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Towards a complete structural deciphering of type VI secretion system

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

The first report on components of the cluster that would become T6SS appeared more than 15 years ago. It was only in 2006 that the identification of a secreted tube component, Hcp, established that the identified cluster was a secretion system [1]. It was coined Type VI Secretion System (T6SS) the following year [2]. The structure of Hcp together with the determination of the crystal structure of the tube tip, VgrG, established the common evolutionary origin of T6SS and myophages (e.g., T4 or Mu) contractile tail [1,3], leading to the vision that T6SS could be some kind of tamed phage. Later on, this hypothesis was completed when T6SS tail sheath proteins VipA and VipB (TssB and TssC) were found to share structural and functional homology with those of myophages, and were at the origin of tail’s contraction [4]. Contrary to what occurs for all other contractile machineries, T6SS tail is recycled after contraction thanks to an ATPase, ClpV, that disassemble the tail sheath into monomeric components, TssB and TssC [5]. This tail sheath recycling constitutes a hallmark of T6S. A group of proteins were found to be attached or embedded in both IM or OM. One of them, TssM, shares sequence identity with a Tss4 component IcmF. This finding completed the view of T6SS as being a phage-like machinery, attached to a secretion membrane system. Between them, another ensemble constitutes the baseplate, which was found to share some similarity with that of myophages.

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

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

The trans-membrane complex spans between cytoplasm and outer membrane

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

In 2015, the TCM biogenesis, structure and function were deciphered using an converging biophysical and biochemical techniques [17] (Fig. 1c-g). The negative stained EM (nsEM) structure of the ternary complex TssJ-TssL-TssM (TssJLM) was determined at ~12 Å resolution (Fig. 1f,g). It assembles 10 IM anchored TssJ-TssM pilars spanning the periplasm and contacting the OM. Five dimers of TssL are anchored via a trans-membrane helix (TMH) in the IM and point in the cytoplasm. Together with the cytoplasmic domain of TssM, they form a hydrophilic platform that serves to dock the baseplate and the rest of the system. Quite surprisingly, the TCM complex obeys a C5 symmetry, forming 5 dimers that could be split between 5 internal and 5 external pilars [17] (Fig. 1f,g). Noteworthy, both the reduced size of the internal channel (15 Å) and the absence of pore did not allow the nsEM structure to elucidate the mysterious secretion mechanism. However, the X-ray structure of the C-terminal domain of TssM in complex with TssJ made it possible to identify loops at the extremity of TssM as candidates that could eventually cross the OM. Residues of TssM C-terminal domain were mutated in cysteines and assayed in a functional T6SS. Loops at the tip of TssM are labelled during T6SS secretion, establishing that they reach the cell exterior [17]. Furthermore, it was demonstrated that TssJ dissociates from TssM during the process. It was therefore proposed that the stroke of the Hcp/VgrG occurring during tail contraction pilum would lead the C-terminal TssM domain through the OM by brute force and drive it towards the cell exterior. The TCM would thus form a transient pore of sufficient size. T6SS TCM domain would thus oscillate between a closed rest state and a forced-open state during secretion.
The T6SS C5 symmetry was quite a surprise as the tail symmetry is C6 for the tail tube and the sheath (C3 for VgrG) (see below). Indeed, the immediate partner of TCM is the baseplate whose structure is unknown. However, due to faint similarity with myophages baseplates [18] it has been proposed that the overall baseplate structure may also obey a C6 symmetry. Therefore, a symmetry mismatch should certainly occur between the TssJLM 5-fold symmetry and its partner component.

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

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

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

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

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

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

The baseplate secrets

The baseplate is formed of TssE, F, G, H, and represents an adaptor between the TMC and the tail. Indeed, due to the drastic conformational changes between extended and contracted tail sheath, its role might be also to accommodate these changes in order to keep the system Strongly assembled.
The baseplate has been the least studied part of T6SS. Sequence analysis by bioinformatics studies suggested that components of the baseplate – if not the complete baseplate – resemble those of myophages, T4 or Mu [18,30,31]. TssE is clearly the counter part of gp25 that binds strongly the tail sheath. TssF is similar with T4 gp6 or Mu ORF47 [31], and it has been proposed that TssF and TssG assembly resemble that of gp6-gp53 in T4 [18,30].

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

We recently determined the X-ray structure of full-length TssK in complex with camelid nanobodies [35] and analyzed its attachment to TssFG [36]. Each monomer of TssK trimer has three domains: shoulders, neck and head (Fig. 3). The N-terminal shoulder domain of TssK is structurally similar to the shoulder domain of siphophage receptor binding proteins, such as phages p2 and 1358. Nanobodies competition assays showed that TssK binds to TssFG using the bottom of the shoulders domain. The C-terminal head domain bind to the TMC. The crude stoichiometry of the TssKFG complex returned from SDS gel analysis 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 TssKFG complex stoichiometry might be either K₃:F₂:G (K₃)₂:F₂:G, i.e. each TssK binding a TssK trimer.

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

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

Conclusion

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

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References
**Legend to Figures**

**Figure 1. Structure of the T6SS trans-membrane complex.** (a) The cluster of conserved T6SS genes in the genome of *E. coli* EAEC. (b) Tomographic slice of an extended *V. cholerae* T6SS structure (adapted from ref. [6]). (c) Crystal structure of the TssM26Ct–TssJ complex represented as ribbons [17]. TssJ is coloured orange, while TssM26Ct is coloured cyan (α-domain) and blue (β-domain). The C-terminal α5-helix and the extended stretch are coloured magenta. (d) Orthogonal relative to (c). (e) View of the TssM26Ct–TssJ complex represented as surface representation (same orientation and colour as in (d)). (f) Segmentation of the TssJLM complex reconstruction [17]. Each pillar within the TMC is segmented in five different domains (shown in different colours). Domains 3, 4 and TssJ correspond to the TssM26Ct–TssJ complex. (g) TssM26Ct–TssJ structure docked into the EM volume corresponding to TssJ and the TssM periplasmic domains. (h) View of the T6SS TMC (grey) modelled with 6/12 instead of 5/10 symmetry, forming a complex with TssA dodecamer (blue, 6 fold symmetry) (adapted from [20] and [19]). (i) Same model as in (h), but viewed from the cytoplasm, showing the fit of TssA arms in between the protruding TssL dimers.

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

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

**Figure 4. Topology model of extended T6SS.** (a) The cluster of conserved T6SS genes in the genome of *E. coli* EAEC. (b) Tomographic slice of an extended *Myxococcus xanthus*
T6SS structure (adapted from ref. [28]). (c) The position of the different modules with known structures have been tentatively assigned in the tomographic slice. Those with unknown structure (TssFGE) have been assigned according to ref. [28]. From the 13 conserved proteins forming T6SS (see (a), 12 have been topologically assigned. The 13th, ClpV, is soluble cytoplasmic ATPase involved in contracted tail disassembling.
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