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1 Priming and polymerization of a bacterial contractile tail structure.

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26
27 **Contractile tails are composed of an inner tube wrapped by an outer sheath assembled**
28 **in an extended, metastable conformation that stores mechanical energy necessary for its**
29 **contraction. Contraction is used to propel the rigid inner tube towards target cells for**
30 **DNA or toxin delivery. Although recent studies have revealed the structure of the Type**
31 **VI secretion system contractile sheath, the mechanisms by which its polymerization is**
32 **controlled and coordinated with inner tube assembly remain unsolved. In this study, we**
33 **report that the starfish-like TssA dodecameric complex interacts with tube and sheath**
34 **components. Fluorescence microscopy experiments revealed that TssA binds first to the**
35 **T6SS membrane core complex and then initiates tail polymerization. TssA remains at**

1 **the tip of the growing structure and incorporates new tube and sheath blocks. Based on**
2 **these results, we propose that TssA primes and coordinates tail tube and sheath**
3 **biogenesis.**

4 Contractile injection machines are fascinating nano-structures evolved to deliver
5 macromolecules into target cells¹. These machines have been elaborated for different
6 purposes such as the injection of DNA into host cells in the case of bacteriophages, for the
7 delivery of protein effectors into bacterial or eukaryotic cells in the case of R-pyocins,
8 *Photorhabdus* Virulence Cassettes, Anti-feeding prophages or Type VI secretion systems
9 (T6SS) or for inducing metamorphosis in invertebrates¹⁻⁶. These machines are constituted of a
10 tubular edifice called a tail^{1,7,8}. The tail is essentially composed of a rigid inner tube wrapped
11 by a contractile structure – the sheath – that is assembled in an extended conformation that
12 stores mechanical energy necessary for its contraction and to propel the inner tube towards the
13 target⁸. The tail is assembled on the baseplate that varies in terms of composition and number
14 of subunits; however, a minimal baseplate is constituted of the hub protein surrounded by
15 wedges^{1,7,8}. The baseplate is not only the platform for the assembly of the tube/sheath but also
16 an important component of the signaling cascade that triggers sheath contraction^{1,8}. Tails are
17 usually completed by terminator proteins that stabilize the sheath and maintain tube and
18 sheath together at the distal end to prevent energy dissipation during sheath contraction and to
19 permit proper ejection of the inner tube⁸⁻¹⁰.

20 The T6SS is composed of a contractile structure anchored to the cell envelope by the
21 TssJLM membrane complex that serves as docking station as well as channel for the passage
22 of the inner tube during sheath contraction¹¹⁻¹⁴ (Extended Data Fig. 1a). The contractile
23 structure is composed of the tail tube made by stacks of Hcp hexameric rings, wrapped by a
24 sheath-like structure constituted of the TssB and TssC subunits (Extended Data Fig. 1a)¹⁴.
25 During T6SS biogenesis, the assembly of the tube and sheath are coordinated: the insertion of
26 a tube ring immediately preceding that of a sheath block¹⁵. This tail polymerizes on a
27 baseplate-like complex composed of the VgrG hub, and the TssE, TssF, TssG and TssK
28 subunits¹⁶⁻¹⁹ (Extended Data Fig. 1a). The TssBC sheath polymerizes in tens of seconds to
29 build a ~ 600-nm long structure that contracts in a few milliseconds¹⁷. Contraction of the
30 sheath propels the Hcp inner tube towards the target cell, such as a nano-crossbow,¹⁴ and is
31 responsible for the delivery of toxin effectors as it correlates with lysis of the competitor
32 bacterium^{20,21}. Recent cryo-electron microscopy (cryo-EM) studies have revealed the atomic
33 structure of the T6SS sheath in its contracted conformation^{22,23}. The sheath is a helical

1 structure composed of 6-TssB/TssC heterodimer strands, each heterodimer being stabilized by
2 an intra- and inter-strand handshake domain²³. In addition, a cryo-EM study of the pyocin R2
3 has provided information regarding the atomic structure of this contractile nanotube in its
4 extended conformation and on how it interacts with the inner tube². Although the general
5 mechanism of T6SS assembly and the structure of the T6SS sheath are now well documented,
6 critical details are missing such as how the polymerization of the sheath is controlled, how
7 tube and sheath assembly is coordinated and how tail polymerization is stopped.

8 **TssA initiates tail tube/sheath polymerization.**

9 During T6SS tail biogenesis, (i) the recruitment and assembly of Hcp hexamers and TssBC
10 sheath blocks should be coordinated and (ii) the tail tube and sheath should be firmly attached
11 together at the distal end to allow proper tube throwing during contraction. We therefore
12 hypothesized that at least one of the T6SS core proteins must be required to coordinate and/or
13 terminate Hcp/TssBC tail assembly. Such candidate subunit(s) should interact with both the
14 tube protein (Hcp) and with at least one component of the sheath (TssB and/or TssC). We
15 therefore performed a systematic bacterial two-hybrid analysis in which Hcp, TssB and TssC
16 were used as baits to identify prey partners within T6SS subunits. Extended Data Fig. 1b
17 shows that a number of baseplate components (TssE, TssF, TssG and VgrG) interact with
18 either Hcp or TssC. However, a unique protein, TssA (gene accession number: 284924261),
19 interacts with both tube and sheath components. Recent data showed that TssA is required for
20 proper formation of Hcp tube¹⁹, arguing against a role of TssA as a terminator-only protein. In
21 agreement with this conclusion, fluorescence microscopy experiments show that T6SS
22 sheathes do not assemble in *tssA* cells (Extended Data Fig. 1c). In addition, Hcp tube proteins
23 are not released in *tssA* cells (Extended Data Fig. 1d). Collectively, these data suggest a
24 critical role of TssA as a regulator of T6SS tail biogenesis.

25 Given that T6SS tube/sheath assembly is initiated on the baseplate complex which is
26 docked at the cytoplasmic side of the TssJLM complex¹⁹, we hypothesized that TssA interacts
27 with baseplate or membrane complex components. First, fractionation experiments showed
28 that the TssA protein mainly localizes in the cytoplasm but a significant amount of the protein
29 is associated with the membrane fraction (Extended Data Fig. 1e). TssA association with the
30 inner membrane is likely dependent on the T6SS membrane complex as co-purification and
31 gel filtration experiments showed that TssA binds to the detergent-solubilized TssJLM
32 complex (Extended Data Fig. 2a-b). Negative-stain electron microscopy (EM) of the TssJLM-
33 TssA complex further demonstrated the presence of a ~300 Å-large complex associated to the

1 cytoplasmic base of the TssJLM rocket-like structure (Extended Data Fig. 2c-d). To gain
2 further information on TssA localization and dynamics, we fused TssA to a fluorescent
3 reporter, the superfolder-Green Fluorescent Protein (sfGFP). sfGFP was inserted at the *tssA*
4 locus on the chromosome, to engineer cells producing a functional sfGFP-TssA chimera
5 protein. Time-lapse microscopy recordings showed that TssA does not distribute randomly
6 but rather assembles 1-3 discrete foci, located close to the membrane (Fig. 1a). Most of these
7 foci are not fixed and show directional movement (Extended Data Fig. 3a). Kymographic
8 analyses confirmed that TssA foci move with an unidirectional trajectory, at a constant
9 velocity (see the schematic representation in the inset of the Fig. 1a panel, and the kymograph
10 in Extended Data Fig. 3b). Based on these trajectory, we wondered whether TssA foci might
11 be pushed by the elongation of the sheath. In $\Delta tssBC$ cells, TssA foci were still assembled at
12 close proximity to the membrane, but their dynamics was completely abolished: the TssA foci
13 remained static (Fig. 1b and Extended Data Fig. 3a). This experiment defined that the TssBC
14 sheath does not interfere with TssA recruitment and primary localization but rather pushes the
15 TssA cluster during its elongation. We further monitored TssB-mCherry and sfGFP-TssA
16 dynamics. Time-lapse recordings and kymographic analyses confirmed that TssA-containing
17 complexes assemble first close to the membrane and are then pushed by the sheath towards
18 the opposite side of the bacterium (Fig. 1c and Extended Data Fig. 3b). In addition,
19 Fluorescence Lifetime Imaging Microscopy (FLIM) assays demonstrated that TssA molecules
20 do not appear to turn over between the foci and the intracellular pool therefore suggesting that
21 the same TssA or TssA-containing complex remains at the distal end of the sheath (Extended
22 Data Fig. 3c-d). However, because sheath contraction is a very fast event that occurs in a few
23 msec¹⁷ and is immediately followed by ClpV-mediated disassembly²⁴ our experiments could
24 not define whether TssA remains associated to the sheath during contraction.

25 To provide insights onto the assembly of the T6SS and particularly on the early events
26 prior to tube/sheath elongation, we tested the localization and dynamics of sfGFP-TssA in
27 various mutant backgrounds. The biogenesis of the T6SS begins with the initial positioning of
28 the TssJ outer membrane lipoprotein and progresses with the sequential recruitment of the
29 inner membrane TssM and TssL proteins¹³, and then the TssEFGK-VgrG baseplate¹⁹.
30 Deletion of baseplate components or Hcp did not affect TssA localization but abolished its
31 dynamics (Fig. 1d and Extended Data Fig. 3e-h). By contrast, the sfGFP-TssA fluorescence
32 was diffuse in *tssM* cells but remained clustered in *tssL* cells (Fig. 1d and Extended Data Fig.
33 3e-h). Taken together, these results showed that TssA is recruited in the early stages of T6SS

1 biogenesis, after formation of the TssJ-TssM complex (Extended Data Fig. 3i). The
2 interaction of TssA with the TssJM complex was further confirmed by co-purification
3 experiments (Extended Data Fig. 2a).

4 To identify TssA additional partners, the interaction of TssA with all the T6SS soluble
5 core components and the soluble domains of the TssL, TssM and TssJ proteins, was first
6 tested by a bacterial two-hybrid approach. Extended Data Fig. 4a shows that in addition to
7 Hcp and TssC, and the previously described TssA-TssK interaction²⁵, TssA interacts with the
8 TssE and VgrG baseplate subunits. To validate these results by an alternative approach, native
9 TssA, Hcp, TssE, VgrG and the TssBC complex were purified and the interactions were
10 assessed by Surface Plasmon Resonance (Extended Data Fig. 4b-e). In the four cases, we
11 observed interactions between the two partners, with dissociation constants ranging from ~ 2
12 to ~ 50 μ M (Extended Data Fig. 4b-e). The affinities of these complexes are rather low but
13 have to be replaced in the context of multiple interactions that probably act synergistically.
14 The interaction of TssA with Hcp or TssBC are of better affinities compared to that of TssA
15 with VgrG and TssE, whereas TssA dissociates more rapidly from VgrG or TssE than from
16 Hcp or TssBC (Extended Data Fig. 4b-e). This suggests that the TssE and VgrG baseplate
17 proteins may first interact to TssA and that these interactions will be displaced by the tube
18 and sheath components (Hcp, TssBC).

19 Based on the interaction network and on the fluorescence microscopy recordings, we
20 conclude that positioning of TssA on the membrane complex recruits the baseplate and
21 initiates polymerization of the tube and sheath. However, TssA remains associated with the
22 distal end of the polymerized structure during the elongation process (see closing remarks).

23 **Structural organization of the TssA protein**

24 The EM analyses of the TssJLM-TssA complex (Extended Data Fig. 2d) suggest that
25 TssA assembles a ~ 300 Å-large complex. A 6 \times His-tagged thioredoxin-TssA fusion (TRX-
26 TssA) was produced, purified to homogeneity by ion-metal affinity and size exclusion
27 chromatographies (Extended Data Fig. 5a). Gel filtration (Extended Data Fig. 5b) and on-line
28 multi-angle laser light scattering/quasi-elastic light scattering/absorbance/refractive index
29 (MALS/QELS/UV/RI, Extended Data Fig. 5c) analyses defined a mass of 891 kDa, which
30 corresponds to the mass of a TRX-TssA dodecamer (theoretical mass = 888 kDa).
31 Crystallization attempts with the purified full-length TssA protein obtained after tag cleavage
32 by the TEV protease failed. We therefore examined the protein sample using small angle X-
33 rays scattering (SAXS, Extended Data Fig. 5d-j) and EM after negative staining (EM,

1 Extended Data Fig. 5k-r). The ~ 19 -Å resolution single particle reconstruction of TssA from
2 the electron micrographs (Extended Data Fig. 5k-n) showed that TssA assembles two stacked
3 hexamers with arm-like short extensions (Extended Data Fig. 5p-r). However, we noted that
4 the EM density does not account for the complete mass of a TssA dodecamer and therefore
5 suspected that the arms may represent a flexible domain, shortened by the averaging
6 procedure. This was confirmed by SAXS studies demonstrating that TssA is composed of a
7 central hexameric core bearing six long arms yielding a starfish-like structure (Extended Data
8 Fig. 5h-j and Extended Data Table 1). Whereas the SAXS model allows to better visualize the
9 arm length compared to the EM reconstruction, its low resolution impairs the visual
10 separation of the dimeric arms.

11 Limited proteolysis of full-length TssA using proteinase K yielded three stable
12 fragments of ~ 45 , ~ 33 and ~ 19 kDa (Extended Data Fig. 6a) corresponding to the N-
13 terminal 1-392 and C-terminal 221-531 and 393-531 regions of TssA respectively (Extended
14 Data Fig. 6b). Based on this result, two domains corresponding to fragments 1-400 (TssA_{Nt},
15 theoretical molecular weight: 44.2 kDa) and 395-531 (TssA_{Ct}, theoretical molecular weight
16 15.1 kDa) were designed. Bacterial two hybrid experiments showed that these two domains
17 oligomerize independently (Extended Data Fig. 6c). MALS/QELS/UV/RI analyses of the
18 purified TssA_{Nt} and TssA_{Ct} domains revealed that the N-terminal fragment is a dimer in
19 solution (Extended Data Fig. 6d) whereas the C-terminal domain is dodecameric (Extended
20 Data Fig. 6e).

21 The N-terminal and C-terminal domain crystallized, and 3.37-Å and 3.35-Å resolution
22 datasets were collected respectively (Extended Data Table 2). In agreement with the
23 MALS/QELS/UV/RI data, TssA_{Nt} is a dimer in the crystal. The peptidic chain starts at
24 residue 221 (instead of 1) and stops at residue 377 (instead of 401). This fragment, named
25 TssA_{Nt2}, results from protein cleavage during crystallization. The TssA_{Nt2} domain is
26 constituted of 3 pairs of helices (1-2, 3-4 and 5-6) structured as a bundle followed by a 7th
27 helix perpendicular to the others (Extended Data Fig. 6f-g). The TssA_{Ct} structure features two
28 stacked head-to-head hexamers 30-Å thick, with an external diameter of 100 Å (Fig. 2a). The
29 overall form of the hexamer is unique and resembles that of a kaleidocycle with 6 triangular
30 wedges that contact each other via α -helix hinges (Fig. 2a). Docking of these X-ray structures
31 onto the starfish-shaped EM and SAXS reconstructions (Fig. 2b and Extended Data Fig. 7a-n)
32 showed that the TssA_{Ct} diameter and width coincide exquisitely with the central core of TssA
33 whereas TssA_{Nt2} dimers fit in the arms at close proximity to the TssA_{Ct} central core. Large

1 density regions remain available at the extremity of the arms and likely correspond to TssA_{Nt1}
2 domains.

3 To understand the contribution of the TssA central core and arm domains to the T6SS
4 assembly mechanism, we tested interactions between these domains and TssA partners.
5 Bacterial two-hybrid analyses demonstrated that TssA_{Nt1} interacts with TssBC and TssE
6 whereas TssA_{Ct} is sufficient to make contacts with Hcp and VgrG (Extended Data Fig. 6h).
7 SPR analyses further confirmed the TssA_{Nt1}-TssBC and TssA_{Ct}-Hcp interactions with K_{DS} of ~
8 4.2 and ~ 48 μ M respectively (Extended Data Fig. 6i-j).

9 **Closing remarks**

10 In this work we combined structural, functional and microscopy analyses to reveal the
11 specific role of TssA subunit during T6SS biogenesis. TssA is recruited to an early stage of
12 T6SS biogenesis, positions the baseplate, and initiates and guides tail tube/sheath
13 polymerization.

14 A TssA dodecamer is recruited to the TssJM complex and clusters in discrete foci that
15 represent the site of assembly of the T6SS tail tube and sheath. Previous studies have shown
16 that TssA is not required for proper recruitment of TssL to the TssJM complex¹³, and hence
17 the T6SS assembly pathway is branched on TssM (Extended Data Fig. 3i). Protein-protein
18 interaction and fluorescence microscopy analyses defined that TssA recruits two components
19 of the baseplate (VgrG and TssE). Once the baseplate is positioned, the polymerization of the
20 tube/sheath structure is initiated. Further co-localization studies demonstrated that the initial
21 static TssA clusters are then pushed towards the opposite side of the cell by the elongation of
22 the sheath structure. FLIM experiments suggested that the same TssA particle remains
23 associated to the distal extremity of the sheath. This result is also in agreement with (i) the
24 interaction of TssA with both tube (Hcp) and sheath (TssC) components, and with the higher
25 affinity of TssA for these two partners compared to the two baseplate components, suggesting
26 that the recruitment of Hcp and TssBC displaces TssA from the baseplate.

27 TssA exhibits fascinating structural characteristics. Twelve TssA proteins assemble a
28 6-fold symmetry starfish-like particle composed of a central core bearing six elongated 170-Å
29 long arms. The TssA central core, corresponding to the C-terminal domain, has a size and a
30 shape comparable to that of an Hcp hexamer (Extended Data Fig. 7o) and interacts with the
31 N-terminal gp27-like VgrG module and Hcp, two proteins whose structures have been shown
32 to be superimposable²², suggesting that TssA recognizes the same fold. The TssA arms

1 interact with TssE and TssC. TssE is the homologue of gp25, a component of the
2 bacteriophage baseplate wedges that assemble around the gp27 hub^{1,8}. Therefore the
3 positioning of the TssA central core on VgrG or Hcp allows the arms to contact the outer
4 wedges or sheath rings. Indeed, molecular docking of the TssA EM volume to the sheath
5 model in the extended conformation shows that the TssA arms are interdigitated with sheath
6 subunits resulting in very complementary shapes and tight contacts (Extended Data Fig. 8a-b).
7 Such an efficient complementarity cannot be modelled with the contracted tail sheath,
8 suggesting that TssA does not bind, or more weakly, to this conformation (Extended Data Fig.
9 8c).

10 The TssA C-terminal domain is a 6-wedge kaleidocycle in which the six wedges are
11 contacting each other by hinge-like helices at the outward of the structure. We hypothesize
12 that large conformational modifications happen, such as the displacement of these wedges to
13 the exterior, opening a large central lumen. This lumen will have a diameter (~ 90 Å)
14 sufficient to accommodate a hexameric Hcp ring. We therefore propose a functional model in
15 which TssA controls the coordinated polymerization of the tube and sheath structure (Fig. 3
16 and Supplementary Video file): the recruitment of an Hcp ring by TssA will open the lumen
17 allowing the incorporation of this Hcp ring to the growing structure. Then the TssA arms
18 might be involved in the recruitment and proper positioning of the TssBC strands around this
19 Hcp ring, before the insertion of a new Hcp ring, etc (Fig. 3a). This model implies that the
20 Hcp hexameric unit is incorporated immediately before the TssBC sheath building block, a
21 hypothesis consistent with data showing that in both bacteriophage T4 and T6SS, tube and
22 sheath grow up from the baseplate together, with the tube protein leading and directing sheath
23 assembly^{15,26}. However, the current data cannot rule out that a pre-formed Hcp-TssBC
24 complex is recruited to the growing structure (Fig. 3b). In these models, the TssA arms secure
25 the sheath under an extended state by connecting it to the rigid Hcp tube, explaining how the
26 sheath is maintained in a metastable conformation during elongation. In bacteriophages,
27 sheath contraction is initiated at the baseplate and progresses to the head. We therefore
28 propose that TssA remains attached to the distal end during T6SS sheath contraction until the
29 last TssBC row contracts to prevent energy dissipation and permit proper propulsion of the
30 Hcp tube, before being released. This model is in agreement with the docking simulations
31 showing tight contacts of TssA with the extended sheath only (Extended Data Fig. 8a-b). The
32 function of the TssA protein is therefore different from that of the distal tail proteins (Dit) of
33 *Siphoviridae* that prime tube/sheath polymerization but remain attached to the baseplate²⁷. It

1 is however closely related to the function of the gp3/gp15 proteins of *Myoviridae*. Although
2 the TssA and gp15 folds are highly divergent, the overall architecture of the central core of
3 TssA is similar in terms of size and diameter to gp15 or gp3^{28,29} (Extended Data Fig. 8d-f).
4 Interestingly, the gp3/gp15 proteins, also known as tail terminators, do not only complete tail
5 assembly: pulse-chase studies of bacteriophage T4 biogenesis demonstrated that gp15 is not
6 recruited once the tail tube/sheath has been polymerized but, conversely, that it is assembled
7 on the baseplate prior to the gp19 tube and gp18 sheath proteins⁹. However, it is found at the
8 opposite extremity of the baseplate once the tail is completed and it stabilizes the sheath²⁶.
9 This dynamic is very similar to that observed for TssA, that is first bound to the baseplate but
10 found at the distal end once the tail is completed. Taken together these data suggest that TssA
11 and gp15 may both prime, control the polymerization, complete and stabilize the T6SS and
12 bacteriophage T4 tails respectively. Finally, according to this model, the T6SS tail tube/sheath
13 is growing by the incorporation of building blocks at the distal end of the structure, a point
14 that remains to be experimentally addressed. Although the newly incorporated subunits do not
15 transit through the Hcp lumen, this mechanism is similar to that of the flagellar cap complex,
16 which binds to the hook and incorporates new flagellin subunits at the distal end by a rotary
17 mechanism³⁰. Defining the TssA conformational modifications that occur during tail
18 elongation will shed light on the molecular mechanism of T6SS tail assembly and how the
19 sheath is maintained in the extended state.

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28

1 **Supplementary Information** includes eight Extended Data Figures, two Extended Data Tables, one
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17 supervised the execution of the experiments. A.Z., E.D., Y.R.B., S.S., B.D. & M.G. performed the experiments.
18 A.Z. performed the *in vivo* experiments (BACTH, fluorescence microscopy) with the help of Y.R.B., M.G., N.F.,
19 L.J. & T.M. E.D. performed the *in vitro* experiments (protein purification and characterization, SAXS, electron
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22 **Author Information** Coordinates and structure factors have been deposited in the Protein Data Bank under
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27 **Figure 1 | *In vivo* imaging of sfGFP-TssA.** a, TssA localizes in mobile foci. Fluorescence
28 microscopy time-lapse recording of wild-type EAEC cells producing sfGFP-TssA. Individual

1 images were taken every 30 sec. The localization of TssA is indicated by the red arrowhead.
2 A schematic diagram representing the localization of TssA (from light to dark green as a
3 function of time) is shown in the inset. Mean square displacement and kymograph analyses are
4 shown in Extended Data Fig. 3a-b. Scale bar is 1 μm . **b**, TssA forms static foci in absence of
5 the TssBC sheath proteins. Fluorescence microscopy time-lapse recording of ΔtssBC cells
6 producing sfGFP-TssA . **c**, TssA is associated with the distal end of the TssBC sheath during
7 elongation. Fluorescence microscopy time-lapse recording of wild-type EAEC cells
8 producing sfGFP-TssA and $\text{TssB}_{\text{mCherry}}$. The mCherry channel (left), GFP channel (middle) and
9 merge channels (right) are shown. Individual images (from top to bottom) were taken every
10 30 sec. The initiation, polymerization and contraction/disassembly stages of the T6SS sheath
11 dynamics are indicated on the right, with a schematic diagram of the observed events. **d**, TssA
12 initial localization requires TssM but not VgrG or TssL. Fluorescence microscopy time-lapse
13 recording of the indicated ΔvgrG , ΔtssL or ΔtssM cells producing sfGFP-TssA . Individual
14 images were taken every 30 sec. The scale bar is 1 μm . Recordings of sfGFP-TssA in ΔtssE ,
15 ΔtssF , ΔtssG , ΔtssK and Δhcp cells are shown in Extended Data Fig. 3e. Large fields,
16 statistical analyses and kymographs analyses are shown in Extended Data Fig 3f, 3g and 3h
17 respectively.

18 **Figure 2 | High-resolution structures of TssA domains.** **a**, X-ray structure of the C-terminal
19 domain of TssA (TssA_{Ct}) (PDB: 4YO5). Top (left panel) and side (right panel) views of the
20 TssA_{Ct} dodecamer structure, shown in ribbon representation with each monomer differently
21 colored. The inset of the right panel shows the rainbow colored (blue to red from the N-
22 terminus) structure of one TssA_{Ct} monomer. The consecutive α -helices are numbered $\alpha 1$ to
23 $\alpha 7$. The crystal structure of TssA_{Nt2} is shown in Extended Data Fig. 6f-g. **b**, Fitting of the
24 TssA_{Ct} (red ribbon) and TssA_{Nt2} (blue ribbon) X-ray structures into the TssA EM
25 reconstruction top (top panel) or side (bottom panel) views (EMDB-3282; grey volume). The
26 scale bar is 10 nm. SAXS and negative-stain EM models of TssA are shown in Extended Data
27 Fig. 5h-j and 5p-r, respectively. The scale bar is 5 nm.

28 **Figure 3 | Model of the assembly of the Type VI secretion system.** Schematic
29 representation of the different stages of the assembly and mechanism of action of the T6SS
30 (from left to right) highlighting the role of TssA. The TssA dodecamer (red) is recruited to the
31 T6SS membrane complex. A negative-stain EM image of the TssJLM-TssA complex is
32 shown in Extended Data Fig. 2d. TssA then recruits the baseplate and initiates polymerization

1 of the tail by the incorporation of Hcp (black rectangles) and TssBC (blue rectangles)
2 building blocks (a) or the incorporation of Hcp-TssBC building blocks (b). During the
3 polymerization, TssA remains at the distal end of the structure. TssA is released after sheath
4 contraction. Docking experiments of TssA at the distal extremity of the extended and
5 contracted sheath are shown in Extended Data Fig. 8a-c. A dynamic representation of this
6 working model is shown in the Supplementary Video.

7

8 METHODS

9 **Bacterial strains, growth conditions and chemicals.** The strains, plasmids and oligonucleotides used in this
10 study are listed in the Supplementary Table. The enteroaggregative *E. coli* EAEC strain 17-2 and its $\Delta tssA$,
11 $\Delta tssBC$, $\Delta tssE$, $\Delta tssF$, $\Delta tssG$, $\Delta tssK$, $\Delta tssL$, $\Delta tssM$, Δhcp , $\Delta vgrG$, and *tssB-mCherry* isogenic derivatives were
12 used for this study^{11,15,25}. The *E. coli* K-12 DH5 α , W3110, BTH101 and T7 Iq pLys strains were used for cloning
13 steps, co-immunoprecipitation, bacterial two-hybrid and protein purification respectively. Strains were routinely
14 grown in LB rich medium (or Terrific broth medium for protein purification) or in Sci-1 inducing medium (SIM;
15 M9 minimal medium, glycerol 0.2%, vitamin B1 1 $\mu\text{g}/\text{mL}$, casaminoacids 100 $\mu\text{g}/\text{mL}$, LB 10%, supplemented
16 or not with bactoagar 1.5%) with shaking at 37°C³¹. Plasmids were maintained by the addition of ampicillin (100
17 $\mu\text{g}/\text{mL}$ for *E. coli* K-12, 200 $\mu\text{g}/\text{mL}$ for EAEC), kanamycin (50 $\mu\text{g}/\text{mL}$) or chloramphenicol (30 $\mu\text{g}/\text{mL}$).
18 Expression of genes from pBAD, pETG20A/pRSF or pASK-IBA vectors was induced at $A_{600\text{nm}} \sim 0.6$ with
19 0.02% of L-arabinose (Sigma-Aldrich) for 45 min, 0.5-1 mM of isopropyl- β -D-thio-galactopyranoside (IPTG,
20 Eurobio) for 14 hours or 0.02 $\mu\text{g}/\text{mL}$ of anhydrotetracyclin (AHT, IBA Technologies) for 45 min. respectively.
21 For BACTH experiments, plates were supplemented with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
22 (X-Gal, Eurobio, 40 $\mu\text{g}/\text{mL}$).

23 **Strain construction.** The *tssA* gene was deleted into the enteroaggregative *E. coli* 17-2 strain using a modified
24 one-step inactivation procedure³² as previously described¹¹ using plasmid pKOBEG³³. Briefly, a kanamycin
25 cassette was amplified from plasmid pKD4⁴⁶ using oligonucleotides carrying 50-nucleotide extensions
26 homologous to regions adjacent to *tssA*. After electroporation of 600 ng of column-purified PCR product,
27 kanamycin resistant clones were selected and verified by colony-PCR. The kanamycin cassette was then excised
28 using plasmid pCP20³². The deletion of *tssA* was confirmed by colony-PCR. The same procedure was used to
29 introduce the *mCherry*-coding sequence upstream the stop codon of the *tssB* gene (vector *pmCh*-KD4 as
30 template for PCR amplification) or the *super-folder GFP*-coding sequence downstream the start codon (vector
31 pKD4-*sfGFP* as template) or upstream the stop codon (vector *psfGFP*-KD4 as template) of the *tssA* gene to yield
32 strains producing TssB-mCherry, sfGFP-TssA or TssA-sfGFP from their original chromosomal loci.

33 **Plasmid construction.** All bacterial two-hybrid plasmids and the plasmid producing the TssJLM membrane
34 core complex (pRSF-TssJ^{ST-FL}TssL-^{6His}TssM, pRSF-TssJLM) have been described previously^{13,25}. Polymerase
35 Chain Reactions (PCR) were performed using a Biometra thermocycler using the Q5 (New England Biolabs) or
36 Pfu Turbo (Agilent Technologies) DNA polymerases. Restriction enzymes were purchased from New England
37 Biolabs and used according to the manufacturer's instructions. Custom oligonucleotides were synthesized by
38 Sigma Aldrich and are listed in the Supplementary Table. Enteroaggregative *E. coli* 17-2 chromosomal DNA
39 was used as a template for all PCRs. *E. coli* strain DH5 α was used for cloning procedures. All the plasmids
40 (except for pETG20A and pDEST17 derivatives) have been constructed by restriction-free cloning³⁴ as
41 previously described²⁵. Briefly, the gene of interest was amplified using oligonucleotides introducing extensions
42 annealing to the target vector. The double-stranded product of the first PCR has then been used as
43 oligonucleotides for a second PCR using the target vector as template. PCR products were then treated with
44 *DpnI* to eliminate template plasmids and transformed into DH5 α -competent cells. For protein purification,
45 the sequences encoding the full-length TssA (residues 1-542), the TssA N-terminal (residues 1-392), the TssA Nt2
46 (residues 221-377) and C-terminal (residues 393-542) domains, the N-terminal domain of VgrG (residues 1-
47 490), the full-length TssE or both TssB and TssC were cloned into the pETG-20A (TssA, TssA_{Ct}, VgrG_{Nt}, TssE)
48 or pDEST17 (TssA_{Nt}, TssBC) expression vector (kind gifts from Dr Arie Geerlof, EMBL, Hamburg) according
49 to standard Gateway protocols. Proteins produced from pETG20A derivatives are fused to an N-terminal 6 \times His-
50 tagged thioredoxin (TRX) followed by a cleavage site for the Tobacco etch virus (TEV) protease whereas
51 proteins produced from pDEST17 are fused to an N-terminal 6 \times His tag followed by a TEV protease cleavage
52 site. All constructs have been verified by restriction analyses and DNA sequencing (Eurofins or MWG).

1 **Bacterial two-hybrid assay (BACTH).** The adenylate cyclase-based bacterial two-hybrid technique³⁵ was used
2 as previously published³⁶. Briefly, the proteins to be tested were fused to the isolated T18 and T25 catalytic
3 domains of the *Bordetella* adenylate cyclase. After introduction of the two plasmids producing the fusion
4 proteins into the reporter BTH101 strain, plates were incubated at 30°C for 48 hours. Three independent colonies
5 for each transformation were inoculated into 600 μ L of LB medium supplemented with ampicillin, kanamycin
6 and IPTG (0.5 mM). After overnight growth at 30°C, 10 μ L of each culture were dropped onto LB plates
7 supplemented with ampicillin, kanamycin, IPTG and X-Gal and incubated for 16 hours at 30 °C. The
8 experiments were done at least in triplicate and a representative result is shown.

9 **Fluorescence microscopy and image treatment.** Fluorescence microscopy experiments have been performed
10 essentially as described^{13,15,21,25}. Briefly, cells were grown overnight in LB medium and diluted to an $A_{600nm} \sim$
11 0.04 into Sci-1 inducing medium (SIM). Exponentially growing cells ($A_{600nm} \sim 0.8-1$) were harvested, washed in
12 phosphate buffered saline buffer (PBS), resuspended in PBS to an $A_{600nm} \sim 50$, spotted on a thin pad of 1.5%
13 agarose in PBS, covered with a cover slip and incubated for one hour at 37°C prior to microscopy acquisition.
14 For each experiment, ten independent fields were manually defined with a motorized stage (Prior Scientific) and
15 stored (X, Y, Z, PFS-offset) in our custom automation system designed for time-lapse experiments. Fluorescence
16 and phase contrast micrographs were captured every 30 sec. using an automated and inverted epifluorescence
17 microscope TE2000-E-PFS (Nikon, France) equipped with Perfect Focus System (PFS). PFS automatically
18 maintains focus so that the point of interest within a specimen is always kept in sharp focus at all times despite
19 mechanical or thermal perturbations. Images were recorded with a CoolSNAP HQ 2 (Roper Scientific, Roper
20 Scientific SARL, France) and a 100 \times /1.4 DLL objective. The excitation light was emitted by a 120 W metal
21 halide light. All fluorescence images were acquired with a minimal exposure time to minimize bleaching and
22 phototoxicity effects. The sfGFP images were recorded by using the ET-GFP filter set (Chroma 49002) using an
23 exposure time of 200-400ms. The mCherry images were recorded by using the ET-mCherry filter set (Chroma
24 49008) using an exposure time of 100-200ms. Slight movements of the whole field during the time of the
25 experiment were corrected by registering individual frames using StackReg and Image Stabilizer plugins for
26 ImageJ. sfGFP and mCherry fluorescence channels were adjusted and merged using ImageJ
27 (<http://rsb.info.nih.gov/ij/>). For statistical analyses, fluorescent foci were automatically detected. First, noise and
28 background were reduced using the ‘Subtract Background’ (20 pixels Rolling Ball) plugin from Fiji³⁷. The
29 sfGFP foci were automatically detected by a simple image processing: (1) create a mask of cell surface and
30 dilate (2) detect the individual cells using the “Analyse particle” plugin of Fiji (3) sfGFP foci were identified by
31 the “Find Maxima” process in Fiji. To avoid false positive, each event was manually controlled in the original
32 data. Box-and-whisker representations of the number of foci per cell were made with R software. T-tests were
33 performed on R to statistically compare each population. Kymographs were obtained after background
34 fluorescence subtraction and sectioning using the Kymoreslicewise plug-in under Fiji³⁷. Sub-pixel resolution
35 tracking of fluorescent foci: Fluorescent foci were detected using a local and sub-pixel resolution maxima
36 detection algorithm and tracked over time with a specifically developed plug-in for ImageJ. The X and Y
37 coordinates were obtained for each fluorescent focus on each frame. The Mean Square Displacement (MSD) was
38 calculated as the distance of the foci from its location at $t=0$ at each time using R software and plotted over time.
39 For each strain tested, the MSD of at least 10 individual focus trajectories was calculated. For statistical analyses
40 of mobile trajectories, kymograph analyses were performed and the percentage of fixed, mobile with random
41 dynamics and mobile with unidirectional trajectory foci are reported.

42 **Fluorescence Lifetime Imaging (FLIM).** FLIM experiments were carried on the same microscope device used
43 for the time-lapse microscopy experiments except with a laser of 488 nm. For each cell a region of interest that
44 corresponds to the size of the laser beam was focused away from TssB_{mCh} sheath-labelled sfGFP-TssA for a time of
45 3 seconds at a maximum intensity of 100%. The extinction of the complete sfGFP-TssA pool was checked by (i) the
46 absence of recovery of bleached sfGFP-TssA-membrane clusters and (ii) by the overall drop and lack of recovery in
47 intracellular intensity.

48 **Protein purification.** *E. coli* T7 Iq pLysS cells bearing pETG20A or pDEST17 derivatives were grown at 37°C
49 in Terrific Broth to an $A_{600} \sim 0.9$ and gene expression was induced with 0.5 mM IPTG for 16 hours at 17°C.
50 Cells were harvested, resuspended in Tris-HCl 20 mM pH8.0, NaCl 150 mM and lysozyme (0.25 mg/mL) and
51 broken by sonication. Soluble proteins were separated from inclusion bodies and cell debris by centrifugation 30
52 min at 20,000 $\times g$. The His-tagged fusions were purified using ion metal Ni²⁺ affinity chromatography (IMAC)
53 using a 5-mL HisTrap column (GE Healthcare) and eluted with a step gradient of imidazole. The fusion proteins
54 were further digested overnight at 4°C by a Hexahistidine-tagged TEV protease using a 1:10 (w/w)
55 protease:protein ratio. The TEV protease and contaminants were retained by a second IMAC and the purified
56 proteins were collected in the flow through. Proteins were further separated on preparative Superdex 200 or
57 Superose 6 gel filtration column (GE Healthcare) equilibrated in Tris-HCl 20 mM pH 8.0, NaCl 150 mM. The
58 fractions containing the purified protein were pooled and concentrated by ultrafiltration using the Amicon
59 technology (Millipore, California, USA). The seleno-Methionine (SeMet) derivatives of TssA_{Nt} and TssA_{Ct} were
60 produced in minimal medium supplemented with 100 mg/L of Lysine, Phenylalanine and Threonine, 50 mg/L of

1 Isoleucine, Leucine, Valine and Seleno-Methionine. Gene induction and protein purification were performed as
2 described above.

3 **Limited proteolysis.** The full-length TssA protein was subjected to Proteinase K limited proteolysis (1/10
4 protease:protein ratio). The reaction was quenched at different time points by the addition of 1mM PMSF and
5 further boiling for 10 min at 96°C. Samples were analyzed by SDS-PAGE and Coomassie blue staining.
6 Digested products were identified by Edman N-terminal sequencing and electrospray mass spectrometry
7 (Proteomic platform, Institut de Microbiologie de la Méditerranée, Marseille, France).

8 **Analytical gel filtration analysis and MALS/QELS/UV/RI-coupled size exclusion chromatography.** Size
9 exclusion chromatography (SEC) was performed on an Alliance 2695 HPLC system (Waters) using KW803 and
10 KW804 columns (Shodex) run in Tris-HCl 20 mM pH 8.0, NaCl 150 mM at 0.5 mL/min. MALS, UV
11 spectrophotometry, QELS and RI were monitored with MiniDawn Treos (Wyatt Technology), a Photo Diode
12 Array 2996 (Waters), a DynaPro (Wyatt Technology) and an Optilab rEX (Wyatt Technology), respectively, as
13 described¹². Mass and hydrodynamic radius calculation were performed with the ASTRA software (Wyatt
14 Technology) using a dn/dc value of 0.185 mL/g.

15 **Surface Plasmon Resonance analysis.** Steady state interactions were monitored using a BIAcore T200 at
16 25°C¹². All the buffers were filtered on 0.2 μm membranes before use. The HC200m sensor chip (Xantech) was
17 coated with purified Hcp, VgrG, TssE or TssBC complex, immobilized by amine coupling ($\Delta RU=4000-4300$). A
18 control flow-cell was coated with thioredoxin immobilized by amine coupling at the same concentration
19 ($\Delta RU=4100$). Purified TssA, TssA_{Nt} and TssA_{Ct} (six concentrations ranging from 3.125 to 100 μM) were
20 injected and binding traces were recorded in duplicate. The signal from the control flow cell and the buffer
21 response were subtracted from all measurements. The dissociation constants (K_D) were estimated using the
22 GraphPad Prism 5.0 software on the basis of the steady state levels of ΔRU , directly related to the concentration
23 of the analytes. The K_D were estimated by plotting on X axis the different concentration of analytes and the
24 different ΔRU at a fixed time (5 s before the end of the injection step) on the Y axis. For K_D calculation,
25 nonlinear regression fit for XY analysis was used and one site (specific binding) as a model which corresponds
26 to the equation : $Y = B_{max} * X / (K_d + X)$.

27 **Co-purification experiments.** Different combinations of plasmids were transformed in BL21(DE3): (i) pRSF-
28 TssJLM + pIBA37(+), (ii) pRSF + pIBA37-^{FL}TssA, (iii) pRSF-TssJLM + pIBA37-^{FL}TssA and (iv) pRSF-TssJM
29 + pIBA37-^{FL}TssA. Transformed BL21(DE3) cells were grown at 37°C in 200 mL LB medium supplemented
30 with kanamycin and ampicillin until $A_{600} \sim 0.6$ and gene induction was achieved by the addition of IPTG (1 mM)
31 and anhydrotetracycline (0.02 μg/mL) during 15 hours at 16°C. After cell lysis through three passages at the
32 French press, total membranes were isolated as described previously¹³. Membranes were solubilized by the
33 addition of 1% Triton X100 (Affimatrix). Solubilized membrane fractions were purified on a 1 mL Streptactin
34 column (GE Healthcare). The column was washed with buffer S (Hepes 50 mM pH 7.5, NaCl 50 mM, Triton
35 X100 0.075%) and bound proteins were eluted with buffer S supplemented with desthiobiotin (2.5 mM) and
36 visualized by Coomassie blue staining and immunoblotting. For EM analyses, BL21(DE3) cells producing
37 TssJLM and FLAG-tagged TssA were grown and the TssJLM-A complex was purified as described for the
38 TssJLM membrane core complex¹³. After the two consecutive affinity columns (His- and Strep-Trap-HP), the
39 pooled fractions were injected onto a Superose 6 10:300 column equilibrated in Hepes 50 mM pH 7.5, NaCl 50
40 mM supplemented with 0.025% DM-NPG.

41 **Electron microscopy observation of the TssJLMA complex.** Nine microlitres of the purified TssJLMA
42 complex (~ 0.01 mg/mL) were incubated to glow-discharged carbon-coated copper grids (Agar Scientific) for 30
43 sec. After absorption, the sample was blotted, washed with three drops of water and then stained with 2% uranyl
44 acetate. Images were collected on an FEI Tecnai F20 FEG microscope operating at a voltage of 200 kV,
45 equipped with a direct electron detector (Falcon II) at 50,000 magnification.

46 **Transmission electron microscopy, single particle analysis and image processing.** Nine microliters of the
47 purified full-length TssA protein (~ 0.01 mg/mL) were incubated on a glow-discharged carbon-coated copper
48 grid (Agar Scientific) for 30 sec. After absorption, the sample was blotted, washed with three drops of water and
49 then stained with 2% uranyl acetate. Images were recorded automatically using the EPU software on a FEG
50 microscope operating at a voltage of 200 kV and a defocus range of 0.6–25 nm, using a FEI Falcon-II detector
51 (Gatan) at a nominal magnification of 50,000, yielding a pixel size of 1.9 Å. A dose rate of 25 electrons per Å²
52 per second, and an exposure time of 1 sec were used. A total of 100,000 particles were automatically selected
53 from 500 independent images and extracted within boxes of 180 pixels × 180 pixels using EMAN2/BOXER³⁸.
54 The CTF was estimated and corrected by phase flipping using EMAN2 (e2ctf). All two- and three-dimensional
55 (2D and 3D) classifications and refinements were performed using RELION 1.3^{39,40}. The automatically selected
56 dataset was clean up by three rounds of reference-free 2D class averaging, and highly populated classes
57 displaying high-resolution features were conserved and a final dataset of 20,000 particles was assembled. An
58 initial 3D-model was generated in EMAN2 using using 30 classes. 3D classification was then performed in
59 Relion with 5 classes. The particles corresponding to most populated class ($\sim 18,000$) were used for refinement.
60 The Relion auto-refine procedure was used to obtain a final reconstruction at ~ 19 -Å resolution after masking

1 and with D6 symmetry imposed. Reported resolutions are based on the gold-standard Fourier shell correlation
2 (FSC) 0.143 criterion, and FSC curve were corrected for the effects of a soft mask on the FSC curve using high-
3 resolution noise substitution (Extended Data Fig. 5o)⁴¹. All density maps were corrected for the modulation
4 transfer function of the detector and then sharpened by applying a negative B-factor (-1000) that was estimated
5 using automated procedures. The electron microscopy map of the EAEC TssA full-length protein has been
6 deposited in the Electron Microscopy Data Bank under accession number EMD-3282.

7 **Small-Angle X-ray Scattering analysis and *ab initio* three-dimensional shape reconstruction.** Small-Angle
8 X-ray Scattering (SAXS) analyses were performed at the ID29 beamline (European Synchrotron Radiation
9 Facility, Grenoble, France) at a working energy of 12.5 keV ($\lambda = 0.931$ Å). Thirty microlitres of protein solution
10 at 1.6, 3.7, 7.1, 9.8 and 14.9 mg/mL in Tris-HCl 20 mM pH 8.0, NaCl 150 mM were loaded by a robotic system
11 into a 2-mm quartz capillary mounted in a vacuum and ten independent 10-sec exposures were collected on a
12 Pilatus 6M-F detector placed at a distance of 2.85 m for each protein concentration. Individual frames were
13 processed automatically and independently at the beamline by the data collection software (Bsx-CUBE), yielding
14 radially averaged normalized intensities as a function of the momentum transfer q , with $q = 4\pi \sin(\theta) / \lambda$, where 2θ
15 is the total scattering angle and λ is the X-ray wavelength. Data were collected in the range $q = 0.04 - 6$ nm⁻¹. The
16 ten frames were combined to give the average scattering curve for each measurement. Data points affected by
17 aggregation, possibly induced by radiation damage, were excluded. Scattering from the buffer alone was also
18 measured prior to and after each sample analysis and the average of these two buffer measures was used for
19 background subtraction using the program PRIMUS⁴² from the ATSAS package⁴³. PRIMUS was also used to
20 perform Guinier analysis⁴⁴ of the low q data, which provides an estimate of the radius of gyration (R_g).
21 Regularized indirect transforms of the scattering data were carried out with the program GNOM⁴⁵ to obtain $P(r)$
22 functions of interatomic distances. The $P(r)$ function has a maximum at the most probable intermolecular
23 distance and goes to zero at D_{max} , the maximum intramolecular distance. The values of D_{max} were chosen to
24 fit with the experimental data and to have a positive $P(r)$ function. Tridimensionnal (3D) bead models that fit
25 with the scattering data were built with the program DAMMIF⁴⁶. Ten independent DAMMIF runs were
26 performed using the scattering profile of TssA, with data extending up to 0.35 nm⁻¹, using slow mode settings,
27 assuming P6 symmetry and allowing for a maximum 500 steps to grant convergence. The models resulting from
28 independent runs were superimposed using the DAMAVER suite⁴⁷ yielding an initial alignment of structures
29 based on their axes of inertia followed by minimisation of the normalized spatial discrepancy (NSD)⁴⁸. The NSD
30 was therefore computed between a set of ten independent reconstructions, with a range of NSD from 0.678 to
31 0.815. The aligned structures were then averaged, giving an effective occupancy to each voxel in the model, and
32 filtered at half-maximal occupancy to produce models of the appropriate volume that were used for all
33 subsequent analyses. All the models were similar in terms of agreement with the experimental data, as measured
34 by DAMMIF χ parameter and the quality of the fit to the experimental curve (calculated SQRT Chi value =
35 1.774). The SAXS data parameters are provided in Extended Data Table 1.

36 **Crystallization, data collection, processing and refinement.** Seleno-methionine (SeMet)-labelled TssA_{Nt2} and
37 TssA_{Ct} crystallization trials were carried out by the sitting-drop vapor diffusion method in 96-well Greiner
38 crystallization plates at 20°C, using a nanodrop-dispensing robot (Cartesian Inc.). Crystals of SeMet-labelled
39 TssA_{Ct} grew in a few days after mixing 300 nL of protein at 4.7 mg/mL with 100 nL of 20% PEG 8000, 0.2M
40 Calcium Acetate, 0.1 M MES pH 6.8. Crystals of SeMet-labelled TssA_{Nt} grew in a few days after mixing 300 nL
41 of protein at 4.7 mg/mL with 100 nL of 29% PEG 3350, 0.1 M Hepes pH 7.5. Crystals were cryoprotected with
42 mother-liquor supplemented with 20 % polyethylene glycol and flash frozen in liquid nitrogen. Datasets were
43 collected at the SOLEIL Proxima 1 beamline (Saint-Aubin, France). After processing the data with XDS⁴⁹, the
44 scaling was performed with SCALA and the structures were solved using the SHELXD program⁵⁰. The structure
45 was refined with AutoBUSTER⁵¹ alternated with model rebuilding using COOT⁵². The final data collection and
46 refinement statistics are provided in Extended Data Table 2. The Ramachandran plots of the TssA_{Nt} and TssA_{Ct}
47 structures exhibit 90.7/3.3 and 91.8/2.9 residues in the favored and outlier areas, respectively. Figures were made
48 with PyMOL⁵³.

49 **Tail sheath modeling and TssA docking to contracted and extended sheaths.** The tail sheath modelling was
50 performed using the *Vibrio cholerae* VipAB (TssBC) complex as starting structure²³ (PDB: 3J9G) and the
51 contracted tail sheath structures of *Vibrio cholerae*²³. To date, however, the molecular structure of the extended
52 (non contracted) sheath is not available. In a recent paper a low resolution model of the extended VipAB sheath
53 was modelled using the low resolution EM map of the extended T4 phage tail sheath²². By superimposing the
54 VipAB EM map to the gp18 bacteriophage T4 sheath protein structure, gross features of the sheath structure
55 were obtained²². A similar approach was applied with Chimera⁵⁴ using the VipAB molecular model in the
56 extended T4 phage tail sheath instead of using the low resolution VipAB EM map, yielding a model similar to
57 that of Kube et al.²², but with molecular details. The sheath internal channel diameter shrinks from 110 to ~95 Å
58 diameter, and the external diameter from ~290 Å to ~190 Å. The internal diameter of the tail sheath makes it
59 possible to fit stacked Hcp hexamers that are in contact with the tail sheath internal wall. Both extended and
60 contracted tail sheath conformations were used to explore the faisability of sheath complexes with TssA using its

1 EM map. TssA being at the distal end of the sheath, the polarity of the sheath was taken into consideration. It
2 was suggested that the polarity of T6SS tail sheath is similar to that of bacteriophage T4 and therefore that the
3 VipA (TssB) N-terminal and VipB (TssC) C-terminal helices point to and contact the baseplate³¹. TssA was
4 therefore docked at the opposite extremity of the tail sheath using Chimera⁵⁴.
5 **Miscellaneous.** Hcp release^{11,25} and fractionation assays^{11,19,25} have been performed as previously described.
6 SDS-Polyacrylamide gel electrophoresis was performed using standard protocols. For immunostaining, proteins
7 were transferred onto 0.2 µm nitrocellulose membranes (Amersham Protran), and immunoblots were probed
8 with primary antibodies, and goat secondary antibodies coupled to alkaline phosphatase, and developed in
9 alkaline buffer with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. The anti-TolB polyclonal
10 antibodies are from our laboratory collection, while the anti- FLAG (M2 clone, Sigma Aldrich) and anti-EFTu
11 (Roche) monoclonal antibodies and alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse secondary
12 antibodies (Beckman Coulter) have been purchased as indicated.

13 **Accession numbers.** Coordinates and structure factors have been deposited in the Protein Data Bank under
14 accession numbers 4YO3 and 4YO5 for TssA_{N12} and TssA_{Ct} respectively. Electron microscopy map for full-
15 length TssA has been deposited in the Electron Microscopy Databank (EMDB) under accession code EMD-3282.
16

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18

19 **Legend to Extended Data**

20

21 **Extended Data Table 1 | SAXS parameters of TRX-TssA.**

22 **Extended Data Table 2 | Data collection, phasing and refinement statistics for SAD (SeMet)** 23 **structures.**

24 **Extended Data Figure 1 | Hcp and TssC interact with TssA, a cytoplasmic protein required for**
25 **sheath assembly and Hcp release. a**, Schematic representation of the architecture of the bacterial
26 Type VI secretion system. The scheme highlights the membrane complex anchoring the tail structure
27 composed of the assembly baseplate, the spike, the tube and the sheath (cyto, cytoplasm; IM, inner
28 membrane; PG, peptidoglycan layer; OM, outer membrane). **b**, Bacterial two-hybrid assay. BTH101
29 reporter cells producing the indicated proteins or domains (TssLc, cytoplasmic domain of the TssL
30 protein; TssMc and TssMp, cytoplasmic and periplasmic domain of the TssM protein respectively)
31 fused to the T18 or T25 domain of the *Bordetella* adenylate cyclase were spotted on plates
32 supplemented with IPTG and the chromogenic substrate X-Gal. Interaction between the two fusion
33 proteins is attested by the dark blue color of the colony. The TolB-Pal interaction serves as a positive
34 control. **c**, The absence of TssA prevents T6SS sheath dynamics. Fluorescence microscopy time-lapse
35 recordings showing sheath dynamics using the chromosomally-encoded *tssB-mCherry* fusion in WT
36 (*tssB_{mCherry}* pBAD33), $\Delta tssA$ ($\Delta tssA$ *tssB_{mCherry}* pBAD33) and complemented $\Delta tssA$ ($\Delta tssA$ *tssB_{mCherry}*
37 pBAD33-TssA_{VSV-G}) cells. Individual images were taken every 30 sec. Assembly and
38 contraction/disassembly events are indicated above the time-lapse images. The scale bars are 1 μ m. **d**,
39 The absence of TssA prevents Hcp release. Hcp release was assessed by separating whole cells (C)

1 and supernatant (SN) fractions from 17-2 (WT), $\Delta tssA$ ($\Delta tssA$ pBAD33, *tssA*) and complemented
2 $\Delta tssA$ ($\Delta tssA$ pBAD33-TssA_{VSV-G}, *tssA*^{WT}) cells producing FLAG epitope-tagged Hcp. A total of 1×10^8
3 cells and the TCA-precipitated material from the supernatant of 2×10^8 cells were analysed by Western
4 blot using anti-FLAG monoclonal antibody (lower panel) and anti-TolB polyclonal antibodies as a
5 lysis control (upper panel). The molecular weight markers (in kDa) are indicated on the left. The
6 uncropped scans of the western blots are provided in the Supplementary Figure. **e**, TssA co-
7 fractionates with cytoplasmic and membrane proteins. A fractionation procedure was applied to
8 EAEC $\Delta tssA$ cells producing FLAG-tagged TssA. Whole cells (T) were fractionated to isolate the
9 supernatant (SN), periplasmic (P), cytoplasmic (C) and total membrane (M) fractions. Extracts from
10 10^9 (T) or 2×10^9 (SN, P, C, M) cells were separated by SDS-PAGE and immunodetected with anti-
11 FLAG monoclonal (TssA), anti-EF-Tu (cytoplasmic marker) and TolB (periplasmic marker)
12 antibodies. The molecular weight markers (in kDa) are indicated on the left. The uncropped scans of
13 the western blots are provided in the Supplementary Figure.

14 **Extended Data Figure 2 | Purification and negative-stain electron microscopy analyses of the**
15 **TssJLM-TssA complex.** **a**, TssA interacts with the TssJM complex. The total solubilized membrane
16 extract (T) of 4×10^9 cells producing the indicated proteins was subjected to affinity chromatography
17 using Streptactin resin. Bound proteins (E) were separated by SDS-PAGE and immunodetected with
18 anti-FLAG (TssA and TssL), anti-Streptag (TssJ) and anti-5His (TssM) monoclonal antibodies. The
19 molecular weight markers are indicated on the left. The uncropped scans of the western blots are
20 provided in the Supplementary Figure. **b**, Superose 6 10/300 gel filtration profile of the purified
21 TssJLM-TssA complex. The asymmetry of the peak probably reflects the co-purification of different
22 complexes or the dissociation of TssA from the TssJLM complex. **c**, Examples of representative raw
23 particles observed for the purified TssJLM-TssA complex sample using negative-stain electron
24 microscopy. A typical TssJLM complex is shown in red (number of particles observed $n=240$)
25 whereas a TssA-bound TssJLM complex is shown in white ($n=95$). Scale bar is 10 nm. **d**,
26 Magnification of the two complexes shown in panel c. Scale bar is 10 nm.

27 **Extended Data Figure 3 | TssA localization and dynamics.** **a**, Mean square displacement (MSD; in
28 arbitrary units (a.u)) of a representative _{sfGFP}TssA clusters in a wild-type strain (red line) or its $\Delta tssBC$
29 isogenic derivative (black line) were measured by sub-pixel tracking of fluorescent foci and plotted
30 over time (in sec). **b**, Kymographic analysis reporting representative _{sfGFP}TssA (green) and TssB_{mCherry}
31 (red) positions within the cell as a function of time. **c**, Representative Fluorescence lifetime imaging
32 microscopy (FLIM) of _{sfGFP}TssA clusters in the _{sfGFP}TssA-TssB_{mCh} strain. A membrane-associated
33 _{sfGFP}TssA cluster was chosen to define the bleached area (red circle). The laser (488 nm) was set to
34 maximum power and focused for 3 s to ensure complete bleaching of the GFP diffusible pool. Images
35 were taken every 30 s to follow recovery dynamics. The scale bar is 1 μ m. **d**, Quantification of

1 $_{sfGFP}TssA$ fluorescence dynamics over time after bleaching. The dynamics of fluorescence intensity is
2 shown over time for $n=10$ independent $_{sfGFP}TssA$ foci after FLIM (blue line). The fluorescence
3 intensity of the bleached focus was also followed over time (FRAP, red line). As a control for laser
4 focussing and intensity, membrane-associated clusters were systematically bleached in these
5 experiments and showed no recovery suggesting the total intracellular $_{sfGFP}TssA$ has been bleached by
6 the laser. **e**, Representative fluorescence microscopy time-lapse recordings of the indicated $\Delta tssK$,
7 $\Delta tssE$, $\Delta tssF$, $\Delta tssG$ or Δhcp cells producing $_{sfGFP}TssA$. Individual images were taken every 30 sec.
8 Red arrowheads indicate the localizations of TssA foci. The scale bar is 1 μm . **f**, Representative large
9 fields of fluorescence microscopy analyses showing localization of $_{sfGFP}TssA$ in the indicated strains.
10 The scale bars are 1 μm . **g**, Box-and-whisker plots of the measured number of $_{sfGFP}TssA$ foci per cell
11 for each indicated strain. The lower and upper boundaries of the boxes correspond to the 25% and
12 75% percentiles respectively. The black bold horizontal bar represents the median values for each
13 strain and the whiskers represent the 10% and 90% percentiles. Outliers are shown as open circles. A
14 Student *t*-test was used to report significant differences (ns, non significant; ***, $p < 0.0001$). The
15 number of cells studied per strain (n) is indicated on top. **h**, Statistical analyses of $_{sfGFP}TssA$ dynamics.
16 $_{sfGFP}TssA$ dynamics were categorized as ‘fixed’, ‘mobile with unidirectional trajectory’ and ‘mobile
17 with random dynamics’ and the number of $_{sfGFP}TssA$ (n , on top) foci in each category is represented as
18 percentage for each indicated strain. Kymographs for the two first categories are shown on bottom. **i**,
19 Schematic representation of the assembly pathway of the T6SS based on this study and available
20 data^{13,15,19,24,25,55}. The biogenesis starts with the initial positioning of the TssJ outer membrane
21 lipoprotein and the sequential recruitment of the indicated subunits (from left to right). The
22 recruitment of TssA is dependent on TssM, and that of TssK is dependent on both TssL and TssA. The
23 exact positions of VgrG and TssE (blue) in the pathway are not known but these two subunits are not
24 required for TssA recruitment but necessary for Hcp and TssBC polymerization.

25 **Extended Data Figure 4 | TssA interacts with tail and baseplate components.** **a**, TssA interaction
26 network identified by bacterial two-hybrid analysis (see legend to Extended Data Fig. 1b). **(b-e)**
27 Surface Plasmon Resonance interaction study of TssA with its partners identified by BACTH.
28 Sensorgrams (variation of Plasmon Resonance in arbitrary unit (ΔRU) as a function of reaction time
29 (in sec)) were recorded upon injection of the purified native TssA protein (concentrations of 3.125
30 (dark grey), 6.25, 12.5, 25, 50 and 100 (light grey) μM) on HC200m chips coated with the purified N-
31 terminal domain of VgrG (**b**), purified TssE (**c**), Hcp (**d**) or TssBC complex (**e**) (upper panels). The
32 graph reporting ΔRU as a function of the TssA concentration (lower panel) was used to estimate the
33 indicated apparent dissociation constants (K_D). Off-rates (percentage of dissociation 400 s after ligand
34 injection) are indicated.

1 **Extended Data Figure 5 | TssA oligomerization and SAXS and EM structural models.** **a**, 10 μg of
2 purified TssA were analysed by SDS-PAGE and Coomassie blue staining. The molecular weight
3 markers (in kDa) are indicated on the left, and TssA and its theoretical size are indicated on the right.
4 **b**, Superose 6 10/300 gel filtration profile of purified TssA (black line) and protein markers of known
5 size (colored lines). **c**, MALS/QELS/UV/RI analysis of purified TssA. The molecular mass of the
6 TssA complex is indicated. **d-j**, Low-resolution SAXS model of TssA. **d**, Experimental scattering data
7 calculated from an *ab initio* model of TssA. The square root χ value of the ‘best representative’ model
8 is indicated. **e**, Representation of the Guinier plot calculated from the experimental curve. **f**, Pair
9 distance distribution. **g**, Kratky plot representative of a multi-domain protein with flexible linkers. **h-j**,
10 SAXS envelope of the “best representative” model of TssA, with top (**h**), side (**i**) and tilted (**j**) views.
11 The scale bar is 100 \AA . **k-r**, Low resolution EM model of TssA. **k**, Representative micrograph of the
12 dataset used for image processing. White circles indicate isolated TssA dodecamers. **l**, Representative
13 selected TssA particles. **m-n**, Gallery of representative top (**m**) and side (**n**) class averages generated
14 after reference free 2D classification using Relion³⁹. **o**, Fourier Shell Correlation (FSC) curve of the
15 TssA reconstruction. The Gold standard FSC curve was calculated in Relion³⁹ using the masked
16 reconstruction of TssA. **p-r**, Top (**p**), side (**q**) and tilted (**r**) views of the tri-dimensional reconstruction
17 model of the TssA dodecamer obtained by electron microscopy (accession number: EMD-3282). The
18 scale bar is 50 \AA . Whereas the SAXS model allows to better visualize the arm length compared to the
19 EM reconstruction, its low resolution impairs the visual separation of the dimeric arms.

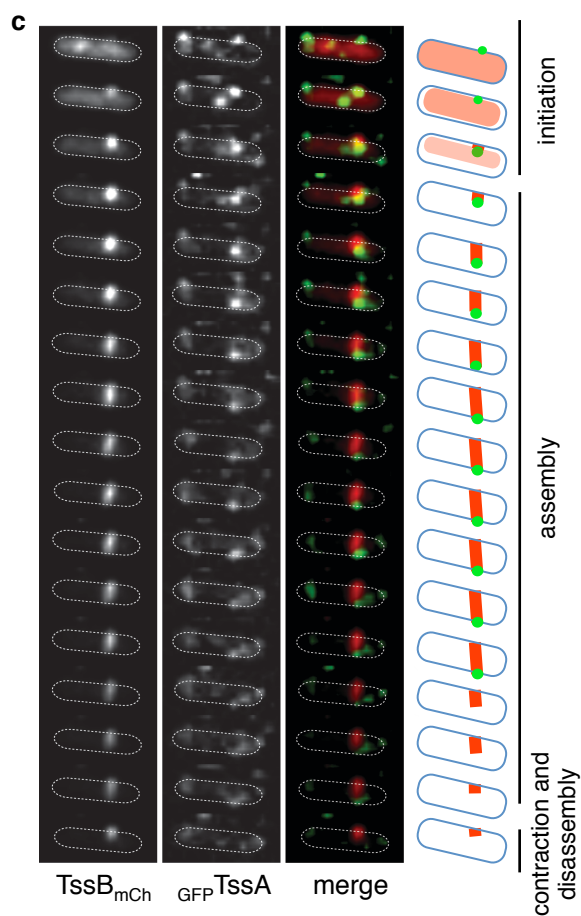
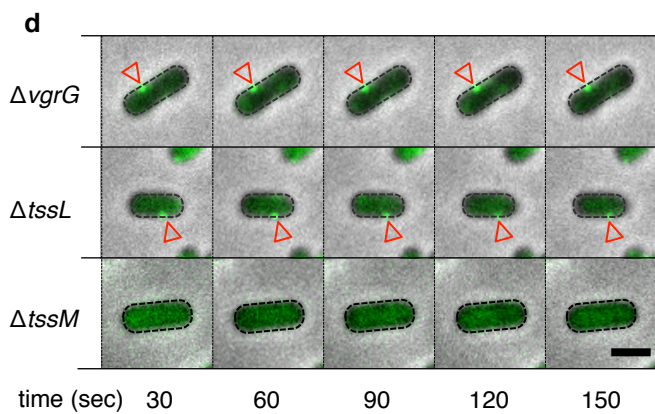
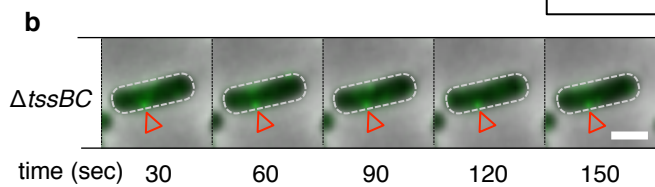
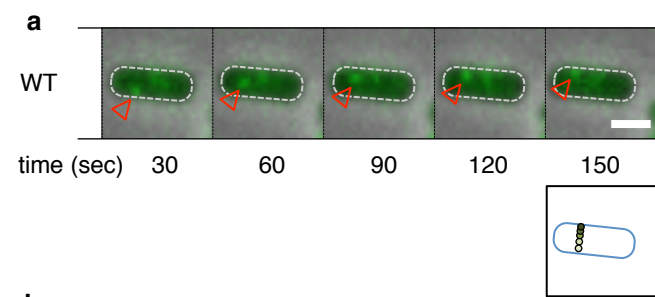
20 **Extended Data Figure 6 | Identification, oligomerisation and interaction analysis of TssA**
21 **domains.** **a**, Limited proteolysis of purified TssA. The purified full-length TssA protein (first lane)
22 was submitted to proteinase K limited proteolysis for the time indicated on top of each lane and
23 analysed by SDS-PAGE and Coomassie blue staining. Stable fragments are indicated on the right with
24 their boundaries (numbers identified in the sequence in panel **b**) and the corresponding fragment. The
25 uncropped scan of the Coomassie blue stained gel is provided in the Supplementary Figure. **b**, TssA
26 protein sequence. The localisation of the boundaries of the stable fragments obtained after Proteinase
27 K limited proteolysis and electrospray mass spectrometry analyses are arrowed. The secondary
28 structures observed in the crystal structures (Figure 2a and Extended Data Fig. 6f-g) are indicated on
29 top of the corresponding sequence. **c**, Bacterial two-hybrid analysis of TssA_{Nt} and TssA_{Ct} interactions
30 (see legend to Extended Data Fig. 1b). **d-e**, MALS/QELS/UV/RI analysis of the purified TssA_{Nt} (**d**)
31 and TssA_{Ct} (**e**) fragments. **f-g**, X-ray structure of the TssA_{Nt2} domain (PDB: 4YO3). The rainbow
32 colored ribbon representation of the TssA_{Nt2} monomer is shown (**f**, consecutive α -helices numbered
33 $\alpha 1$ to $\alpha 7$) whereas the dimeric structure (**g**) highlights the helices at the interface ($\alpha 1$, $\alpha 2$ and $\alpha 6$). **h**,
34 The TssA central core interacts with Hcp and VgrG whereas the TssA arms interact with TssE and
35 TssC. Bacterial two-hybrid analysis of TssA_{Nt} and TssA_{Ct} interactions (see legend to Extended Data
36 Fig. 1b). **(i-j)** Surface Plasmon Resonance interaction study of the purified TssA_{Ct} (**i**) or TssA_{Nt} (**j**)

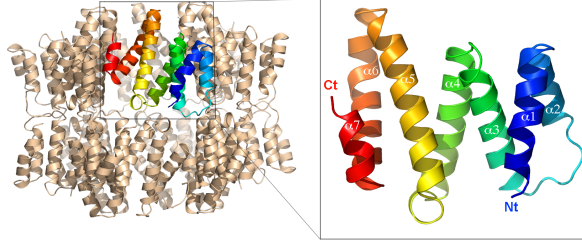
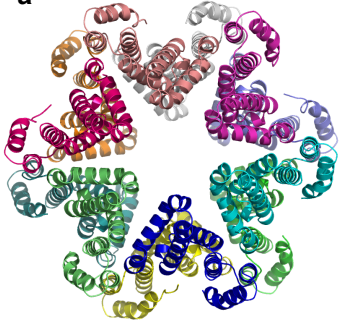
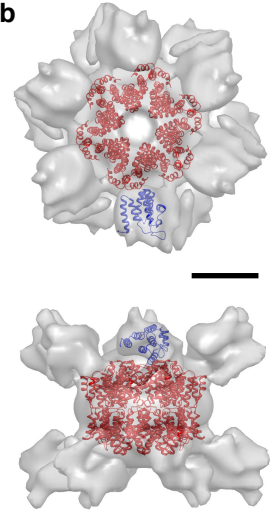
1 domains with the Hcp protein (**i**) or the TssBC complex (**j**). Sensorgrams (variation of Plasmon
2 Resonance in arbitrary unit (Δ RU) as a function of reaction time (in sec)) were recorded upon
3 injection of the purified TssA_{Ct} (**i**) or TssA_{Nt} (**j**) domains (concentrations of 3.125 (dark grey), 6.25,
4 12.5, 25, 50 and 100 (light grey) μ M) on HC200m chips coated with the purified Hcp protein (**i**) or the
5 TssBC complex (**j**) (upper panels). The graph reporting Δ RU as a function of the TssA domain
6 concentration (lower panel) was used to estimate the indicated apparent dissociation constants (K_D).

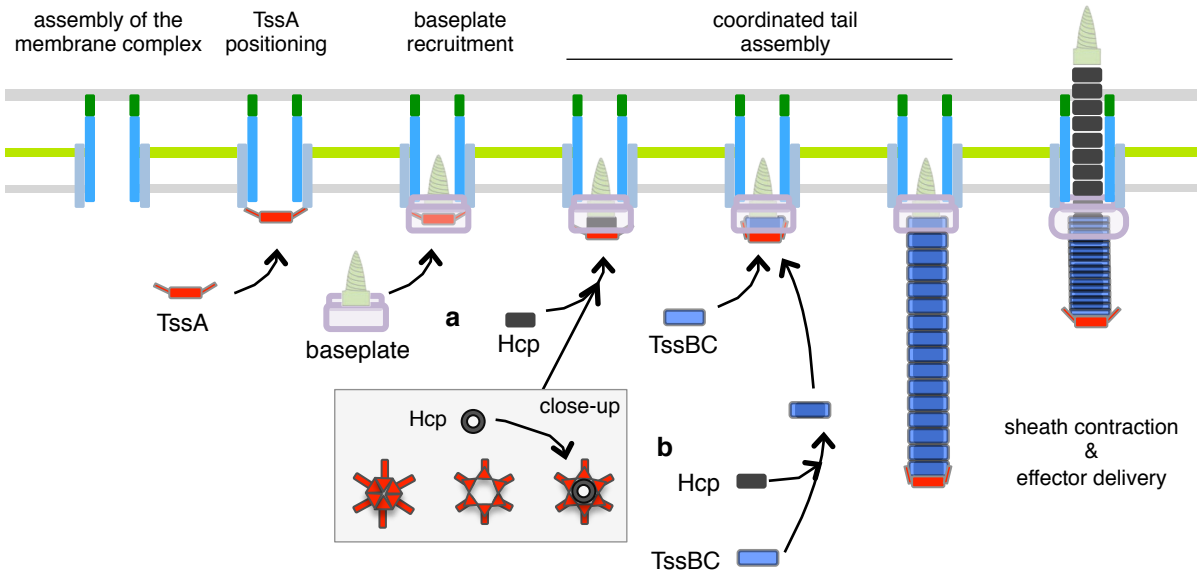
7 **Extended Data Figure 7 | Comparison of the SAXS, EM and X-ray structures of TssA. a,**
8 Schematic representation and color code of the constructs used for SAXS (grey), electron microscopy
9 (light blue) and X-ray (TssA_{Nt2}, dark blue; TssA_{Ct}, red) analyses. The epitopes and theoretical
10 molecular masses of the domains are indicated. TRX, thioredoxine; *N*, N-terminus; *C*, C-terminus. **b,**
11 Fit between the experimental data (green dots) and the calculated scattering curves for TssA_{Nt2} and
12 TssA_{Ct} generated by CRY SOL (red line). **c-f,** SAXS/X-ray comparison. Top (**c**), side (**d**) and bottom
13 (**f**) views of the fitting of TssA_{Nt2} (bleu ribbon) and TssA_{Ct} (red ribbon) X-ray structures into the TssA
14 SAXS envelope (transparent grey surface). Scale bars are 10 nm. Panel **e** is a magnification of a cut-
15 away section of the fitting shown in **d**. Scale bar is 5 nm. **g-i,** SAXS/EM/X-ray comparison. Top (**g**)
16 and side (**h**) views of the superimposition of SAXS (grey surface), EM (transparent light-bleu surface)
17 and X-ray structures of TssA. Scale bars are 10 nm. Panel **i** is a magnification of a cut-away section of
18 the superimposition shown in **h**. **j-n,** EM/X-ray comparison. Top (**j**), side (**k**) and bottom (**l**) views of
19 the fitting of TssA_{Nt2} (bleu ribbon) and TssA_{Ct} (red ribbon) X-ray structures into the TssA EM
20 envelope (transparent grey surface). Scale bars are 10 nm. Panels **m** and **n** are magnifications of the
21 top and bottom views of the docking of the TssA domain X-ray structures into the TssA EM map
22 highlighting the interface between the TssA central core (TssA_{Ct}, red ribbon) and arms (TssA_{Nt2}, blue
23 ribbon). The C-terminal helix of TssA_{Nt2} (ends at position 377) and N-terminal helix of TssA_{Ct} (starts
24 at position 395) are shown in yellow. **o,** Top view of the fitting of the X-ray structure of EAEC Hcp
25 (green ribbon, PDB 4HKH⁵⁶) into the TssA SAXS envelope (grey surface). The scale bar is 10 nm.

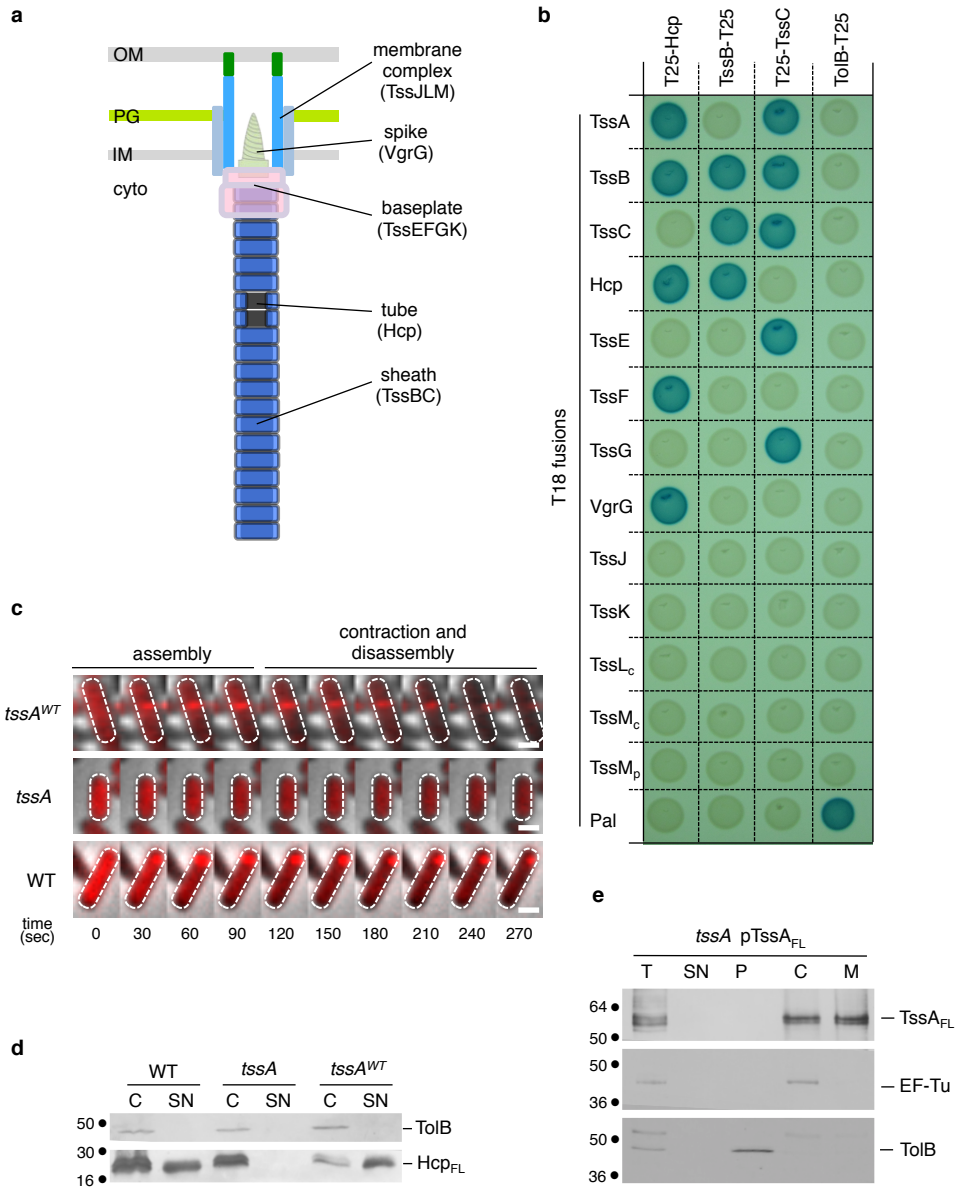
26 **Extended Data Figure 8 | Models of tail sheath-TssA complexes and comparison between the**
27 **bacteriophage T4 gp15 and T6SS TssA subunits. a-b,** Surface (**a**) and cross-section (**b**) views of the
28 complex of TssA (EM map, blue) with the extended tail sheath model (the last four rows shown in
29 different colors). In the cut-away section, four stacks of Hcp rings are visible. As shown by bacterial
30 two-hybrid and SPR analyses, Hcp contacts the TssA_{Ct} central core whereas TssBC contacts the
31 TssA_{Nt} arms. The TssA arms fit between the TssBC monomers of the last row. **c,** Surface view of the
32 complex of TssA (EM map, blue) with the contracted tail sheath model (the last four rows shown in
33 different colors), highlighting the loose packing between TssA and the tail sheath in this conformation,
34 suggesting that TssA might dissociate after sheath contraction. **d-f,** comparison between the
35 bacteriophage T4 gp15 and T6SS TssA subunits. **d-e,** Schematic representations of the bacteriophage

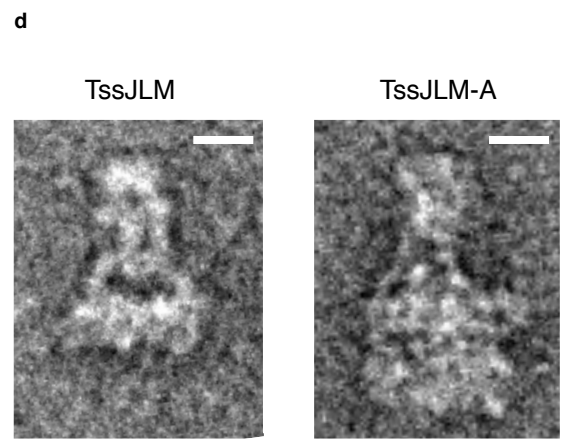
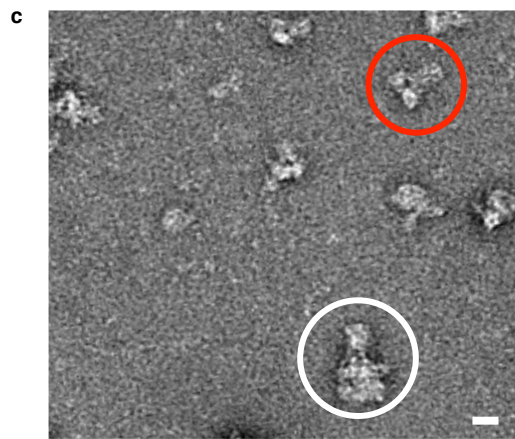
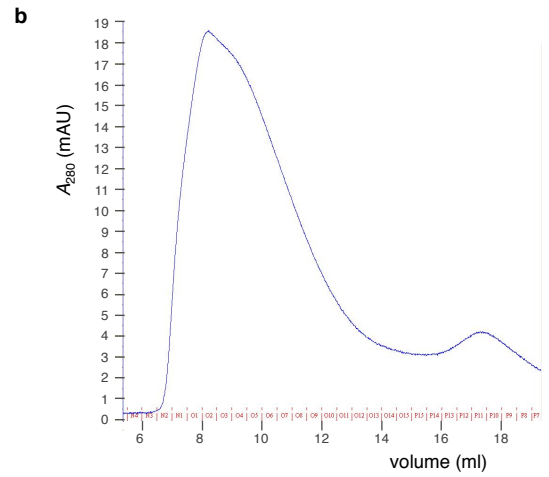
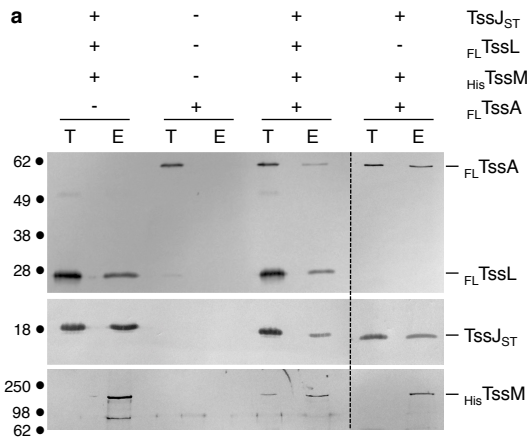
1 T4 tail distal end comprising the gp19 tube (grey) and gp18 sheath (blue) proteins and the gp3 (green)
2 and gp15 (red) neck proteins (**d**) and the T6SS tail distal end comprising the Hcp tube (grey) and
3 TssBC sheath (blue) proteins and the TssA dodecamer (red) (**e**). The possibility that a functional
4 homologue of bacteriophage T4 gp3 exists is shown by the question mark. **f**, Fitting of the model of
5 the gp15 structure in complex with the last row of the gp18 sheath (in purple)²⁹ in the TssA SAXS
6 envelope (grey surface).

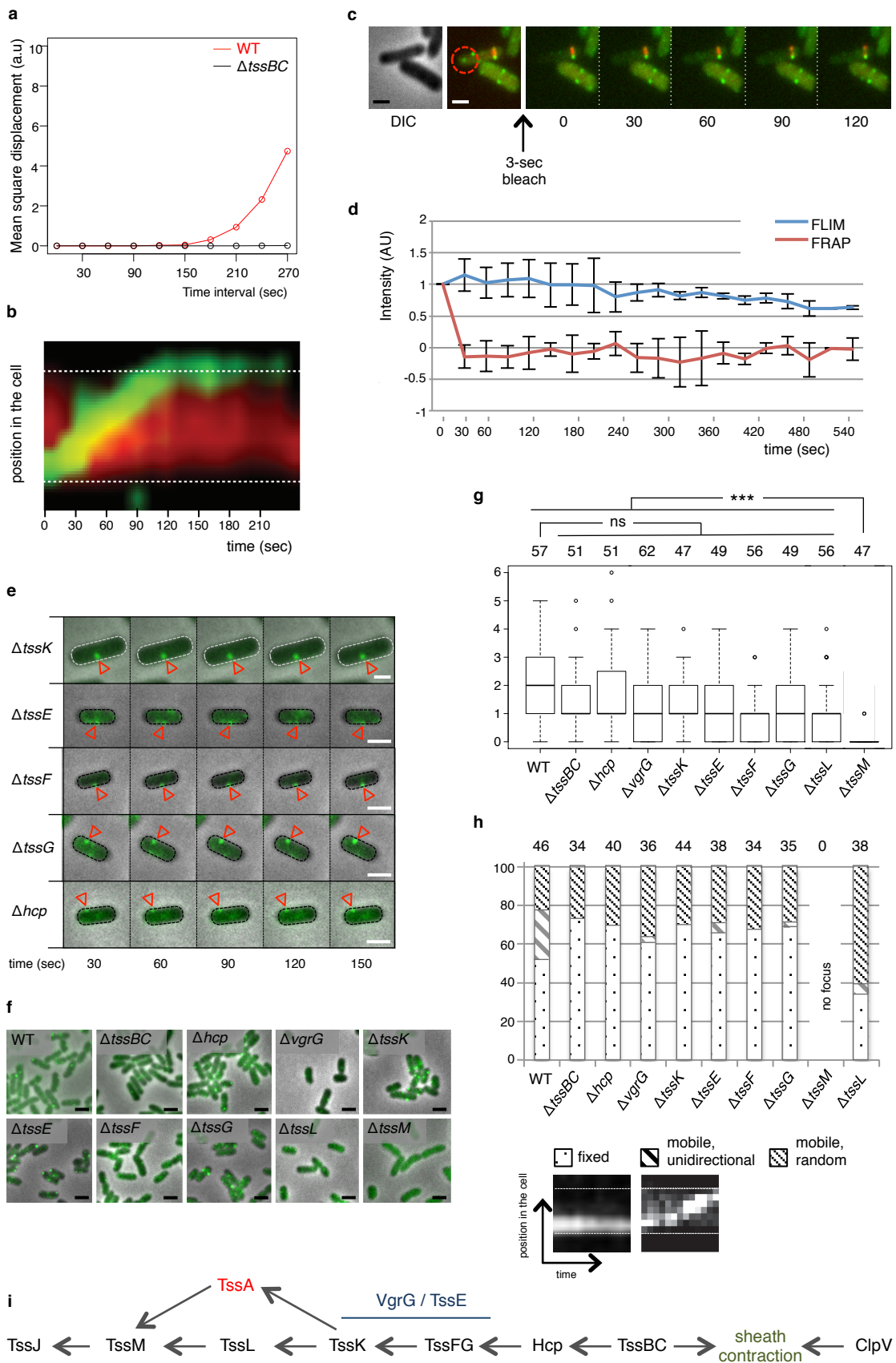


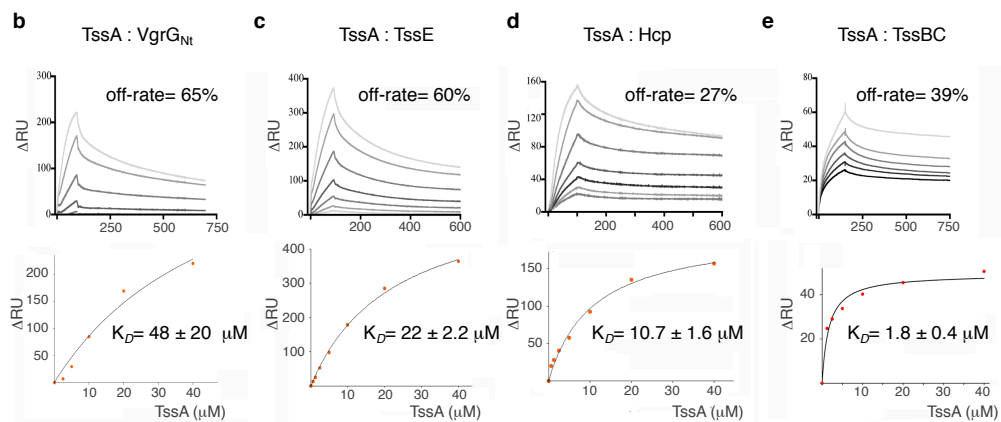
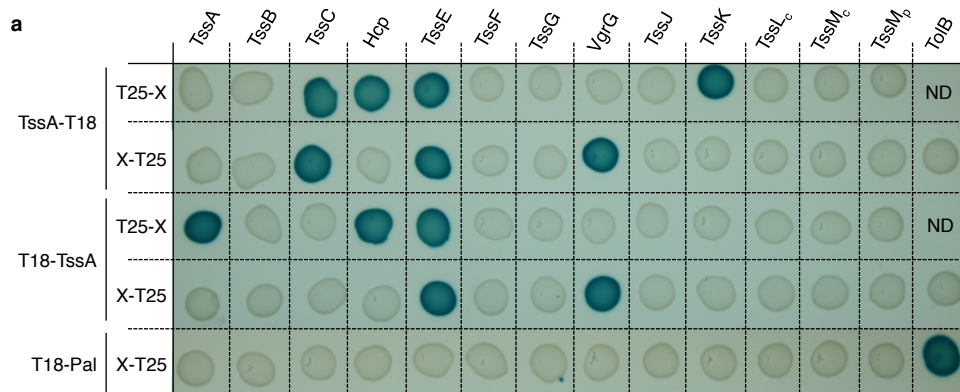
a**b**

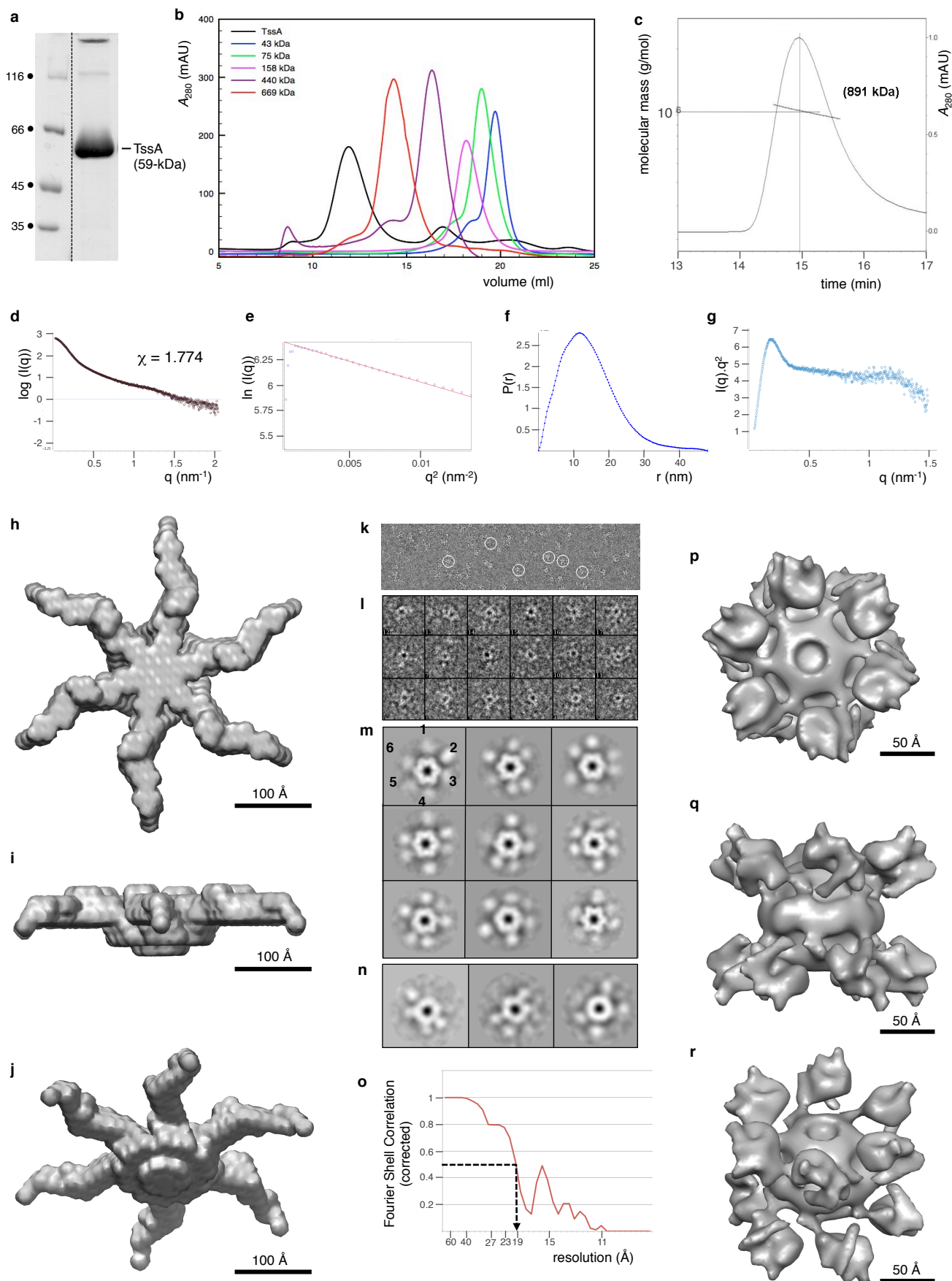


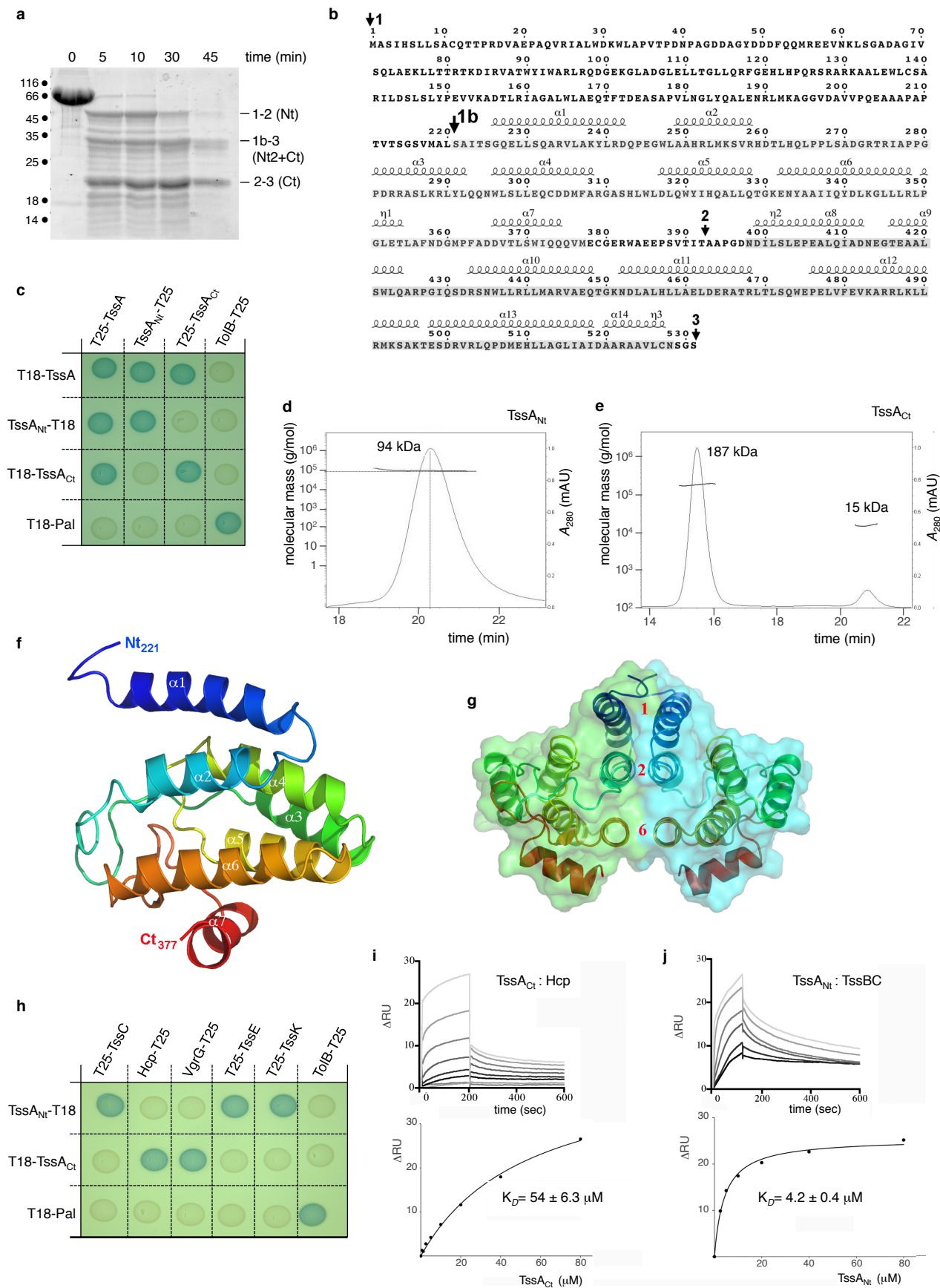




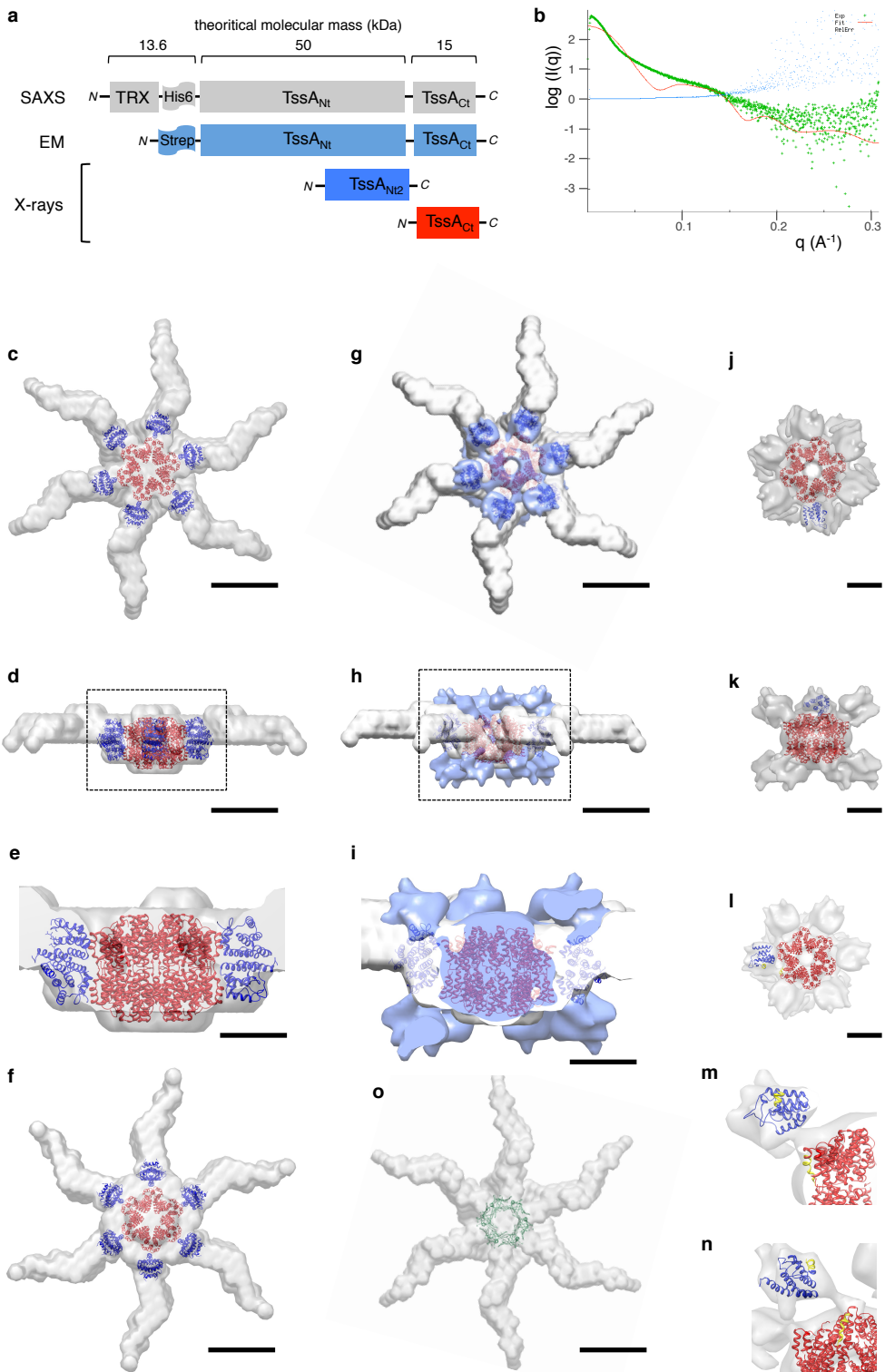


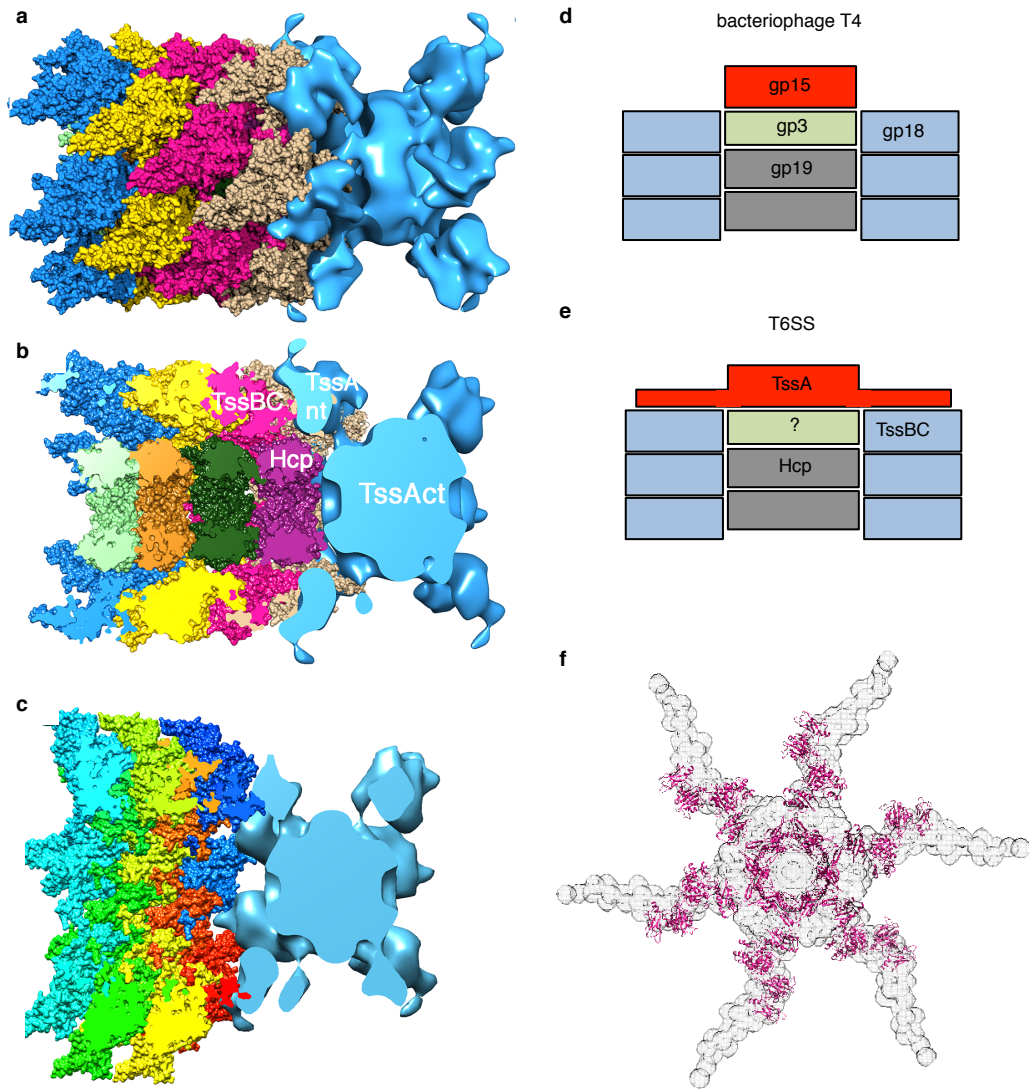






Extended data Fig. 6





Extended Data Table 1 SAXS data parameters of ^{TRX}TssA

Facilities and parameters	Settings and values
Data Collection Parameters	
Beam line	ESRF (Grenoble, France) BM29
Wavelength (Å)	0.992
Detector	Pilatus 1M
q range (nm ⁻¹)	0.028-4.525
Exposure time (s)	1 (10 × 10 sec)
Concentration range (mg/ml)	1.6-14.9
Temperature (°C)	20
Structural Parameters	
R_g from Guinier fitting (Å)	107.1 ± 0.25
$I(0)$ from Guinier fitting	615.49 ± 1.16
R_g from GNOM (Å)	114.5 ± 0.055
$I(0)$ from GNOM	629.7 ± 1.54
Dmax (Å)	420 ± 0.093
V_{porod} from PRIMUS (Å ³)	3081.4 × 10 ³
MW ^{pred} (kDa) ^a	891
MW ^{SEC-MALS} (kDa) ^b	952
MW ^{SAXS} (kDa) ^c	629
NSD of DAMMIF models	0.678-0.815
Software Employed	
Primary Data Processing	PRIMUS
$P(r)$	GNOM
<i>Ab initio</i> Shape Analysis	DAMMIF
Validation and averaging	DAMAVR
SAXS Profile computation	CRYSOL
Molecular Visualization	CHIMERA; PyMol

^a MW^{pred}, theoretical mass of the ^{TRX}TssA protein.

^b MW^{SEC-MALS}, measured from the SEC-MALS experiment.

^c MW^{SAXS}, calculated from the $I(0)$.

Extended Data Table 2 Data collection, phasing and refinement statistics for SAD (SeMet) structures

	TssA _{N12} (PDB: 4YO3) ^a	TssA _{C1} (PDB: 4YO5) ^a
Data collection		
Space group	P2 ₁ 2 ₁ 2	P2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	134.1, 178.0, 106.8	112.3, 90.6, 120.0
α , β , γ (°)	90, 90, 90	90, 102, 90
	<i>Peak</i>	<i>Peak</i>
Wavelength	0.987	0.987
Resolution (Å)*	28.4-3.37 (3.47-3.37)	46.2-3.35 (3.45-3.35)
<i>R</i> _{merge} *	0.15 (0.72)	0.12 (0.76)
<i>I</i> / σ <i>I</i> *	11.4 (2.3)	8.2 (2.0)
Completeness (%)*	99.7 (99.9)	99 (99)
Redundancy*	7.8 (7.9)	4 (4)
Refinement		
Resolution (Å)*	28.4-3.37 (3.47-3.37)	46.2-3.35 (3.45-3.35)
No. reflections*	36772 (2999)	33971 (2906)
<i>R</i> _{work} / <i>R</i> _{free} *	25.1/26.1 (27.6/28.5)	22.6/26.0 (25.1/27.1)
No. atoms		
Protein	14496	12298
Ligand/ion	0	0
Water	0	0
B-factors		
Protein	111	103
Ligand/ion		
Water		
R.m.s deviations		
Bond lengths (Å)	0.01	0.01
Bond angles (°)	1.18	1.17

^a one crystal used for data collection

* highest resolution shell is shown in parenthesis.