapped
464 using HOLE. The atomic model of TssM is coloured according to secondary structure (Helices in pink
465 and strands in brown). A graph shows the pore radius calculated along the centre of the inner pillar
466 of TssMs.

467 **D** Ribbon diagram of the region surrounding the periplasmic gate, with the amino acids involved in
468 the formation of the gate in atom form. Gln779 and Asn780-781 from the inner pillars of TssM
469 (green) are labelled in one of the protomers

470 **E** Fluorescence of prey cells (green) indicating their survival against attacker cells after 4h. The image
471 of a representative bacterial competition spot is shown on the upper part. The relative fluorescent
472 level (in AU) and the number of recovered *E. coli* recipient cells are indicated in the lower graph (in
473 log₁₀ of colony forming units (cfu)). The assays were performed from at least three independent
474 cultures, with technical triplicates and a representative technical triplicate is shown. The circles
475 indicate values from technical triplicate, and the average is indicated by the bar.

476 **F** Fluorescence microscopy recordings showing *sfGFP*TssM foci in the parental (WT) and TssM
477 mutated strains (TssM Q779C/N780C, TssM Δ777-783). TssM foci containing cells are indicated by
478 arrowheads. Microscopy analyses were performed independently three times, each in technical
479 triplicate, and a representative experiment is shown. Scale bars, 1 μm.

480 **G** Fluorescence microscopy recordings showing TssBsfGFP sheath in the parental (WT) and TssM
481 mutated strains (TssM Q779C/N780C, TssM Δ 777-783). Fluorescent sheath containing cells are
482 indicated by arrowheads. Microscopy analyses were performed independently three times, each in
483 technical triplicate, and a representative experiment is shown. Scale bars, 1 μ m.

484

485 **Figure 6. Summary of the Type 6 secretion system cycle of action**

486 The T6SS assembly begins first with the recruitment of the membrane complex (MC) in its resting
487 state (1). The MC recruits the baseplate (BP) and the tail tip complex (TTC) is assembled (2). The
488 recently solved structures of the BP (Cherrak *et al*, 2018) and MC (this paper) in the membrane
489 context are shown in the inset. A conformational change leads to the channel opening (3) and
490 release of the toxin onto the VgrG spike by contraction of the TTC (4). Once the secretion has
491 occurred, the TTC is recycled (5) and the MC can return to its resting state (6).

492

493

494 **Table 1. Bonds between the TssJ and TssM subunits**

Monomer1	Monomer2	Area (Å)	ΔG	Nhb	Nsb
TssM.i	TssM.i-1	1528.78	-5.32	9.2	4.8
TssM.i	TssM.o-1	1168.6	-7.54	4.8	6.2
TssM.i	TssM.o	1126.48	-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

495

496 **Table 2. Cryo-ET data collection, refinement and validation statistics**

	STA_AvgVol_15000 (EMD- 4561)	STA_AvgVol_23500 (EMD- 4562)
Data collection and processing		
Magnification	42000	42000
Voltage (kV)	300	300
Electron exposure (e-/Å ²)	90	90
Defocus range (μm)	0 to -8	0 to -8
Pixel size (Å)	6.898 (binned once), or 3.45 unbinned	6.898 (binned once), or 3.45 unbinned
Symmetry imposed	C5	C5
Initial particle images (no.)	25276	28463
Final particle images (no.)	15000	23500
Map resolution (Å)	20	
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

497

498

499

Table 3. 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	120,000X	120,000X	120,000X	120,000X
Voltage (kV)	200	200	200	200
Electron exposure (e ⁻ /Å ²)	120	120	120	120
Defocus range (μm)	0.4 to 5 μm	0.4 to 5 μm	0.4 to 5 μm	0.4 to 5 μm
Pixel size (Å)	1.24 Å	1.24 Å	1.24 Å	1.24 Å
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)	4.6Å	4.9Å	7.9Å	17Å
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	4.6Å			
Model resolution range (Å)				
Map sharpening <i>B</i> factor (Å ²)				
Model composition				
Non-hydrogen atoms	52890			
Protein residues	6905			
Ligands	N/A			
<i>B</i> factors (Å ²)				
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				
Favored (%)	89.46			
Allowed (%)	10.54			
Disallowed (%)	0			

503

504 **Expanded view Figure legends:**

505 **Figure EV1. The TssJLM complex exhibits a C5 symmetry in *E. coli* BL21 and in EAEC.**

506 **A** Slice (13.8 nm) through an engineered BL21 minicell with a diameter of ~450nm. Scale bar 100 nm
507 **B-J** Slices showing side views (upper row) and top views (lower row) of TssJLM embedded in the cell
508 envelope of BL21(DE3) (expressing TssJLM) and EAEC cells, as indicated by red arrows. The outer and
509 inner membranes are indicated (OM and IM). Scale bars 10 nm
510 **B** Slice (9.7 nm) showing a side view of a TssJLM particle embedded in the cell envelope of a BL21
511 minicell. The particle spanned the periplasm and resembled an inverted Y letter
512 **C** Slice (9.7 nm) showing a top view of a TssJLM particle embedded in the membrane of a BL21
513 minicell. The particle resembles a 5-branched star
514 **D** Slice (9.7 nm) showing a side view of a particle embedded in the cell envelope of a BL21 ghost cell
515 **E** Slice (9.7 nm) showing a top view of a particle embedded in the cell envelope of a BL21 ghost cell
516 **F** Slice (9.7 nm) showing a side view of a particle embedded in the cell envelope of a FIB-milled BL21
517 cell.
518 **G** Slice (13.8 nm) showing a top view of a particle embedded in the cell envelope of a FIB-milled BL21
519 cell
520 **H** Slice (9.9 nm) showing a side view of a particle embedded in the cell envelope of an EAEC cell in
521 which TssJLM was heterologously overexpressed
522 **I** Slice (9.9 nm) showing a side view of a particle embedded in the cell envelope of a wild type EAEC
523 cell
524 **J** Slice (9.9 nm) through the distorted periplasm of an EAEC in which TssJLM was heterologously
525 overexpressed. The particles seen in side view were detached from the OM but still attached to the
526 periplasmic side of the IM

527

528 **Figure EV2. The cryo-EM reconstruction of the core of the membrane complex (MC)**

529 **A** 2D classes of the subtracted SPA cryo-EM density corresponding to the core of the MC
530 **B** FSC curve of the core complex as calculated using postprocess
531 **C** 3D representation of the core 3d reconstruction coloured according to the local resolution. Two
532 views are shown
533 **D** Fitting of 10 TssM-TssJ pseudoatomic models from the crystal structure (4Y7O) in the core cryo-EM
534 density locally sharpened. The cryo-EM density was coloured according to the fitted structure. The
535 five additional densities are highlighted by arrows pointing at them. Two views of the complexes are
536 shown. In orange is the TssJ, in blue the beta sheet-rich region and in cyan the alpha helical domain
537 **E** Transparent representation of the cryo-EM density in which the third TssJ was fitted (ribbon
538 diagram in orange). The colouring follows that in D
539 **F** Representative cryo-EM density, corresponding to the TssJ' sequence 27-33, with the fitted
540 pseudoatomic model in stick form

541

542 **Figure EV3. The TssJ-TssM assembly**

543 **A** Schematic diagram of the TssM-TssJ assembly, labelled according to the nomenclature used
544 throughout this paper.
545 **B** Superimposition of two slices through the MC complex corresponding to TssJ in orange and TssM in
546 light blue, mimicking the diagram A.
547 **C** TssJ.o-TssJ' interface (in orange) with the two residues involved in the interaction as an atom
548 diagram and labelled

549

550 **Figure EV4. The TssM pseudoatomic model**

551 **A** Comparison between the TssM from the PDB (4Y7O) in cyan and the refined TssM on the cryo-EM
552 density in orange

553 **B** Comparison of the pseudoatomic model of TssM in the internal (green) and in the external (blue)
554 pillars
555 **C** Interaction interfaces between the internal TssM.a and the external TssM.A within the complex
556 **D** Interaction interfaces between the internal pillars TssM.a and TssM.a⁺¹. The two pillars are twisted
557 at 76° with respect to one another
558 **E** Interaction interfaces between the internal pillar TssM.a and the external TssM.A⁺¹. The two pillars
559 are twisted at 68° with respect to one another
560 **F** Weak C-terminal density of TssM (1109-1129) in pink
561 **G** The unsharpened density map of the whole complex at a contour level of 0.0055. The TssM foot
562 domain (in blue) between amino acids 382 and 570 predicted structure by RaptorX fits into the
563 density. The two feet converge upon arriving at the membrane level. The rest of the pseudoatomic
564 model of TssM is in pink
565

566 **Expanded view Movie legends**

567
568 **Movie 1. Movie of a cryotomogram of a FIB-milled E. coli BL21(DE3) cell expressing TssJLM.**

569 The average was placed back at the positions and orientations of the individual subvolumes that
570 were used to generate the final average. The movie shows the position of TssJLM within the cell
571 envelope. Some particles were found in cytoplasmic membrane invaginations
572

573 **Movie 2. Movie of a cryotomogram of an E. coli BL21 ghost cell expressing TssJLM.** The average was
574 placed back in the tomogram at the individual positions and orientations that were used to generate
575 the final average
576

577 **Movie 3.** The first part shows the cryotomogram of a FIB-milled E. coli BL21(DE3) cell expressing
578 TssJLM (same cell as in Movie 1) in which the average was placed back at the positions and
579 orientations of the individual subvolumes that were used to generate the final average. The second
580 part shows a morph between the in situ structure and the high resolution cryoEM structure. The
581 third part shows the high resolution structure with the pseudoatomic model, focusing on the
582 position of the periplasmic gate (first pause) and the periplasmic channel (second pause). The last
583 part replaces the cryoEM structure in the cell envelope at the same position as the in situ structure
584 at the beginning of the movie
585
586

587

588

589 **Materials and methods**

590

591 **Strains and media**

592 Strains used in this study are listed in [Appendix Table S1](#). The *E. coli* K-12 W3110 bearing the pUA66-
593 *rrnB* vector (Kan^R and GFP⁺, (Zaslaver *et al*, 2006)) was used as recipient for antibacterial competition
594 assays. Strains were routinely grown in lysogeny broth (LB) rich medium or in Sci-1-inducing medium
595 (SIM; M9 minimal medium, glycerol 0.2%, vitamin B1 1 µg.mL⁻¹, casaminoacids 100 mg.mL⁻¹, LB 10%,
596 supplemented or not with bactoagar 1.5%) (Brunet *et al*, 2011) with shaking at 37°C.

597

598 **Strain construction**

599 *tssM* and *tssJ* point mutations were engineered at the native locus on the chromosome by allelic
600 replacement using the pKO3 suicide vector (Link *et al*, 1997) into the enteroaggregative *E. coli* 17-2
601 strain. Briefly, 17-2 WT strain was transformed with a pKO3 plasmid in which a fragment of the *tssM*
602 or *tssJ* gene carrying the point mutations has been cloned (see below). Insertion of the plasmid into
603 the chromosome was selected on chloramphenicol plates at 42°C. Plasmid sequences removal was
604 then selected on 5% sucrose plates without antibiotic and *tssM* point mutation recombinant strains
605 were screened by PCR and confirmed by DNA sequencing (Eurofins, MWG). Chromosomal fluorescent
606 reporter insertions into the enteroaggregative *E. coli* 17-2 strain mutated in *tssM* or *tssJ* was
607 achieved by using a modified one-step inactivation procedure (Datsenko & Wanner, 2000) as
608 previously described (Aschtgen *et al*, 2008b) using plasmid pKOBEG (Chaverocche *et al*, 2000). Briefly,
609 a kanamycin cassette was amplified from plasmid pKD4 using oligonucleotide pairs carrying 5' 50-
610 nucleotide extensions homologous to regions adjacent to the gene to be deleted. After
611 electroporation of 600 ng of column-purified PCR product, kanamycin-resistant clones were selected
612 and verified by colony-PCR. The kanamycin cassette, inserted at the gene locus on the bacterial
613 chromosome, was then excised using plasmid pCP20, leaving an FRT scars. Gene deletions were
614 confirmed by colony-PCR and sequencing.

615

616 **Fluorescence microscopy, image treatment and analyses**

617 Fluorescence microscopy experiments were performed as described (Brunet *et al*, 2013; Zoued *et al*,
618 2013). Briefly, cells were grown overnight in LB medium and diluted to $A_{600nm} \sim 0.04$ in SIM.
619 Exponentially growing cells ($A_{600nm} \sim 0.8-1$) were harvested, washed in phosphate-buffered saline
620 buffer (PBS), resuspended in PBS to $A_{600nm} \sim 50$, spotted on a 1.5% agarose pad and covered with a
621 cover slip. Fluorescence micrographs were captured using AxioImager M2 microscope (Zeiss)
622 equipped with an OrcaR2 digital camera (Hamamatsu). For time lapse fluorescence microscopy,
623 images were recorded with a Nikon Eclipse Ti microscope equipped with an Orcaflash 4.0 LT digital

624 camera (Hamamatsu) and a perfect focus system (PFS) to automatically maintain focus so that the
625 point of interest within a specimen is always kept in sharp focus at all times despite mechanical or
626 thermal perturbations. Fluorescence images were acquired with a minimal exposure time to reduce
627 bleaching and phototoxicity effects, typically 200 ms for TssB-sfGFP and 300 ms for sfGFP-TssM. For
628 image treatment, noise and background were reduced using the 'Subtract Background' (20 pixels
629 Rolling Ball) and Band plugins of imageJ (Schneider *et al*, 2012). The sfGFP foci were automatically
630 detected using the microbeJ plugin (Ducret *et al*, 2016). Floating bars representing the number of
631 detected foci for each strain were made using GraphPad (<https://www.graphpad.com>). Microscopy
632 analyses were performed at least three times, each in technical triplicate, and a representative
633 experiment is shown.

634

635 **Interbacterial competition assay**

636 The antibacterial growth competition assay was performed as previously described (Flaughnatti *et al*,
637 2016). Wild-type *E. coli* K-12 strain W3110 bearing the pUA66-*rrnB* plasmid (conferring kanamycin
638 resistance and constitutive GFP fluorescence (*gfp* gene under the control of the ribosomal *rrnB*
639 promoter, (Gueguen & Cascales, 2013) was used as recipient. Attacker and recipient cells were
640 grown for 16 h in LB medium, diluted in SIM to allow maximal expression of the *sci-1* gene cluster
641 (Brunet *et al*, 2011). Once the culture reached $A_{600nm} \sim 0.8$, cells were harvested and normalized to
642 $A_{600nm} = 0.5$ in SIM. Attacker and recipient cells were mixed to a 4:1 ratio and 15- μ l drops of the
643 mixture were spotted in triplicate onto a pre-warmed dry SIM agar plate. After incubation for 4 h at
644 37°C, the bacterial spots were resuspended in LB and bacterial suspensions were normalized to
645 $A_{600nm} = 0.5$. For the enumeration of viable prey cells, bacterial suspensions were serially diluted and
646 spotted onto kanamycin LB plates. The assays were performed from at least three independent
647 cultures, with technical triplicates and a representative technical triplicate is shown.

648

649 **Protein preparation**

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

655

656 **Cryo-EM grids preparation and data acquisition**

657 C-flat [™] (CF-2/1-2C) grids were coated with graphene oxide as previously described (Martin *et al*,
658 2016). 3.5 μ l of the sample at 0.2 mg.mL⁻¹, was loaded on the copper side and then blotted on the

659 same side for 2s in a Leica EM GP at 80% humidity and 4 °C, before being plunge frozen in liquid
660 ethane (-184°C). Micrographs ([Appendix Fig S2B](#)) at a nominal magnification of 120,000 X were
661 collected in a Talos Arctica electron microscope equipped with a Falcon 3EC camera (Thermo Fisher,
662 Waltham, MA, USA) in linear mode and with a pixel size of 1.24 Å. Dose-fractionated movie frames
663 20/micrograph were acquired for 1 s with a total electron flux of 120 e/Å/s. The defocus range
664 chosen for the automatic collect was 0.7 to 2 µm, which resulted in an actual range between 0.4 to 5
665 µm.

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

669

670 **Cryo-EM image processing**

671 The 16,000 movies collected were aligned using MotionCor2, with dose weighting (6 e-/Å²/frame)
672 and with 5X5 patches applied (Zheng *et al*, 2017). gCTF was used to estimate the CTF parameters
673 (Zhang, 2016) and low quality images were discarded. Relion 2.1 (Scheres, 2012) autopicked 227,527
674 particles and after several rounds of 2D classification in cryosparc (Punjani *et al*, 2017) and a
675 heterogeneous *ab initio* reconstruction (2 classes), 37,435 particles were converted using the script
676 csparc2star.py (Asarnow, 2016) and selected for a final 2D classification in relion 2.1 ([Appendix Fig](#)
677 [S2C](#)), of which 36,828 particles were selected. An initial unmasked refinement using the *ab initio*
678 model from cryosparc, gave us a resolution of 7.6 Å with 5-fold applied symmetry and a soft mask of
679 450 Å. This refined structure was used to do a movie refinement with all the frames and a polishing
680 step with RELION2.1. The final masked refinement of the full structure gave a final resolution of 4.9 Å
681 with a C5 symmetry applied, and 7.9 Å with no symmetry applied ([Appendix Fig S2E, I](#)), The
682 disordered tip and base were subtracted and a masked refinement around the core structure yielded
683 a final resolution of 4.6 Å ([Fig EV2B](#)). The base focused refinement was also performed on subtracted
684 particles, without the tip and the core regions, to a resolution of 17Å ([Appendix Fig S4F](#)). The
685 resolution for all densities except the base, was calculated by masked postprocessing according to
686 the “gold standard” method using 0.143 as the FSC value cut-off, or 0.5 for the low resolution
687 reconstruction (Rosenthal & Henderson, 2003) and the local resolution of the core was calculated by
688 relion 2.1 ([Fig EV2C](#)).

689 For figures and to build *de novo* pseudoatomic models in Coot (Emsley *et al*, 2010), the cryo-EM
690 density was initially sharpened using phenix.autosharpen (Terwilliger *et al*, 2018) and later with
691 LocalDeblur (Ramírez-aportela *et al*, 2018). Fitting of density, correlation calculations, molecular
692 graphics and analyses were performed on UCSF Chimera (Pettersen *et al*, 2004). For the amphipoles
693 dataset, 2D classes were calculated from a total of 8637 particles ([Appendix Fig S10A](#)).

694

695 **Model building**

696 Model building proceeded by fitting the PDB 4Y7O (Durand *et al*, 2015) into the density 2 times for
697 each pillar, with the cross correlation being calculated using Chimera (Pettersen *et al*, 2004)(Fig
698 EV2D). To complete the structure of TssM beyond the known region, which spanned aa. 869-1129,
699 we used the *de novo* tracing strategy that we introduced in an earlier work (Cherrak *et al*, 2018).
700 Briefly, we iterated between manual model building and structural refinement on Coot (Emsley *et al*,
701 2010) using bulky sidechains and secondary structure predictions obtained by Phyre2 (Kelley *et al*,
702 2015) as guides, and sequence-structure registration based on contact prediction obtained by
703 RaptorX (Källberg *et al*, 2012). The map of the predicted contacts was aligned with those of the built
704 PDB, the algorithm introduced by the MapAlign software (Ovchinnikov *et al*, 2017). Where
705 discrepancies were observed, the register was modified to fit the predicted contact maps (Appendix
706 Fig S6). The model was eventually refined using one round of rosetta.refine (Wang *et al*, 2016) and
707 phenix.real_space_refine (Afonine *et al*, 2018). This procedure allowed us to extend the structure of
708 TssM to the fragment spanning residues 579 to 869, and to produce a model of the fragment
709 between amino acids 390 and 550.

710

711

712 **Validation of the data**

713 The model was validated as in the protocol in Refmac5 (Murshudov *et al*, 2011). The FSC map to
714 model was calculated with the sharpened map (FSC_{sum}). The model was shaken by 0.5 Å and the FSC
715 map to model was calculated with one Half map (FSC_{work}). This refined model was then used to
716 calculate the FSC map to model with the other Half map (FSC_{free}) (Appendix Fig S12A).

717 The cross correlation between each amino acid in the model and map was also calculated with
718 phenix.real_space_refine (Afonine *et al*, 2018) (Appendix Fig S12B) and the Molprobit score (Chen
719 *et al*, 2010). was obtained from the online server (Table 3) Pore radius calculations were carried out
720 using the HOLE (Smart *et al*, 1996) plugin in Coot and the protein interfaces were analysed with PISA
721 (Krissinel & Henrick, 2007).

722

723 **Strains, media and chemicals**

724 The strains, plasmids and nucleotides used in this study are listed in Appendix Tables S1 and S2. For
725 the cryo-ET studies, *E. coli* K-12 BL21(DE3) and enteroaggregative *E. coli* EAEC strain 17-2 were used
726 for protein overexpression before plunge freezing. Strains were routinely grown in LB-Miller or in Sci-
727 1-inducing medium (SIM; M9 minimal medium, glycerol 0.2%, vitamin B1 1 mg ml⁻¹, casaminoacids
728 100 mg ml⁻¹, LB 10%, supplemented or not with bactoagar 1.5%) (Brunet *et al*, 2011) with shaking at

729 37°C. Plasmids were maintained by the addition of ampicillin (100 mg ml⁻¹ for *E. coli* K-12, 200 mg ml⁻¹
730 for EAEC), kanamycin (50 mg ml⁻¹) or chloramphenicol (30 mg ml⁻¹). Expression of genes from pRSF
731 (in BL21) and pBAD33 (in EAEC) vectors was induced for 2-3h with 1 mM of isopropyl-b-D-thio-
732 galactopyranoside (IPTG) or 0.3% L-arabinose, respectively.

733

734 **Preparation of frozen-hydrated specimens**

735 Plunge freezing was performed according to (Weiss *et al*, 2017). *E. coli* BL21 or EAEC cells were
736 concentrated by centrifugation to an OD₆₀₀ of 3 - 20 and then mixed with protein A – 10 nm gold
737 conjugate (Cytodiagnosics Inc.). The higher concentrations of cells were used when preparing grids
738 for cryo-focused ion beam (cryo-FIB) milling to form “bacterial lawns” of several layers of bacteria on
739 top of each other. Bacterial lawns were found to be more amenable to cryo-FIB milling than
740 individual cells. A 3 µL droplet of the sample was applied to a carbon-coated EM copper grid (R2/1,
741 Quantifoil) that had been previously glow-discharged for 90 s at -25 mA using a Pelco easiGlow™
742 (Ted Pella, Inc.). The grid was plunge-frozen in liquid ethane-propane (37 %/63 %) using a Mark IV
743 Vitrobot (Thermo Fisher Scientific). The forceps were mounted in the Vitrobot (27°C, humidity 95%)
744 and the grid was blotted from both sides or only from the backside by installing a Teflon sheet
745 (instead of a filter paper) on the front blotting pad. Grids were stored in liquid nitrogen.

746

747 **Cryo-focused ion beam milling**

748 Cryo-focused ion beam (cryo-FIB) milling was used to prepare samples of plunge-frozen cells that
749 could then be imaged by electron cryotomography (Marko *et al*, 2007). Our cryo-FIB milling workflow
750 has been detailed in (Medeiros *et al*, 2018b). Frozen grids with lawns of *E. coli* BL21 cells
751 overexpressing TssJLM were clipped into modified Autogrids provided by J. Plitzko or a commercial
752 prototype provided by Thermo Fisher. We then transferred the grids into the liquid nitrogen bath of
753 a loading station (Leica Microsystems) and clamped them onto a “40° pre-tilted TEM grid holder”
754 (Leica Microsystems). The holder with grids was shuttled from the loading station to the dual beam
755 instrument using the VCT100 transfer system (Leica Microsystems). The holder was mounted on a
756 custom-built cryo-stage in a Helios NanoLab600i dual beam FIB/SEM instrument (FEI). The stage
757 temperature was maintained below -154°C during loading, milling and unloading procedures. Grid
758 quality was checked by scanning EM (SEM) imaging (5 kV, 21 pA). The samples were then coated with
759 a Platinum (Pt) precursor gas using the Gas Injector System. We adapted a “cold deposition”
760 technique that was published previously (Hayles *et al*, 2007) (needle distance to target of 8 mm,
761 temperature of the precursor gas of 27 °C, and open valve time of 5 s). Lamellae were milled in
762 several steps. We first targeted two rectangular regions to generate a lamella with ~2 µm thickness
763 with the ion beam set to 30 kV and ~400 pA. The current of the ion beam was then gradually reduced

764 until the lamella reached a nominal thickness of 150-400 nm (ion beam set to ~25 pA). Up to 6
765 lamellae were milled per grid. After documentation of the lamellae by SEM imaging, the holder was
766 brought back to the loading station using the VCT100 transfer system. The grids were unloaded and
767 stored in liquid nitrogen.

768

769 **Electron cryomicroscopy and electron cryotomography**

770 *E. coli* BL21 and EAEC cells (overexpressing TssJLM where indicated), cryo-FIB-processed *E. coli* BL21
771 cells overexpressing TssJLM, and purified TssJLM samples were examined by electron
772 cryotomography (cryoET). Images were recorded on a Tecnai Polara TEM (Thermo Fisher Scientific)
773 equipped with post-column GIF 2002 imaging filter and K2 Summit direct electron detector (Gatan),
774 or on a Titan Krios TEM (Thermo Fisher Scientific) equipped with a Quantum LS imaging filter and K2
775 Summit (Gatan). Both microscopes were operated at 300kV and the imaging filters with a 20 eV slit
776 width. The pixel size at the specimen level ranged from 4.93 Å to 4.05 Å. The latter pixel-sized was
777 used for the sub-tomogram average. Tilt series covered an angular range from -60° to +60° with 2°
778 (lamellae, sheath preparations) increments and -10 to -6 µm defocus, or in focus (0 µm defocus)
779 when the data was collected on the Titan Krios with a Volta phase plate (Thermo Fisher Scientific)
780 (Danev & Baumeister, 2016). The total dose of a tilt series was 60-100 e⁻/Å². Tilt series and 2D
781 projection images were acquired automatically using UCSF Tomo (Zheng *et al*, 2007) on the Tecnai
782 Polara and SerialEM (Mastronarde, 2005) on the Titan Krios. Three-dimensional reconstructions and
783 segmentations were generated using the IMOD program suite (Kremer *et al*, 1996a). Table 2
784 summarises the data collection, refinement and validation statistics.

785

786 **Sub-tomogram averaging**

787 Tomograms used for subtomogram averaging were not CTF-corrected, as most of the particles
788 were extracted from tomograms collected in focus with the Volta phase plate. Individual particles
789 were identified visually in tomograms as 5-branched stars shapes in top and bottom views and as
790 inverted-Y shapes in side views and their longitudinal axes were manually modelled with open
791 contours in 3dmod (Kremer *et al*, 1996b). The manual particle picking and first round of sub-
792 tomogram averaging were performed with the PEET software package on tomograms that were
793 binned by 4 (1k reconstructions). Model points, the initial motive list, and the particle rotation axes
794 were generated using the stalkInit program from the PEET package (Nicastro, 2006). This approach
795 allowed the definition of each structure's longitudinal axis as the particle y-axis. 28474 individual
796 particles extracted from cryotomograms of *E. coli* BL21 ghost and FIB-milled cells were averaged
797 using PEET with a box size of 44 pixels in x and z, and 72 pixels in y for the final step on data binned
798 by 2 (2k reconstruction, final pixel size 8.1 Å). A random particle was chosen as a first reference.

799 Missing wedge compensation was activated. The final motive lists obtained after this initial average
800 performed on tomograms that were binned by 4 were then translated and used to perform a new
801 round of sub-tomogram averaging on tomograms that were binned by 2 (2k reconstructions). From
802 individual particles and after analysing the resulting average, C₅ symmetry was imposed. The Fourier
803 shell correlation curves were calculated in PEET to estimate resolution. A cylindrical mask centred on
804 the structure's longitudinal axis was applied to the volumes using imodmop (IMOD package) in order
805 to mask neighbouring structures and the membranes during averaging. 3dmod (IMOD package) and
806 UCSF Chimera (Pettersen *et al*, 2004) were used for visualization of the averages. 3dmod was used
807 for generating all the movies, except for the morph and the atomic model visualization in Movie 3
808 that were generated in UCSF Chimera.

809

810 **Data availability**

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

815

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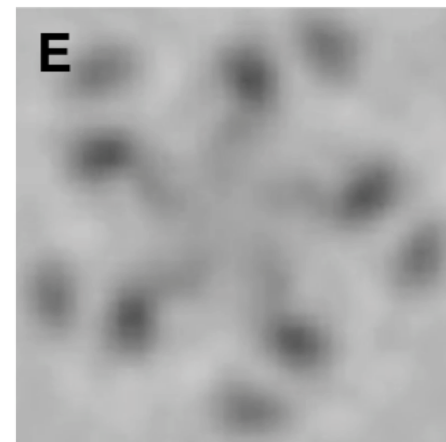
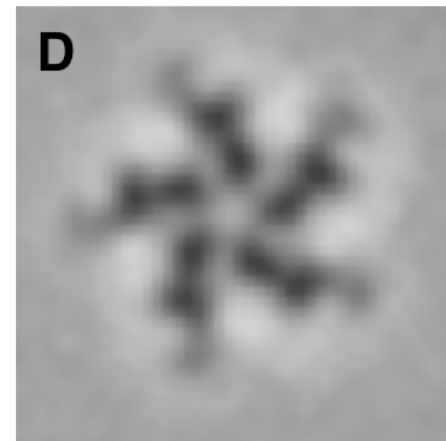
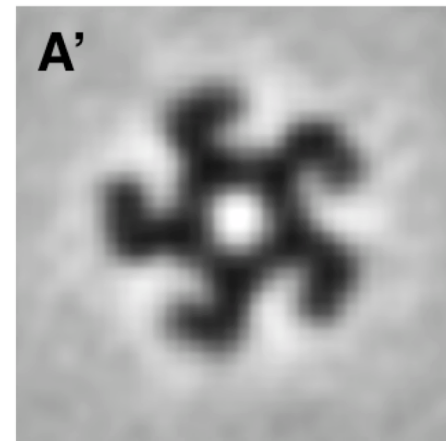
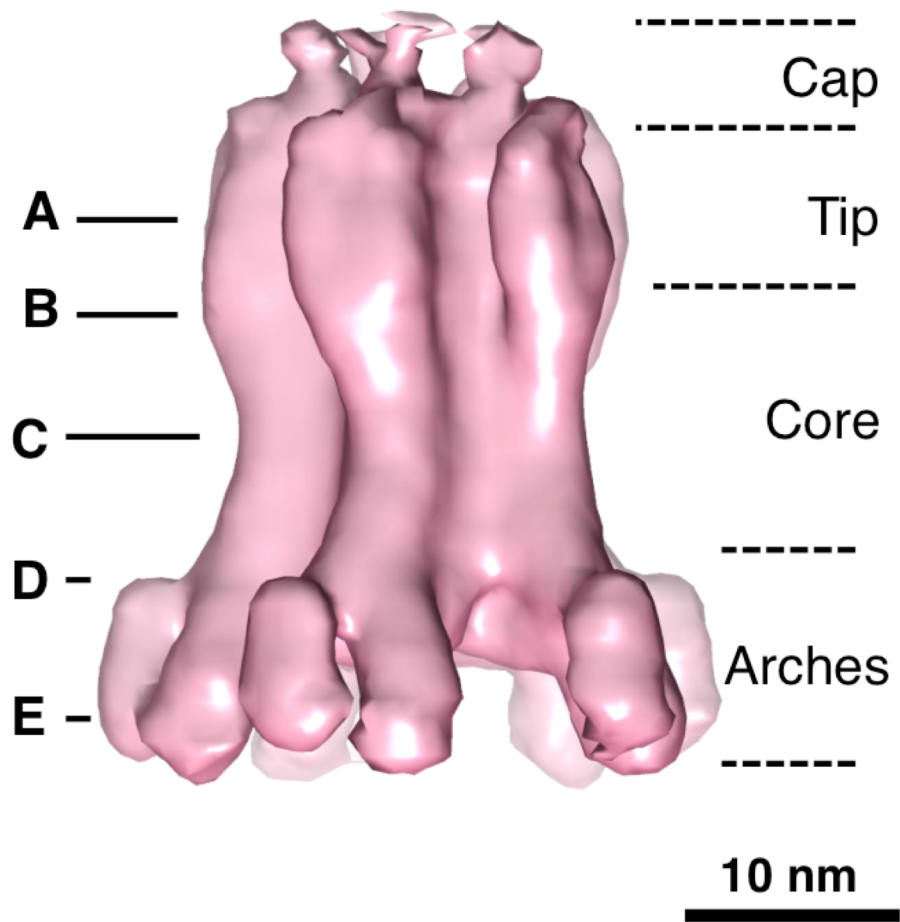
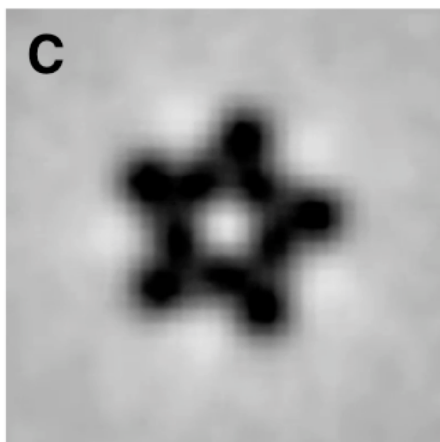
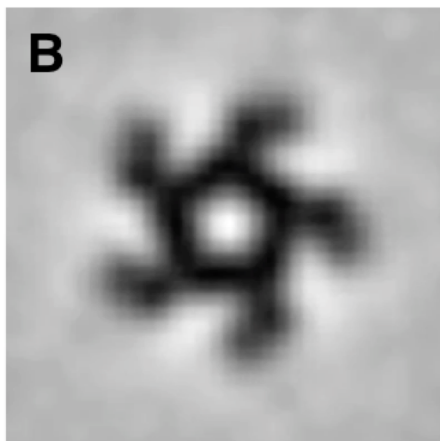
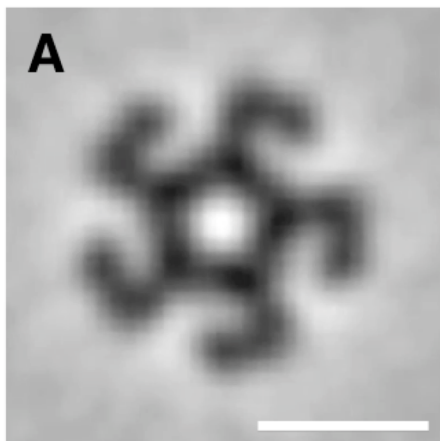
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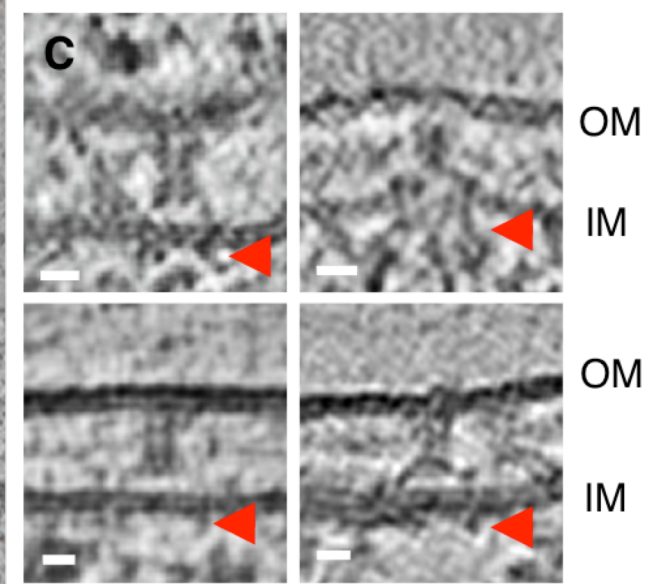
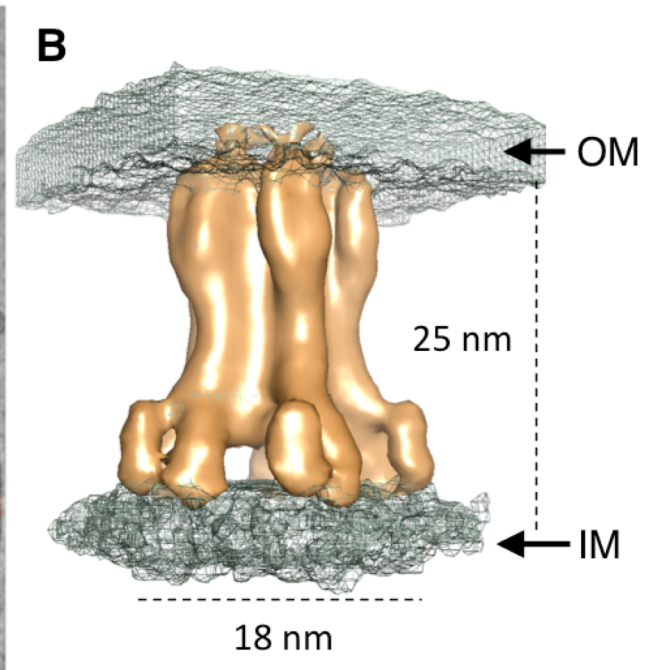
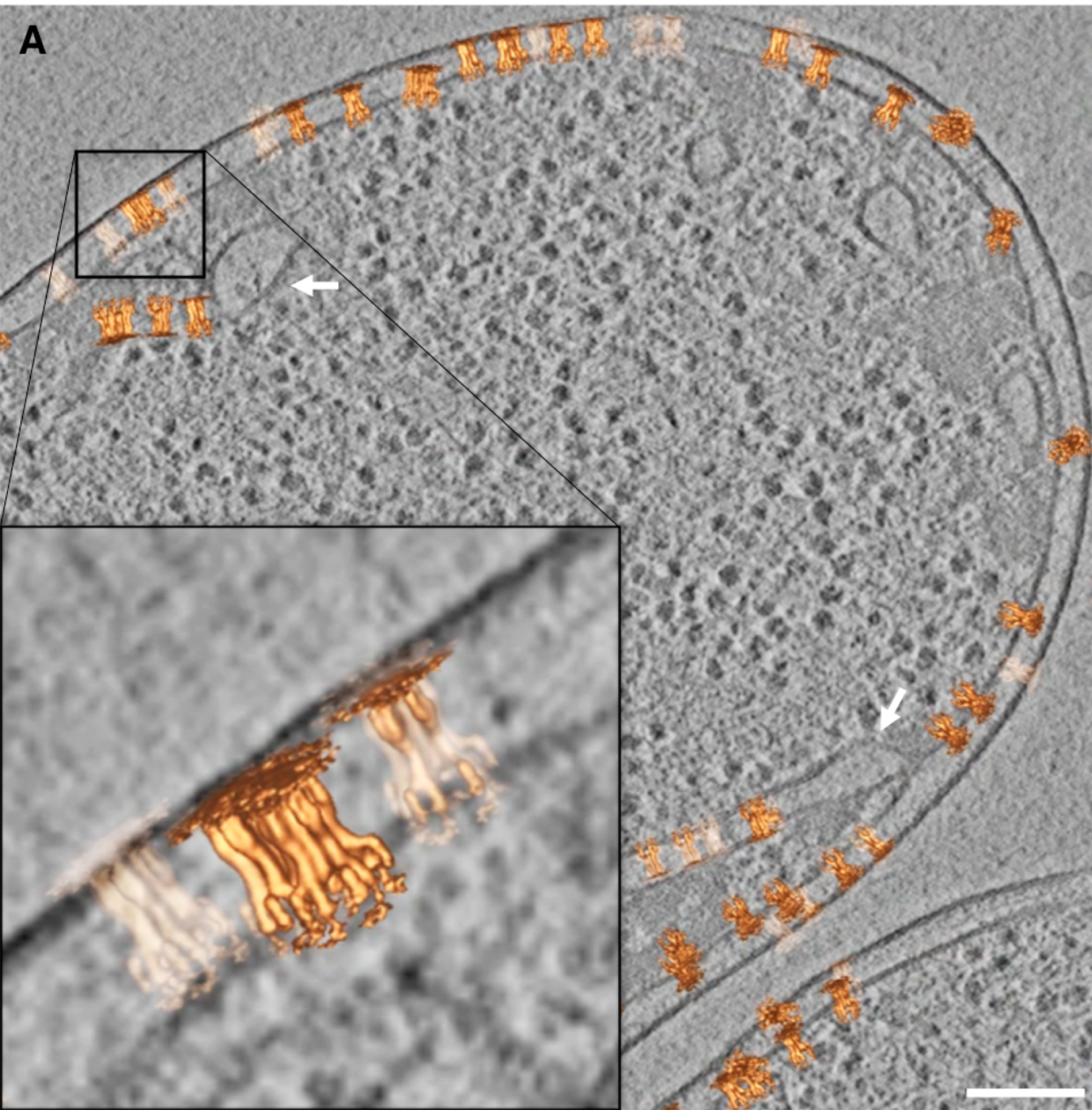
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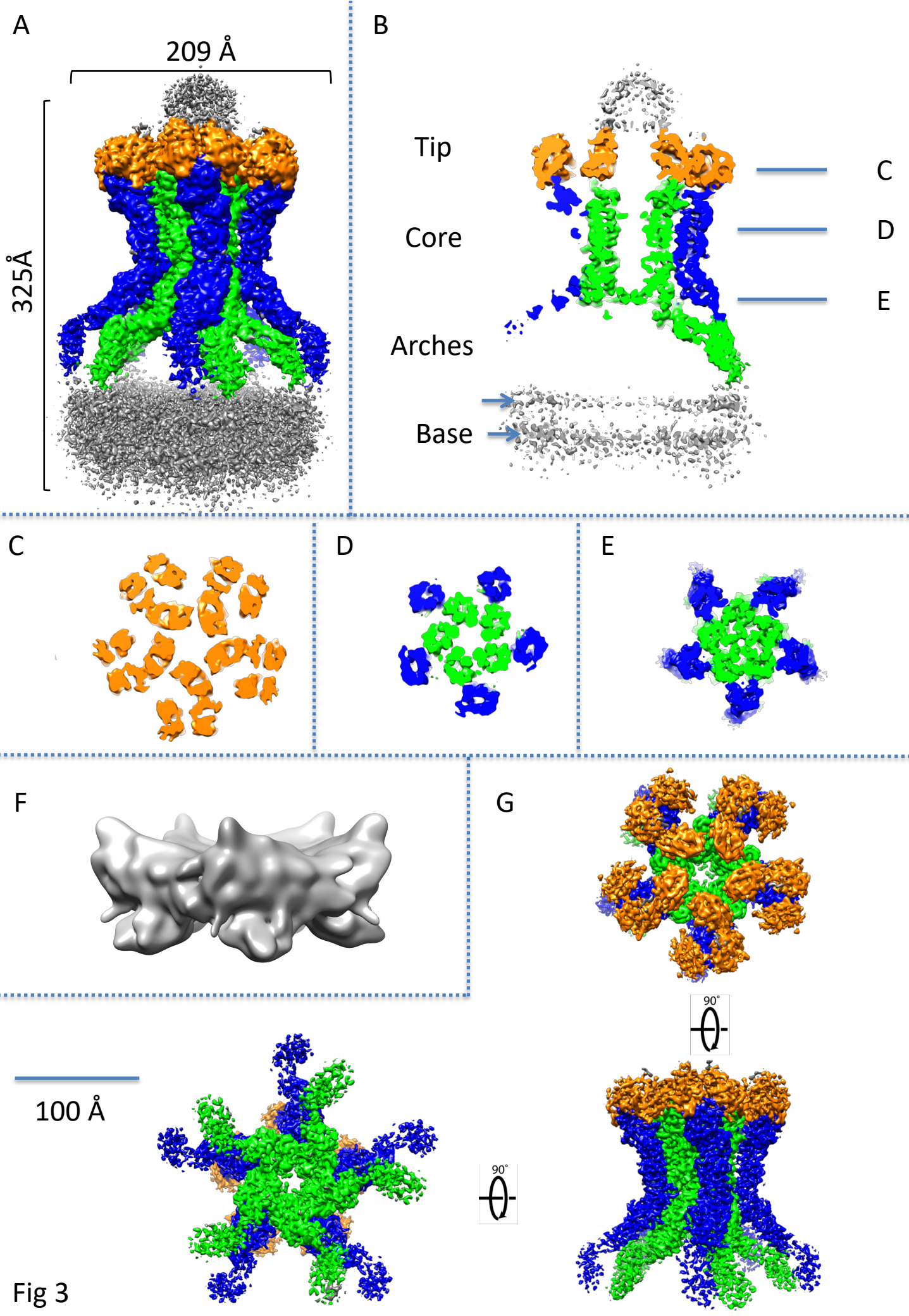
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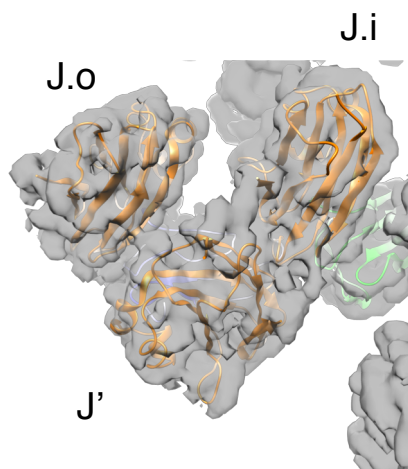
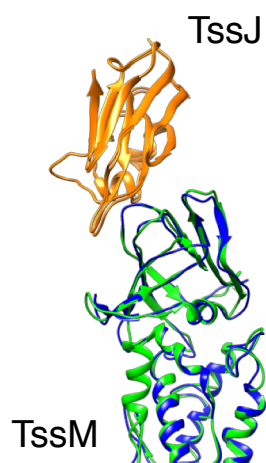
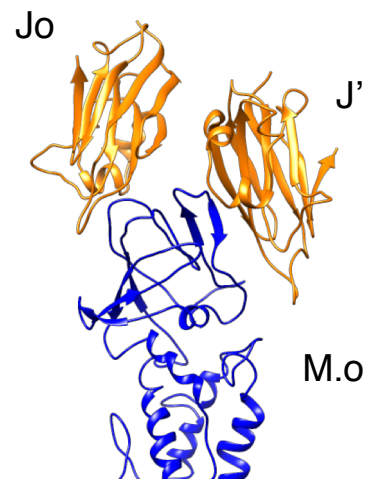
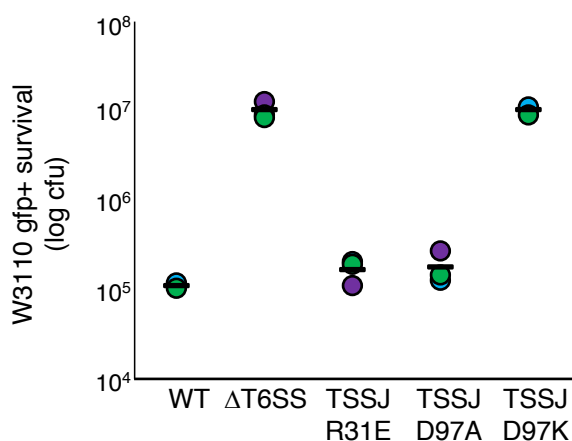
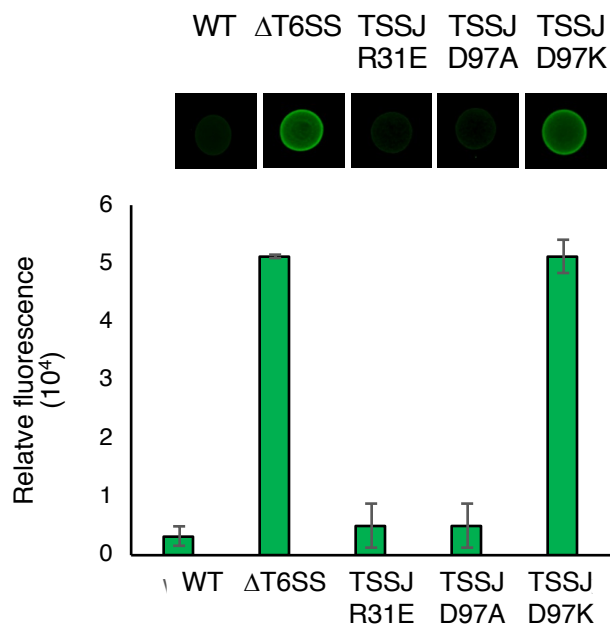
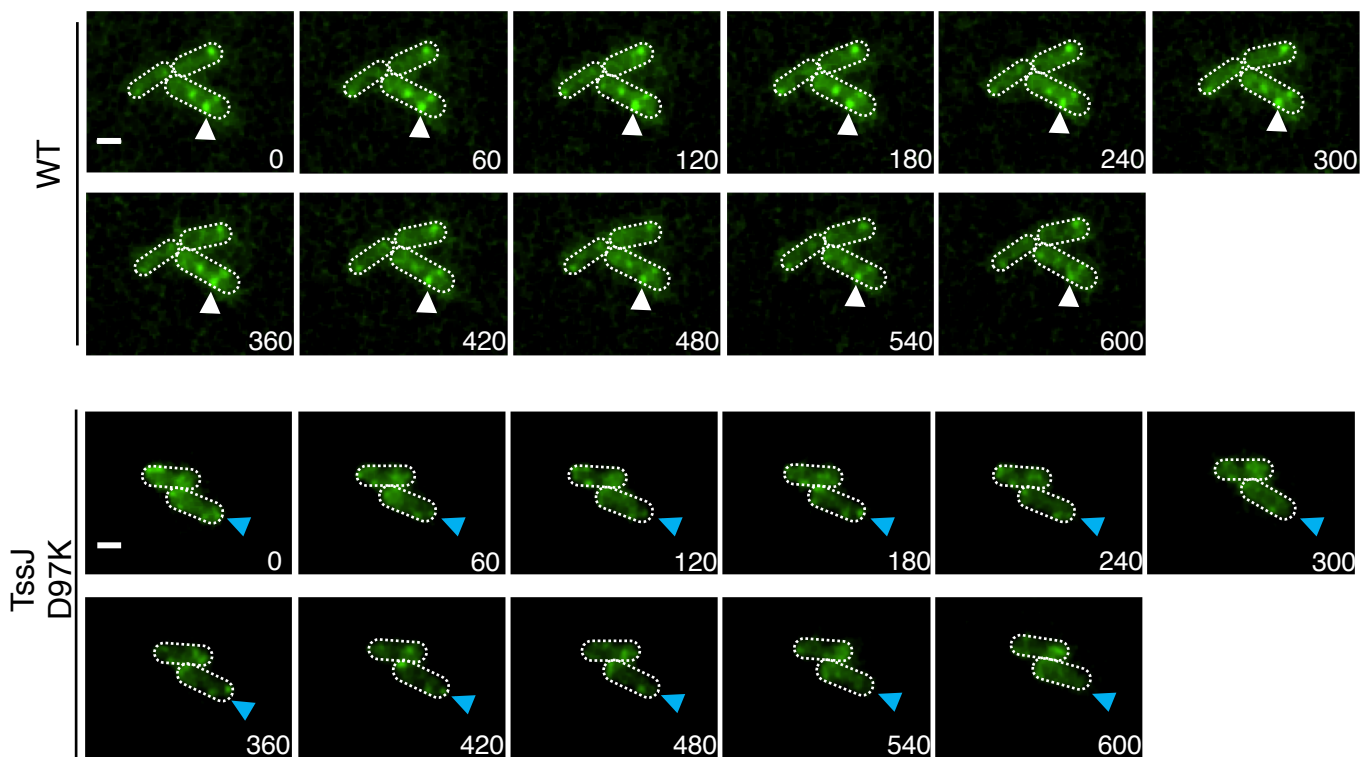
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A**B****C****D****E**

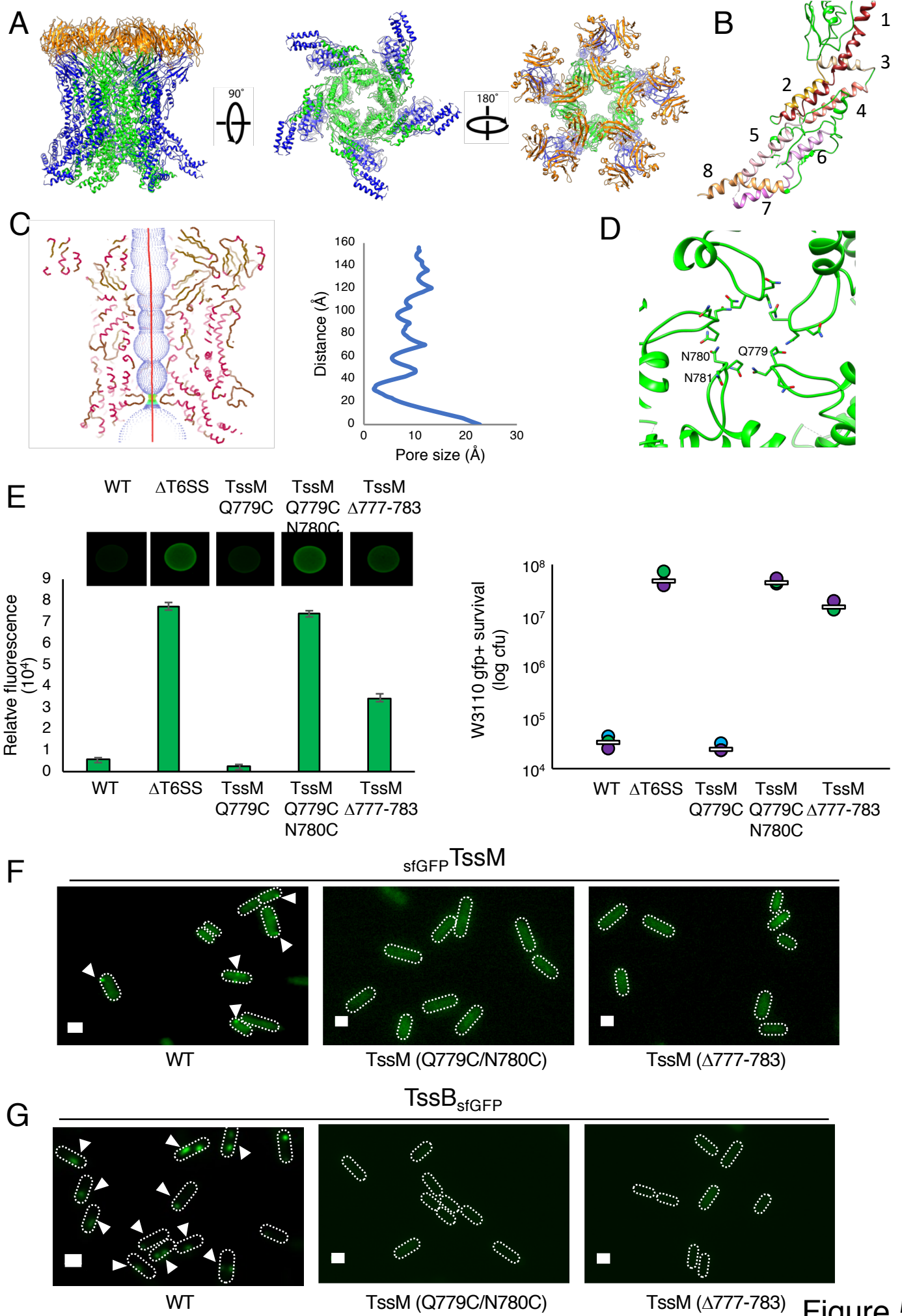


Figure 5

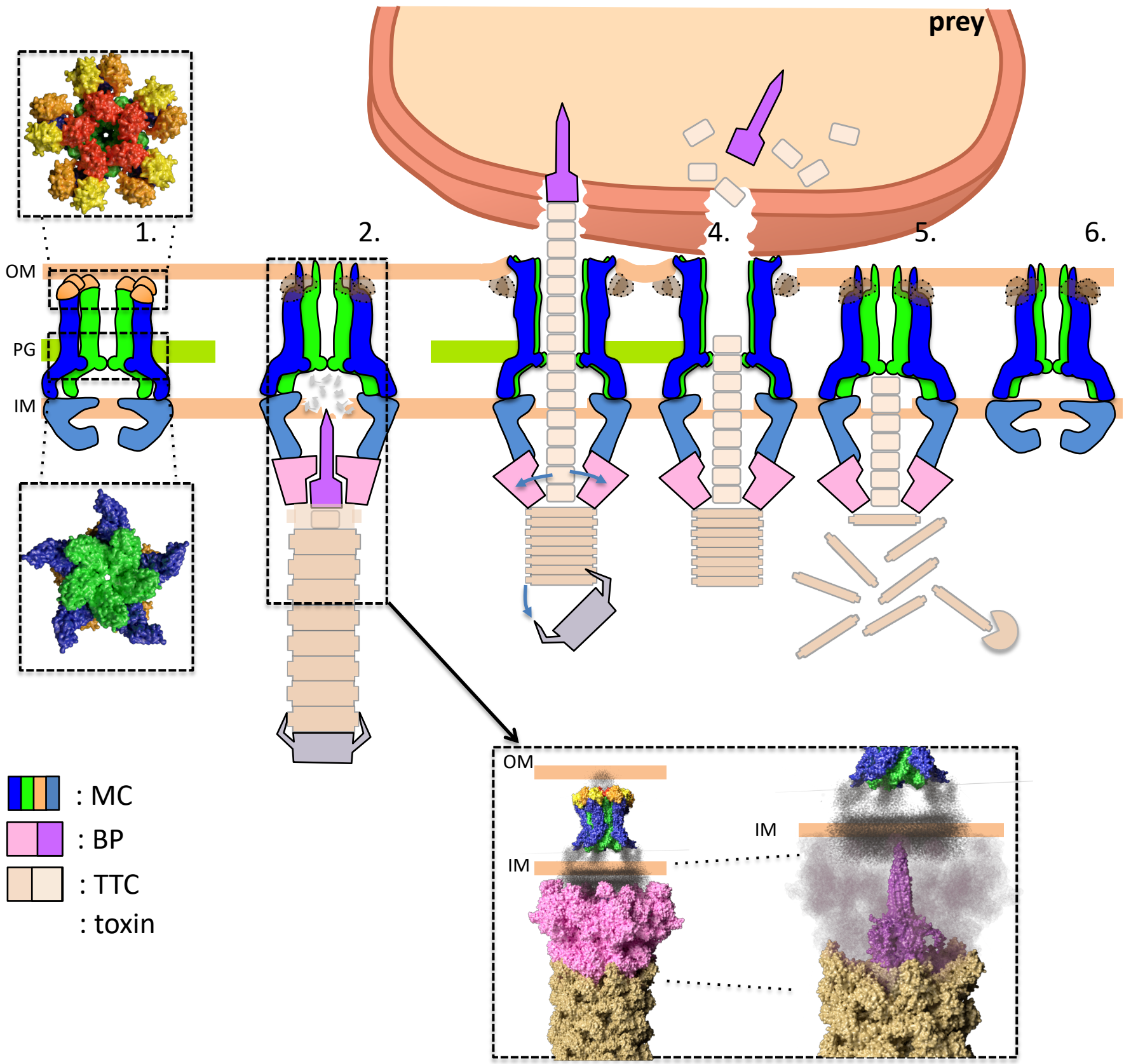
1. MC resting state

2. BP docking & TTC assembly

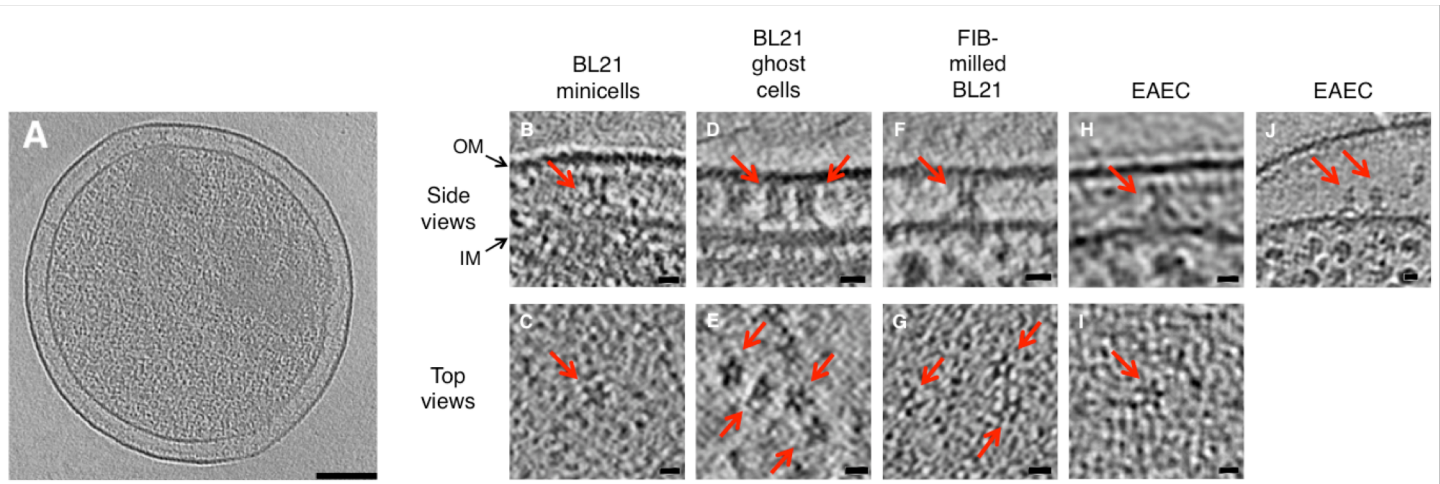
3. OM channel opens, TTC contraction & Hcp-tube and toxin firing

4. TTC recycling & channel closure

5. back to MC resting state

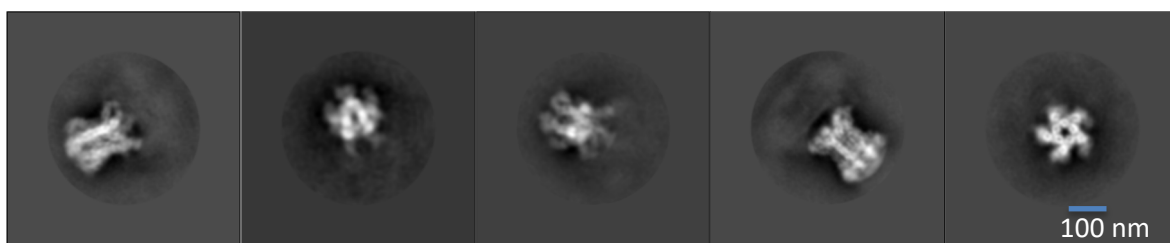


Expanded view Figure 1

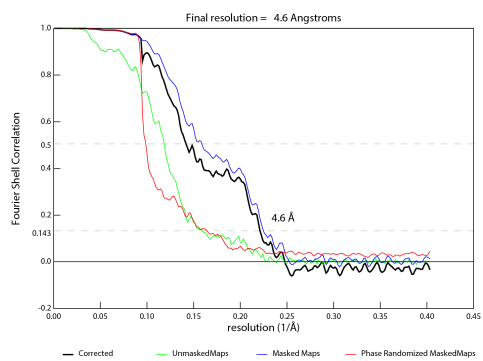


Expanded view Figure 2

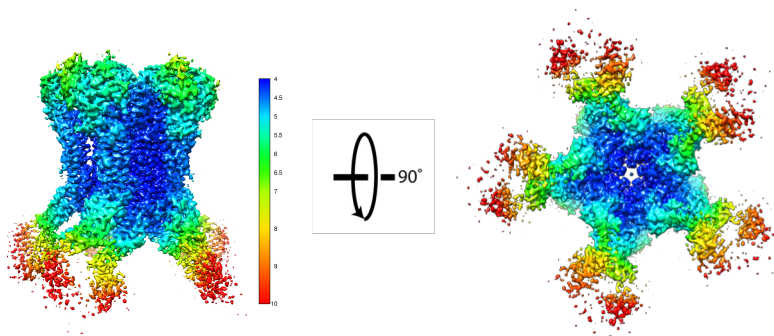
A



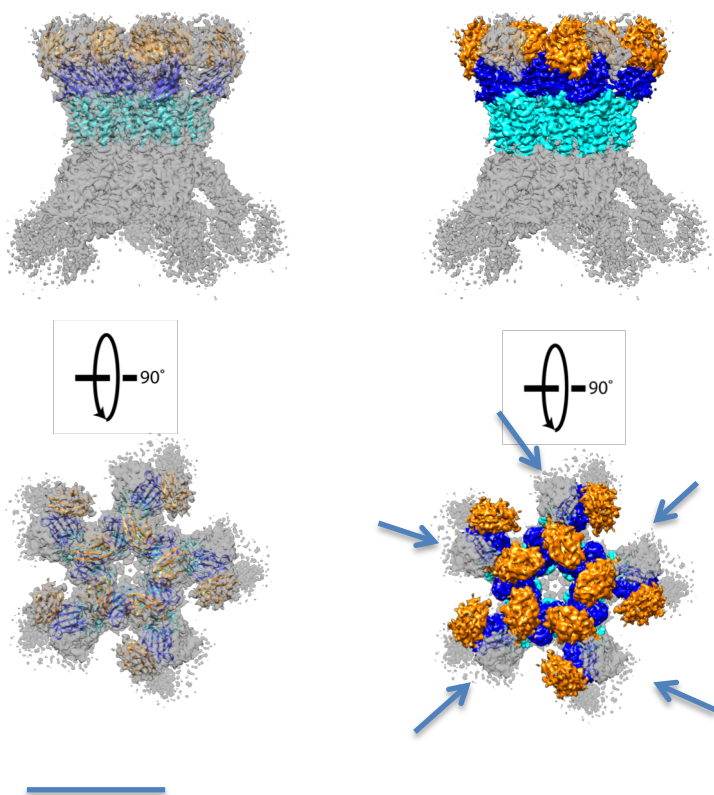
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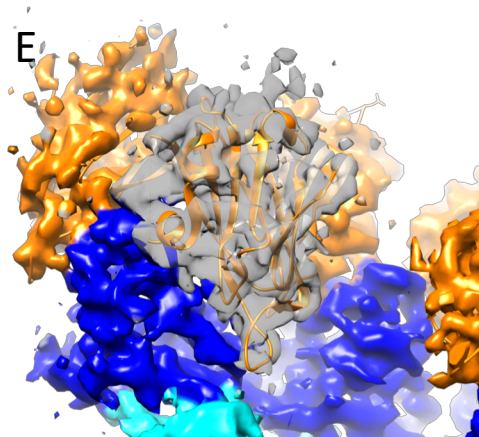
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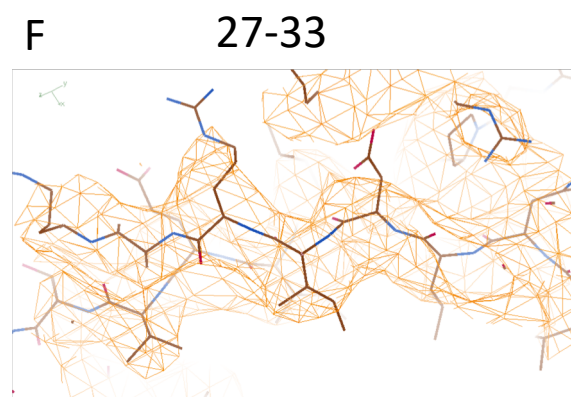
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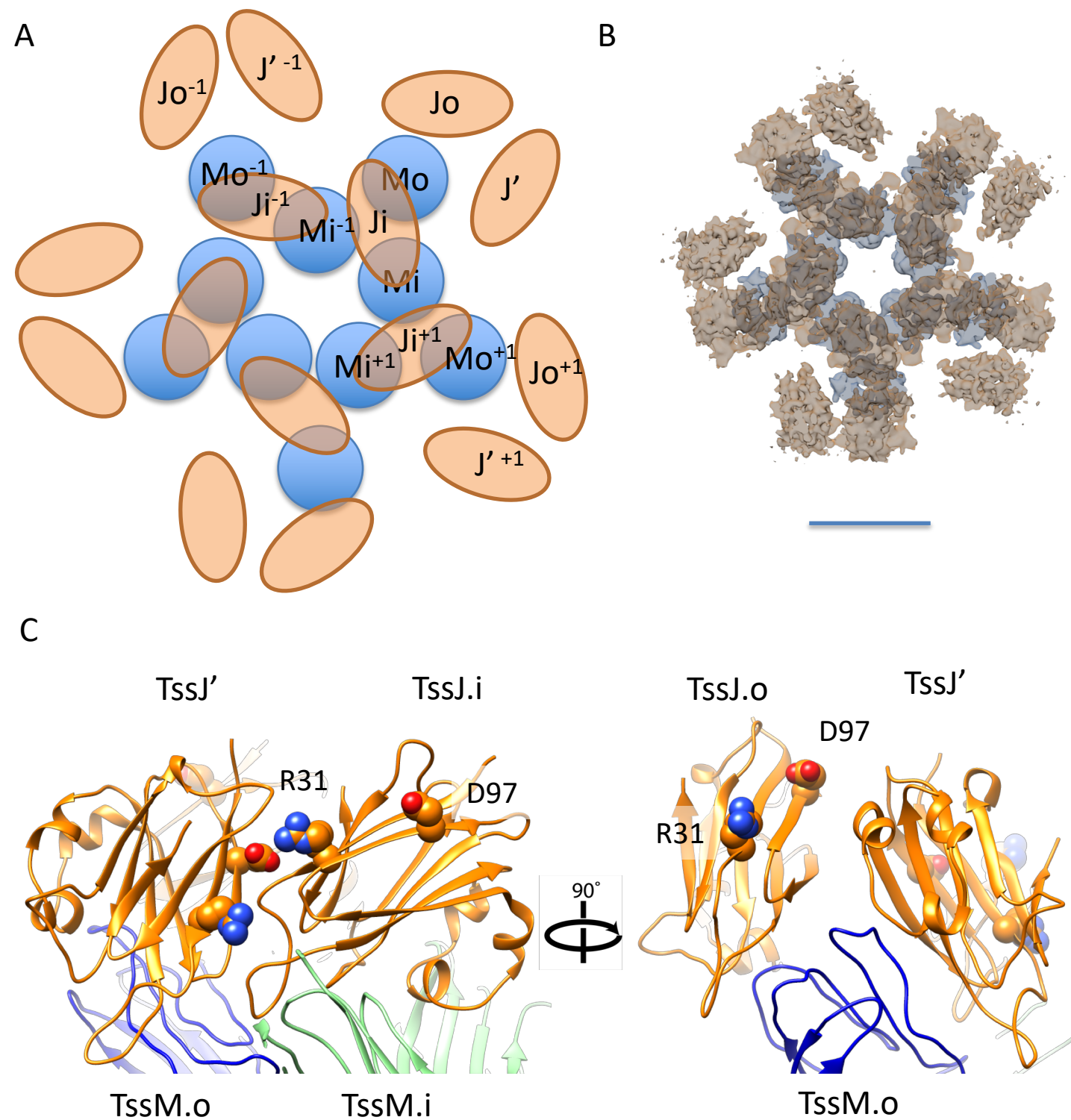
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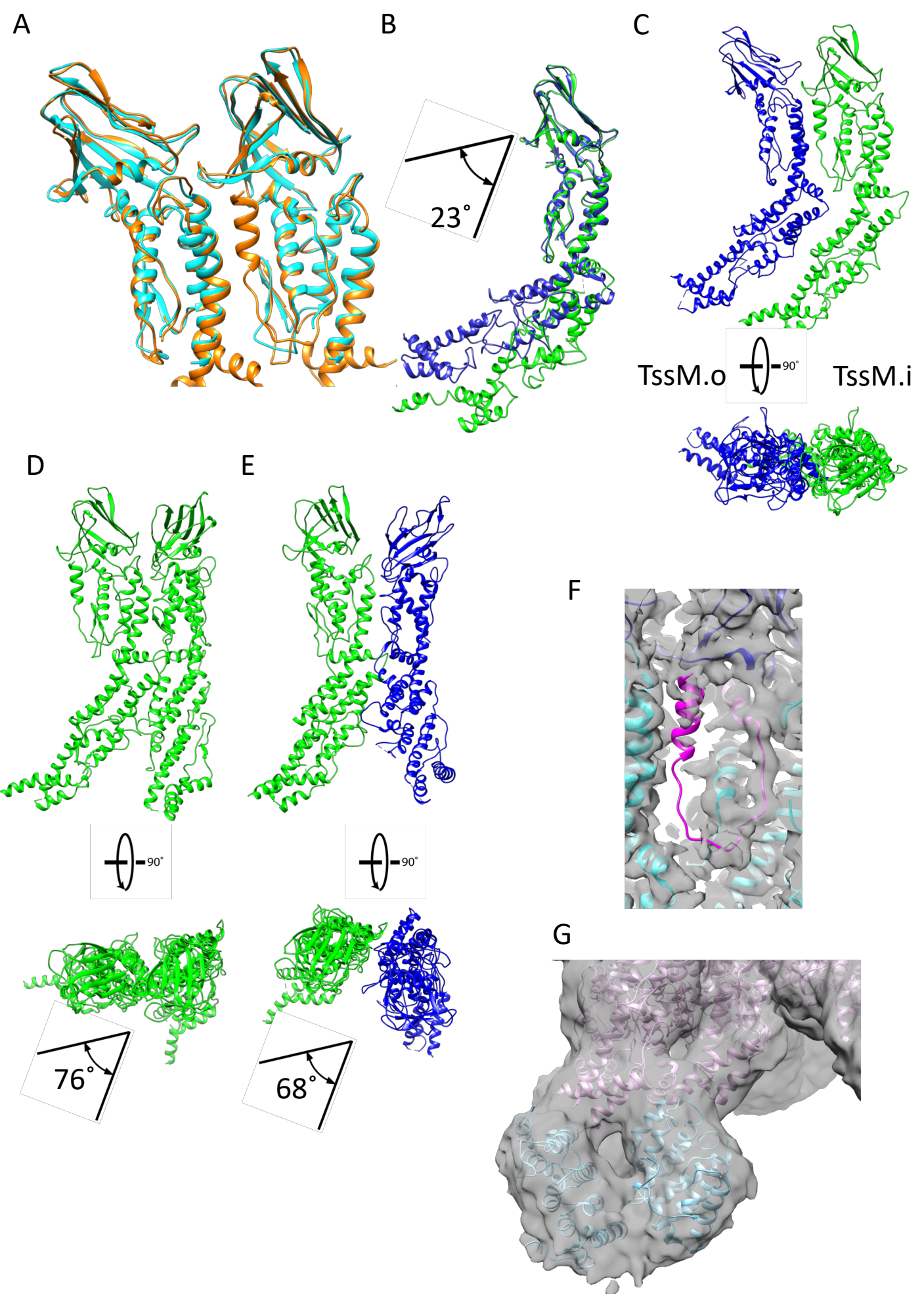


F



Expanded view Figure 3





Expanded view Figure 4