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1 **Towards a complete structural deciphering of type VI secretion system**

2
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15 *Keywords:* Type VI secretion, baseplate, contractile tail, receptor binding protein, tail
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17
18 § present address

20 **The Type VI secretion system (T6SS) is a complex and dynamic nanomachine**
21 **present in ~50% Gram-negative bacteria. Using a contraction mechanism similar to that**
22 **of myophages, bacteriocins or anti-feeding prophages, it injects toxic effectors into both**
23 **eukaryotic and prokaryotic cells. T6SS assembles three large ensembles: the trans-**
24 **membrane complex (TMC), the baseplate and the tail. Within the two last years, the tail**
25 **structure has been elucidated by cryo electron microscopy (cryoEM) both in extended**
26 **and contracted forms. The structure of the trans-membrane complex has been**
27 **deciphered using a combination of X-ray crystallography and EM. However, despite**
28 **recent progress, the structural knowledge on the baseplate lags behind. Baseplate should**
29 **therefore remain the target of future studies, leading then to a complete structural**
30 **description of T6SS.**

31

32

33 INTRODUCTION

34

35 The first report on components of the cluster that would become T6SS appeared more than 15
36 years ago. It was only in 2006 that the identification of a secreted tube component, Hcp,
37 established that the identified cluster was a secretion system [1]. It was coined Type VI
38 Secretion System (T6SS) the following year [2]. The structure of Hcp together with the
39 determination of the crystal structure of the tube tip, VgrG, established the common
40 evolutionary origin of T6SS and myophages (e.g. T4 or Mu) contractile tail [1,3], leading to
41 the vision that T6SS could be some kind of tamed phage. Later on, this hypothesis was
42 completed when T6SS tail sheath proteins VipA and VipB (TssB and TssC) were found to
43 share structural and functional homology with those of myophages, and were at the origin of
44 tail's contraction [4]. Contrary to what occurs for all other contractile machineries, T6SS tail
45 is recycled after contraction thanks to an ATPase, ClpV, that disassembles the tail sheath into
46 monomeric components, TssB and TssC [5]. This tail sheath recycling constitutes a hall mark
47 of T6SS. A group of proteins were found to be attached or embedded in both IM or OM. One
48 of them, TssM, shares sequence identity with a Tss4 component IcmF. This finding
49 completed the view of T6SS as being a phage-like machinery, attached to a secretion
50 membrane system. Between them, another ensemble constitutes the baseplate, which was
51 found to share some similarity with that of myophages.

52 In 2012, a tomography electron microscopy (EM) examination of T6SS yielded the first
53 complete topology and dynamics of the system. It made it possible to visualize the three
54 domains of T6SS, TMC, baseplate and tail, and revealed that the tail crosses completely the
55 cell's cytoplasm. Furthermore, analysis of tail's dynamics established that elongation takes
56 tens of seconds, while contraction is very fast (<5 ms) [6].

57 T6SS is able to kill both prokaryotic or eukaryotic cells [7,8]. To this end, deadly effectors
58 can be loaded at different positions: inside Hcp, docked to VgrG via PAAR module, or can be
59 part of evolved Hcp or VgrGs. These effectors have been the subject of several reviews and
60 are not part of this account [9,10].

61

62 **The trans-membrane complex spans between cytoplasm and outer membrane**

63 On the 13 proteins conserved in the T6SS cluster (Fig. 1a), four have been shown to be
64 inserted in the cell membranes: TssL, TssL and TagL in the IM and TssJ, a lipoprotein, in the

65 OM [11]. TssL is a monotopic membrane protein with a large cytoplasmic domain. Its X-ray
66 structure and further analysis allowed to determine that it forms an essential biological dimer
67 [12]. The main component of the TMC, TssM, was early identified as a T4SS IcmF-like protein
68 [1]. This ~1100 amino-acids large protein was shown to be formed of four structural
69 segments: a large cytoplasmic domain (~250 aa) inserted in the IM through a three helix
70 membrane domain, followed by a periplasmic segment that can be further divided in a large
71 helical domain followed by a C-terminal beta domain [11,13]. This latter beta-domain was
72 shown to interact with the TssJ OM lipoprotein that binds TssM and brings its C-terminus in
73 the vicinity of the OM [13]. Together with TssL [12] and TagL [14,15], TssM and TssJ form
74 the TMC [16]. Despite this knowledge, the functioning of T6SS TMC remained obscure,
75 letting open questions about the presence of an eventual OM pore and its opening.

76 In 2015, the TCM biogenesis, structure and function were deciphered using an converging
77 biophysical and biochemical techniques [17] (Fig. 1c-g). The negative stained EM (nsEM)
78 structure of the ternary complex TssJ-TssL-TssM (TssJLM) was determined at ~12 Å
79 resolution (Fig. 1f,g). It assembles 10 IM anchored TssJ-TssM pilars spanning the periplasm
80 and contacting the OM. Five dimers of TssL are anchored via a trans-membrane helix (TMH)
81 in the IM and point in the cytoplasm. Together with the cytoplasmic domain of TssM, they
82 form a hydrophilic platform that serves to dock the baseplate and the rest of the system. Quite
83 surprisingly, the TCM complex obeys a C5 symmetry, forming 5 dimers that could be split
84 between 5 internal and 5 external pilars [17] (Fig. 1f,g). Noteworthy, both the reduced size of
85 the internal channel (15 Å) and the absence of pore did not allow the nsEM structure to
86 elucidate the mysterious secretion mechanism. However, the X-ray structure of the C-terminal
87 domain of TssM in complex with TssJ made it possible to identify loops at the extremity of
88 TssM as candidates that could eventually cross the OM. Residues of TssM C-terminal domain
89 were mutated in cysteines and assayed in a functional T6SS. Loops at the tip of TssM are
90 labelled during T6SS secretion, establishing that they reach the cell exterior [17].
91 Furthermore, it was demonstrated that TssJ dissociates from TssM during the process. It was
92 therefore proposed that the stroke of the Hcp/VgrG occurring during tail contraction pilum
93 would lead the C-terminal TssM domain through the OM by brute force and drive it towards
94 the cell exterior. The TCM would thus form a transient pore of sufficient size. T6SS TCM
95 domain would thus oscillate between a closed rest state and a forced-open state during
96 secretion.

97 The T6SS C5 symmetry was quite a surprise as the tail symmetry is C6 for the tail tube and
98 the sheath (C3 for VgrG) (see below). Indeed, the immediate partner of TCM is the baseplate
99 whose structure is unknown. However, due to faint similarity with myophages baseplates [18]
100 it has been proposed that the overall baseplate structure may also obey a C6 symmetry.
101 Therefore, a symmetry mismatch should certainly occur between the TssJLM 5-fold
102 symmetry and its partner component.

103 Until a recent paper in 2016, TssA was the most intriguing and less documented component
104 of T6SS. The nsEM map of TssA revealed that it is a dodecamer assembling two back-to-
105 back C6 hexamers [19]. The crystal structure of the core C-terminal domain exhibits the
106 shape of a donut, roughly the diameter of Hcp. Both nsEM and SAXS reveal the presence of 6
107 propeller-like N-terminal domains decorating each hexamer. Noteworthy, co-expressed TssA
108 and TssJLM result in a complex that, examined by nsEM, exhibits a large volume at the base
109 of TssJLM, most likely due to the attachment of TssA. Knowing the tridimensional structures
110 of both partners spurred us on to model their complex. This proved impossible to perform
111 with the C5 TssJLM nsEM structure. A model was put up by assembling TssJLM membrane
112 and cytoplasmic domains with C6 symmetry that exquisitely fits with TssA [20] (Fig. 1h,i).
113 This suggest that TssA acts as a chaperone selecting C6 TssJLM, and displacing C5 TssJLM
114 and eventual other forms towards it. It was also found that TssA, after priming, located at the
115 distal end of the tail where it acts as a stopper [19 ,20].

116

117 **The tail, a highly dynamic and energetic system**

118 T6SS tail is a part that attracted most interest in the last five years. Not a surprise, the first
119 components for which the X-ray structure have been determined were Hcp and VgrG,
120 suggesting the common origin of T6SS and myophage tails [1,3,21,22]. More recently
121 another component of the tail, PAAR, was identified. Its position at the tip of VgrG allows to
122 sharpen the puncturing device and to dock effectors [23].

123 A contractile tail sheath, composed of TssB and TssC (identified as VipA and VipB in *Vibrio*
124 *cholerae*) enveloppes the Hcp/VgrG internal tube. The T6SS tail has been show to be micro-
125 meters long in the metastable extended form, spanning the whole cytoplasm from cell-wall to
126 cell-wall. The assembly of this tube involves TssA that acts both as a chaperone for the TCM
127 as well as a “stopper” at the distal extremity of the tube [19]. Under an external and unknown
128 trigger, TssBC changes conformation and contracts, thus propelling the Hcp/VgrG pilum

129 through TssJLM and to the inter-cellular space, and further into the nearby prey (REFs).
130 Because the extended tail sheath is in a metastable state, the *in vitro* expression of TssBC
131 leads exclusively to the contracted form. Three groups reported cryoEM structure of
132 expressed (contracted) TssBC at medium or high resolution, resulting in atomic models of the
133 tail sheath [24-26] (Fig. 2). VipAB/TssBC form a helix of 6 components per layer. The core
134 domain of the sheath is maintained by four β -strands originating from three monomers, one
135 VipA (TssB) and two VipB (TssC). The internal radius of the contracted sheath is larger than
136 the Hcp diameter, allowing thus tube release during contraction (Fig. 2a,b). While the overall
137 sheath structure resembles that of phage T4 [18], the external part is different. Not surprising,
138 a VipB (TssC) exposed domain interacts with the ClpV ATPase, allowing VipAB recycling, a
139 phenomenon not present in T4 [27]. This TssC domain is indeed hidden in the extended
140 sheath [28].

141 Recently, electron cryotomography and sub-tomogram averaging of T6SS tail *in vivo*, yielded
142 the low-resolution structure of the extended tail sheath loaded with Hcp, together with that of
143 the contracted form [28]. A while after, the cryoEM structure of *Vibrio cholerae* extended tail
144 was determined at ~ 3.5 Å resolution [29] (Fig. 2c,d). This extended form could be obtained
145 thanks to a trick, the addition of three residues to the VipA N-terminal linker connecting
146 sheath strands, blocked the tail in a metastable form. This remarkable work reveals the
147 structure of the extended sheath with its internal Hcp tube, as well as the Hcp/Hcp contacts
148 and the VipAB/Hcp interaction. Comparison of the extended and contracted structure reveals
149 a shrink of the inter disk rise, from 38.0 Å to 21.8 Å ($\sim 42\%$) associated with an increase of
150 the twist helical angle from 23.6 to 29.9 degree [29] (Fig. 2). The authors calculated that
151 contraction of a 1 μm long sheath would release an energy of 10,000 kcal/mole, push the
152 pilum by 420 nm towards the prey, while rotating at $\sim 40,000$ rpm: an incredible powerful
153 machinery that indeed has no problem to open a passage in the TssJLM TCM complex.

154

155 **The baseplate secrets**

156 The baseplate is formed of TssE, F, G, H, and represents an adaptor between the TMC and
157 the tail. Indeed, due to the drastic conformational changes between extended and contracted
158 tail sheath, its role might be also to accommodate these changes in order to keep the system
159 strongly assembled.

160 The baseplate has been the least studied part of T6SS. Sequence analysis by bioinformatics
161 studies suggested that components of the baseplate – if not the complete baseplate – resemble
162 those of myophages, T4 or Mu [18,30,31]. TssE is clearly the counter part of gp25 that binds
163 strongly the tail sheath. TssF is similar with T4 gp6 or Mu ORF47 [31], and it has been
164 proposed that TssF and TssG assembly resemble that of gp6-gp53 in T4 [18,30].

165 The nsEM TssK structure reveals that it is a trimer [32]. It and intracts both with TMC and
166 tail [32]. Subsequently, TssK has been shown to be part of a definite TssKFG sub-complex
167 that may assemble into different multimers [33]. Later on, a larger complex was identified
168 that assemble TssE, F, G, K and VgrG. This complex is recruited at the IM by the TMC. K is
169 the first component to dock the TMC, and it further recruits FG [34]. Finally, TssBC and Hcp
170 elongate with the help of TssA, bolstered by the baseplate. In another report on phage T4
171 baseplate structure, purification of the TssKFG sub-complex was reported [18]. However, the
172 TssKFG stoichiometry and the baseplate symmetry are still pending issues. SDS gel analysis
173 reported a clear 2:1 ration for TssF:TssG, comparable to that of myoviridae counterparts
174 [18,33]. However, contradictory results are reported concerning TssK, proposed to belong to a
175 4:2:1 complex [33] or a 3:2:1 complex [18].

176 We recently determined the X-ray structure of full-length TssK in complex with camelid
177 nanobodies [35] and analyzed its attachment to TssFG [36]. Each monomer of TssK trimer
178 has three domains: shoulders, neck and head (Fig. 3). The N-terminal shoulder domain of
179 TssK is structurally similar to the shoulder domain of siphophage receptor binding proteins,
180 such as phages p2 and 1358. Nanobodies competition assays showed that TssK binds to
181 TssFG using the bottom of the shoulders domain. The C-terminal head domain bind to the
182 TMC. The crude stoichiometry of the TssKFG complex returned from SDS gel analysis
183 reports K:F:G values of 4.04/1.86/1 and 4.6/1.97/1. The high ratio of K suggests that the
184 TssKFG complex stoichiometry might be either $K_3:F_2:G$ ($(K_3)_2:F_2:G$), i.e. each TssK binding a
185 TssK trimer.

186 From an evolutionary viewpoint, these results suggest that even if T6SS tail and baseplate
187 originates from Myophages, the TssK N-terminal domain may have derived from a
188 *Siphoviridae* component to provide the ability to bind the TMC, pointing thus to a complex
189 evolutionary process leading to T6SS in which components of different phage types were
190 combined.

191

192 **The T6SS classes**

193 T6SS is not a totally homogeneous system. Two classes have been distinguished on the base of
194 functional co-evolution between TssB, TssC, TagJ/HsiE, and ClpV [37], while three classes
195 have been identified within human gut bacteroidales [38]. However, in these classes, most of
196 the 13 components of the T6SS cluster are conserved. Recently, the complete low-resolution
197 structure of a contractile secretion system from *Amoebophilus asiaticus*, has been
198 determined by cryo-tomography [39]. This system possesses a T6SS-like tail, a baseplate, an
199 IM anchored module, but no *bona fide* TCM. The tail is shorter than in T6SS, as it is
200 controlled by a tape measure protein (TMP), a feature found in all contractile systems but
201 T6SS. This tail is not recycled by a ClpV ATPase. No effector has been isolated to date, and
202 the action could be only due to mechanical effects. Contrary to T6SS, the system is not
203 isolated and forms packed arrays at the IM surface. The *Amoebophilus asiaticus* secretion
204 system cluster do not share any homology with those of T6SS and its components do not
205 share any sequence identity with T6SS components, but share a strong identity with anti-
206 feeding prophages. Although the authors proposed to class this secretion system as the 4th
207 exemple of T6SS, this proposition seems fulsome and could be misleading for futures studies
208 of T6SS.

209

210 **Conclusion**

211 The structural and functional knowledge of T6SS has made recently huge progresses, thanks
212 to the achievements of cryoEM/tomography. The structural data available to date make it
213 possible to tentatively propose a topological model of T6SS (Fig. 4). In the extended model
214 presented here, 12 proteins out of the 13 conserved have been localized, either based on
215 strong data, or tentatively (TssEFG). The cytoplasmic ClpV ATPase, the 13th conserved
216 protein, interacts with the contracted tail to disassemble it. Obviously, we are not far of
217 having the structures of all the components determined, alone or in groups. No doubt that sub-
218 tomogram averaging of a complete T6SS *in vivo* will allow in a near future to visualize a
219 complete T6SS and its interactions, and shed further light on its secretion mechanism.

220

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229

230

231

232 **References**

233

234 **Legend to Figures**

235

236 **Figure 1. Structure of the T6SS trans-membrane complex.** (a) The cluster of conserved
237 T6SS genes in the genome of *E. coli* EAEC. (b) Tomographic slice of an extended *V.*
238 *cholerae* T6SS structure (adapted from ref. [6]). (c) Crystal structure of the TssM26Ct–TssJ
239 complex represented as ribbons [17]. TssJ is coloured orange, while TssM26Ct is coloured
240 cyan (α -domain) and blue (β -domain). The C-terminal α 5-helix and the extended stretch are
241 coloured magenta. (d) Orthogonal relative to (c). (e) View of the TssM26Ct–TssJ complex
242 represented as surface representation (same orientation and colour as in (d)). (f) Segmentation
243 of the TssJLM complex reconstruction [17]. Each pillar within the TMC is segmented in five
244 different domains (shown in different colours). Domains 3,4 and TssJ correspond to the
245 TssM26Ct–TssJ complex. (g) TssM26Ct–TssJ structure docked into the EM volume
246 corresponding to TssJ and the TssM periplasmic domains. (h) View of the T6SS TMC (grey)
247 modelled with 6/12 instead of 5/10 symmetry, forming a complex with TssA dodecamer
248 (blue, 6 fold symmetry) (adapted from [20] and [19]). (i) Same model as in (h), but viewed
249 from the cytoplasm, showing the fit of TssA arms in between the protruding TssL dimers.

250

251 **Figure 2. Structure of T6SS tail.** (a) Top ribbon view of the *V. cholerae* T6SS contracted tail
252 sheath. Hcp hexamers have been modelled in the internal TssBC pipe in order to evidence that
253 they do not interact with the sheath. (b) View rotated by 90° (lateral view). (c) Top ribbon
254 view of the *V. cholerae* T6SS extended tail sheath, formed of TssBC (VipAB) hexamers, and
255 containing stacked Hcp hexamers forming the tube [26]. (d) View rotated by 90° (lateral
256 view). (a-d): One protofilament of the sheath is colored orange. The TssC recycling domain
257 (that recognized by ClpV) is colored cyan. The Hcp inner tube is colored red.

258

259 **Figure 3. Crystal structure of the TssK trimer.** Ribbon view of TssK structure evidencing
260 the topology of this trimer formed of shoulder, neck and head domains [36]. The binding of
261 TssK to nbK27 (that occupies the binding site of TssFG) is reminiscent of the binding of the
262 Dit arm and hand extension of the phage p2 Dit molecule [40].

263

264 **Figure 4. Topology model of extended T6SS.** (a) The cluster of conserved T6SS genes in
265 the genome of *E. coli* EAEC. (b) Tomographic slice of an extended *Myxococcus xanthus*

266 T6SS structure (adapted from ref. [28]). (c) The position of the different modules with known
267 structures have been tentatively assigned in the tomographic slice. Those with unknown
268 structure (TssFGE) have been assigned according to ref. [28]. From the 13 conserved proteins
269 forming T6SS (see (a), 12 have been topologically assigned. The 13th, ClpV, is soluble
270 cytoplasmic ATPase involved in contracted tail disassembling.

271

272 **References**

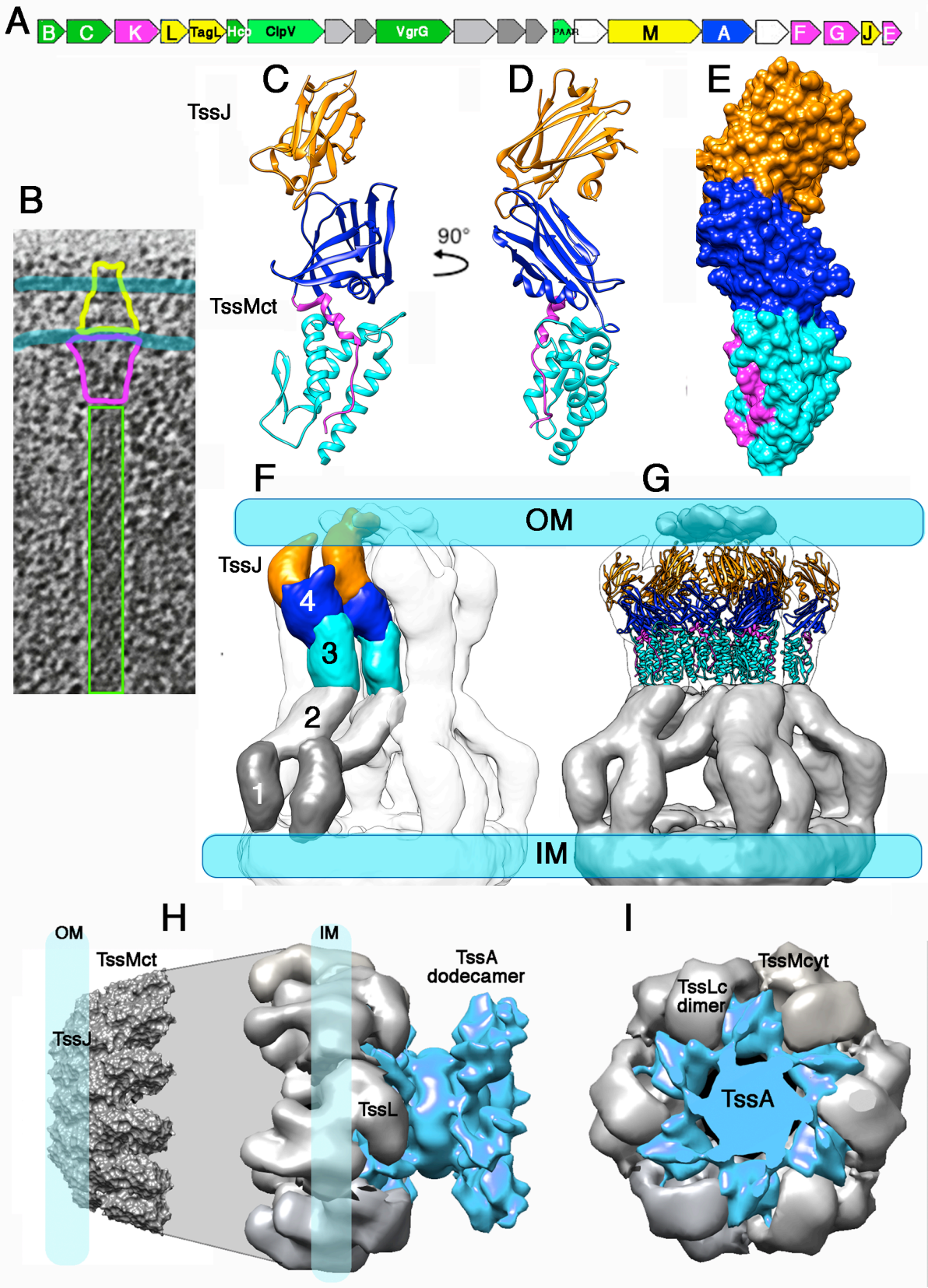
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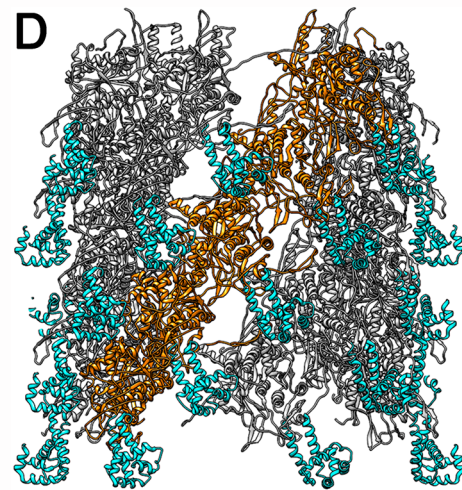
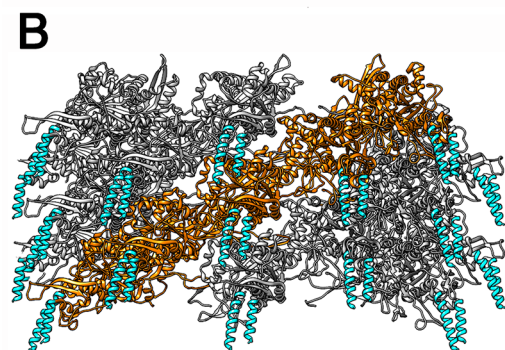
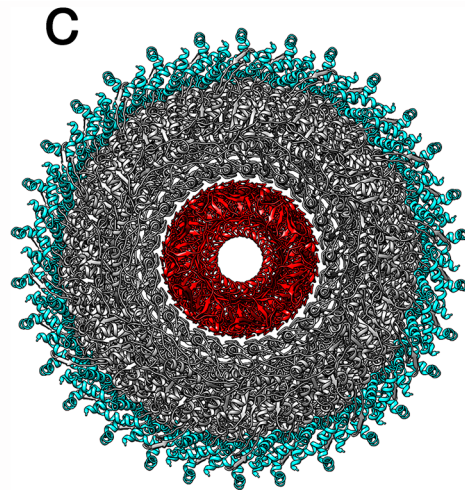
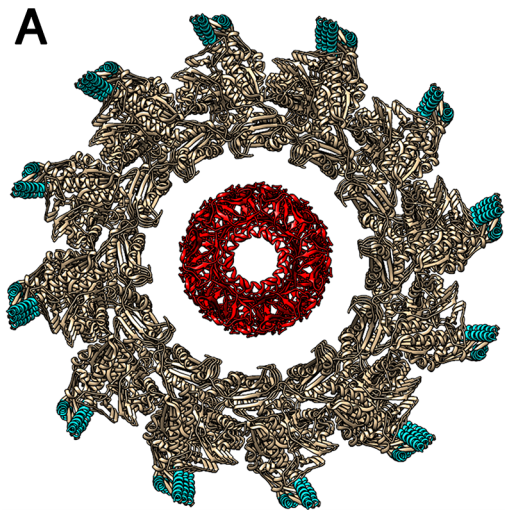
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