Atomic structures of an entire contractile injection system in both the extended and contracted states

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Contractile injection systems are sophisticated multiprotein nanomachines that puncture target cell membranes. Although the number of atomic-resolution insights into contractile bacteriophage tails, bacterial type six secretion systems and R-pyocins is rapidly increasing, structural information on the contraction of bacterial phage-like protein-translocation structures directed towards eukaryotic hosts is scarce. Here, we characterize the antifeeding prophage AFP from Serratia entomophila by cryo-electron microscopy. We present the high-resolution structure of the entire AFP particle in the extended state, trace 11 protein chains denovo from the apical cap to the needle tip, describe localization variants and perform specific structural comparisons with related systems. We analyse inter-subunit interactions and highlight their universal conservation within contractile injection systems while revealing the specificities of AFP. Furthermore, we provide the structure of the AFP sheath–baseplate complex in a contracted state. This study reveals atomic details of interaction networks that accompany and define the contraction mechanism of toxin-delivery tailocins, offering a comprehensive framework for understanding their mode of action and for their possible adaptation as biocontrol agents.

Contractile injection systems (CIS) consist of a long tube with a needle-like tip, a helical sheath surrounding the tube and a baseplate linking external signals with sheath contraction that propels the tube out of the sheath, enabling the needle to perforate the target membrane. In addition to contractile bacteriophage tails, CIS include bacterial contractile phage tail-like particles, which are further subdivided into the intracellular type VI secretion system (T6SS) and extracellular CIS (eCIS). The latter includes R-pyocins, which dissipate the proton motive force of their target bacteria, as well as phage-like protein-translocation structures (PLTS), which inject specific toxic proteins into eukaryotic cells. This major difference with pyocins led to the qualification of PLTS as toxin-delivery tailocins, thereby emphasizing their relationship with both pyocins and T6SS. These systems, bioinformatically identified in hundreds of bacteria and archaea dwelling in different environments, are exemplified by the Serratia antifeeding prophage (AFP) and the Photobacterium virulence cassette (PVC)—targeting insect larvae and haemocytes, respectively—and are similar to the metamorphosis-associated contractile structures and to the intracellular Amoebophilus asiaticus CIS.

The cryo-electron microscopy (cryo-EM) revolution has enabled spectacular progress in the structural characterization of both T6SS and R-pyocin as well as the related myotyphage T4 (refs. 9,17–20). However, structural understanding of PLTS is lagging behind, with only a single recent publication detailing the structures of the entire extended PVC particle and the contracted PVC sheath. Here, we present high-resolution structures of AFP—the archetype PLTS from the soil bacterium Serratia entomophila—in both the extended and contracted states, including the previously unknown structure of a contracted PLTS baseplate. This analysis enables us to propose a structural mechanism of AFP action as a toxin-delivery syringe and to provide a detailed comparison with other CIS. Conservation of the baseplate components among the PLTS suggests that the inferred contraction mechanism is probably shared by all toxin-delivery tailocins.

Results

Overall structure of the AFP particle. The S. entomophila AFP gene cluster comprises 18 open reading frames with translation products denoted as Afp1 to Afp18 (Supplementary Table 1). AFP translocates the insecticidal toxin Afp18 into larvae of the New Zealand pasture pest Costelytra zealandica, thereby causing cessation of feeding activity. The extended AFP is a 110 nm-long bullet-shaped particle built up by a helical trunk with a conical cap at the apical end and a flat base with a mobile fibre network at the proximal end (Fig. 1). To obtain a high-resolution cryo-EM structure of the entire AFP particle, its images were divided into three parts—the trunk (containing the sheath and inner tube), the cap and the baseplate. Each part was reconstructed separately and recombined to give a final composite cryo-EM map (see Methods; Fig. 1a,b,d, Supplementary Figs. 1, 2 and Supplementary Table 2). This approach allowed us to: (1) benefit from the helical symmetry
of the trunk while applying a six- or threefold rotational symmetry to the baseplate and needle and a sixfold symmetry to the cap, and (2) circumvent long-range distortions and separate two populations of AFP particles that differ in terms of the trunk length (Fig. 1a and Supplementary Figs. 1,2). The resolution of most of the structure ranges from 2.8 to 3.3 Å, which is overall slightly higher than
that of the recently published PVC structure and falls off towards the peripheral trunk protrusions, baseplate edges and attachment fibres (Fig. 1d,e, Supplementary Fig. 3 and Supplementary Table 2). De novo atomic model building for nearly all proteins composing the AFP particle (Supplementary Table 3) was made possible by the high quality of the maps (Fig. 1 and Supplementary Fig. 3), which reflects the regularity of the assembly and especially of the best-resolved inner tube, which needs to be rigid enough to penetrate the cell membrane following contraction. In the contracted state (Fig. 1f and Supplementary Figs. 2,4,6), the length of the sheath decreases from 850 to 350 Å and the diameter swells from 170 to 230 Å to eject the inner tube through a rearranged baseplate. The post-contraction baseplate (global resolution of 5.4 Å, ranging from 4 Å for the bottom of the sheath to about 12 Å for the outer rim of the baseplate) is less well resolved than the sheath (resolution of 3.8 Å), presumably because of the loss of the needle–baseplate interactions that are required for the tube and needle release.

The final atomic model of the extended AFP contains a total of 11 different proteins (Fig. 1). Three proteins make up the inner tube (Afp1, Afp5 and Afp7), three others build the surrounding sheath (Afp2, Afp3 and Afp4), two comprise the needle (Afp10 and Afp8), three belong to the baseplate (Afp9, Afp11 and Afp12) and one constitutes the apical cap (Afp16). The fibres formed by Afp13 were not well resolved (Fig. 1e and Supplementary Fig. 3), which is consistent with their proposed function in sensing the target-cell surface, requiring mobility and flexibility to orient the AFP particle and position the baseplate for the power stroke. Mass-spectrometry measurements (see Methods; Supplementary Table 1) identified a single copy of the Afp18 toxin, presumably represented by an unassigned density inside the tube, as well as the small and uncharacterized Afp6, which may either be also inside the tube or at the baseplate periphery associated with fibres. These measurements also confirmed the absence of the tape measure protein Afp14 (refs. 22,23), assembly chaperone AAA+ ATPase Afp15 (ref. 24) and ‘inactive pseudotoxin’ Afp17 (refs. 22,23) in the mature particle. Details about AFP proteins and their equivalents in other CIS are summarized in Supplementary Table 1.

**AFP tube.** As in the T4 phage, the first ring of the AFP tube is assembled at the baseplate from Afp7, followed by a ring of Afp5 and subsequent rings of Afp1 that span the interior of the entire particle up to the apical end formed by the tail-tube terminator cap of Afp16 (Figs. 1a and 2). Afp1, Afp5 and Afp7 have a common fold (Fig. 2b), which is also shared with the T4 gp19, the T6SS Hcp and the R-pyocin, phage lambda gpV3 and T5 pb6 (ref. 25) tube proteins (Supplementary Fig. 5a,b). Similarly to T4 gp19, the inner surface of the tube formed by Afp1 is negatively charged, possibly to allow the efficient release of the Afp18 toxin, which would be negatively charged at pH 11 in the gut of C. giveni larvae (Supplementary Fig. 5c). Like Pvc7, Afp7 possesses a LysM-like peptidoglycan-binding domain (Fig. 2g,h) and links the C6 symmetry of the tube to the C3 symmetry of the needle protein Afp8 (Fig. 2f and Supplementary Fig. 9c). At the apical end, in the cap-bound conformation, the N-terminal Afp1 α-helix unfolds to follow the interdomain loop of Afp16 (Fig. 2h,c). Each apical Afp1 strongly binds the cap by interacting with three Afp16 subunits, presumably to prevent tube release from the opposite end following contraction (as also see below).

The β-hairpins of the adjacent tube subunits constitute a β-sheet barrel with a diameter of 75 nm in the middle of the tube. Each Afp1 monomer interacts with four subunits from the Afp1 layer above and four subunits from the layer below (Fig. 2d); the main interaction occurs through β-sheet intercalation as in other CIS and a continuous mesh of such β-sheet intercalations spreads through the entire particle, from the needle to the cap (Fig. 2e–f). The free energy of these tube-stabilizing interactions and comparisons between the intra-tube, intra-sheet and tube–sheath interactions in AFP, R-pyocin and T6SS are detailed in Supplementary Tables 4 and 5. The observation that the intra-tube interactions, of similar strength in all three assemblies, are much stronger than the consistently weak tube–sheath interactions reflects the need for the rigidity of the tube following perforation of the target cell and the necessity for the tube to slide through the sheath during contraction.

**AFP sheath.** The most striking feature of the AFP sheath is that it is constructed of two alternating hexameric layers, each composed of a different shear protein (Fig. 3). A comparative genomics study revealed that most PLTS contain one sheath-encoding gene, although some have two and Enterobacteria (including Serratia) have three. Afp2, Afp3 and Afp4 have a conserved fold (Fig. 3e and Supplementary Fig. 6) integrating elements of domains 1 and 2 of VipA-B/TssB-C. At the proximal end, the sheath starts with a single hexameric ring of Afp4 followed by a ring of Afp2, and then Afp3 and Afp2 rings alternate up to the apical Afp16 cap. 77% of the AFP particles are built by 11 Afp2 and 10 Afp3 layers surrounding 21 layers of the tube protein Afp1 and resulting in a terminal Afp2–Afp16 interaction (Figs. 1a and 2a); 23% contain only 10 Afp2 layers and 10 Afp3 layers surrounding 20 Afp1 layers, and therefore terminate with Afp3–Afp16 (Supplementary Figs. 1,3). The tube–sheath interactions may be considered identical along the length of the trunk, ignoring negligible differences in the contacts between Afp1 and either Afp2 or Afp3 (Supplementary Fig. 6d).

Afp3 is the longest of the three sheath proteins and forms a poorly resolved N-terminal bulge responsible for the characteristic appearance of the AFP trunk, which is carved by six prominent ridges of Afp3 with deep Afp2 grooves in between (Fig. 1e and Supplementary Fig. 3d). In contrast to AFP, PVC has been described to be built of 10 layers of alternating Pvc2 and Pvc3 followed by 12 layers assembled exclusively of Pvc2. However, examination of the entire PVC map (EMDB ID: EMD-9762) suggests that this interpretation may have resulted from processing a mixed population of PVC particles, some of which may have been composed of Pvc2 and Pvc3 that actually alternated beyond the end of the back-folded fibres, further towards the apical cap, as is the case for AFP. Therefore, the N-terminal bulge domain of Afp3/Pvc3 may play another yet unidentified role in addition to the fibre-docking function proposed based on the patterning of the PVC sheath. The shape of this domain, which is partially conserved between Afp3 and Pvc3 (Supplementary Fig. 6f,g), is to some extent reminiscent of the slightly bigger domain 3 of VipA-B/TssB-C. However, the latter is known to be recognized by the cognate AAA+ ATPase ClpV in the contracted T6SS sheath to specifically trigger its disassembly and recycling, whereas the AAA+ ATPase in the PLTS is proposed to instead be involved in the assembly of the baseplate and the needle.

As in R-pyocins and T6SS, the sheath assembly appears to rely on a β-strand exchange in the innermost layer of the sheath, termed a ‘handshake’ mechanism (Fig. 3a,d). As described above for the intra-tube intercalations, the sheath-stabilizing mesh of β-sheet handshakes propagates from the baseplate of the AFP particle to the cap (Fig. 3d). Afp4 forms the first baseplate-proximal sheath ring by offering the corresponding β-strand to the sheath initiator Afp9 while accepting an analogous β-strand of Afp2, which in turn receives a β-strand from Afp3, and this pattern continues up to the top-most sheath layer. Furthermore, in the cap, the tail-terminator Afp16 mimics this handshake interaction by binding into a cleft in the terminal Afp2/3 (Fig. 3d;i); see below).

Although contraction was experimentally achieved by briefly subjecting AFP particles to guanidine hydrochloride (see Methods), no loss of protein integrity was detected (Supplementary Fig. 6c). Similarly to R-pyocins, the handshake inter-subunit connectivity does not change between the extended and the contracted state,
presumably maintaining the sheath integrity (Fig. 3b,c and Supplementary Fig. 6a,b). Contraction leads to a rigid-body rotation of each sheath subunit by roughly 33° and an outward shift of 40 Å, and the assembly length compresses approximately twofold. The switch from the extended to contracted state results in major displacements of the Afp2 and Afp3 N- and C-termini, with the
Afp2 N-terminus rotating more than 120° (Fig. 3f). Following contraction and radial expansion, the subunits of the upper rings insert in the newly formed gaps of the lower rings. Therefore, in spite of the loss of the contact with the tube, each subunit subunit buries circa 900 Å² more of its surface (Supplementary Table 6), meaning that a total of circa 730 kcal mol⁻¹ of energy would be released during a single contraction, resulting in the tube expulsion and drilling into the target cell membrane (Supplementary Tables 5, 6). Interestingly, the difference in free energy between the extended and contracted AFP sheath is much smaller than in T6SS and R-pyocin (Supplementary Table 5), probably reflecting the remarkable stability of extended AFP at a basic pH in the gut of the host.

The sheath–tube interaction appears universally conserved among CIS and mainly relies on complementary surface charges on contacting interfaces (see Supplementary Fig. 6d,e for the conservation of Afp2 and Afp3 residues involved in Afp1 binding.) The fact that the AFP particle inside the cell assembles into a metastable AFP apical-end cap. The cryo-EM maps of the apex of the major and minor AFP populations are virtually identical, independent of whether the terminal layer under the Afp16 cap is comprised of Afp2 or Afp3 (Fig. 4 and Supplementary Fig. 3c,f). The Afp16 protein is composed of two distinct domains linked by a long β-strand-containing loop and forms a hexameric ring at the apex of the trunk. The N-terminal domain of Afp16, folded similarly to gp15 (T4) and gpU (phage lambda) but differently from TssA (T6SS), constitutes an extra layer of the tube while the PLTS-specific C-terminal domain, slightly shifted inwards in AFP in comparison to PVC, caps the sheath (Fig. 4a–c and Supplementary Fig. 7a,b). The N-terminal Afp16 domains strongly bind to two Afp1 subunits underneath via a β-sheet intercalation, the linker β-strand of Afp16 docks into the C-terminal domain of one Afp2 subunit in a handshake manner (Figs. 3d(i) and 4a,b), while the C-terminal Afp16 domain interacts...
The structure of the needle–baseplate complex (Fig. 5) was solved to an average resolution of 3.1 Å (see Methods and Supplementary Fig. 1 for details). As in T6SS, AFP is a fusion of the hub and the needle genes, whereas the tip of the needle is encoded by afp10 (gp5.4 in T4; VgrG PAAR in T6SS; Supplementary Fig. 9e). Reminiscent of PVC, T4 (ref. 30) and T6SS39, the needle hub is composed of twin β-barrel domains of Afp8 with a fold similar to the core domain of the tube proteins (Fig. 5b,c). This creates a pseudo-hexameric layout that accommodates the transition from the hexameric ring formed by the tube initiator Afp7 (gp48 equivalent) to the C3-symmetry of the rest of the Afp8 needle trimer (Supplementary Fig. 9c). Thus, the top surface of Afp8 packed against the bottom of Afp7 plays the C6-to-C3 symmetry adaptor function attributed to gp27 (ref. 25). Interestingly, the similarity to gp27 extends even beyond the twin domains (Supplementary Fig. 9e). The bottom of Afp8 forms a tapered needle with an intercalated β-helical wall analogous to the T4 gp5 and T6SS VgrG/VgrG1 but notably shorter; this difference in length (Supplementary Fig. 9e) may be related to the specificity of AFP for eukaryotic targets. The tip of the C3-symmetrical needle is sharpened by a single copy of Afp10. Remarkably, the Afp8-bound region of Afp10 follows a pseudo-C3 symmetry, resulting in three clearly resolved β-sheets characteristic of gp5.4, the T4 homologue of Afp10, and thus enabling fitting of gp5.4 into the density (Supplementary Fig. 9f).

The AFP wedge is comprised of the sheath-initiator protein Afp9, featuring an EPR motif conserved over a variety of CIS, and two other baseplate proteins, Afp11 and Afp12 (Fig. 5e,f). In contrast, the T4 wedge is composed of gp25, gp53 and a module built of two copies of gp6 and one copy of gp7 stabilized by an α-helical core bundle20. Despite these apparent differences, both wedges share a remarkably similar architecture. Furthermore, Afp12 can be regarded as a hybrid between gp7 and one of the gp6 copies, whereas the second gp6 is mimicked by Afp11. Indeed, the alignment of Afp9 and gp25 overlays Afp11 and Afp12 with (gp6)_2-gp7* (Supplementary Fig. 9d). This structural conservation, which is unexpected from the sequence analysis, highlights the importance of the Afp11–Afp12 module in the assembly and function of the baseplate. Close to the particle axis, Afp11 folds back to loosely bind the Afp8 needle just above the β-helix. Because of the C3-symmetry of the latter, three copies of Afp11 interact with Afp8 more tightly than the other three (Fig. 5c). This fold-back interaction is different from those observed in T4 (ref. 20) and is mostly polar. The disorder observed in this region of Afp11 may facilitate needle ejection following contraction. Situated at the periphery of the baseplate, Afp12 hosts the proximal end of the Afp13 tail fibre, which can be visualized in the local-resolution-filtered maps and at lower thresholds (Supplementary Fig. 3b,e). As proposed for T4 (ref. 30), recognition of receptors on the target cell surface may trigger rotation of the wedge and the fibres thereby initiating contraction.

**Discussion**

Comparison between the structures of the extended and contracted forms leads us to propose a key role for the sheath initiator protein Afp9. Despite its relatively small 16 kDa size, Afp9 would act as a stabilizer and multivalent connector protein, crucial for the assembly and structural integrity of the whole AFP particle. It is located at the inwards-pointing apex of the baseplate wedge and links the helical trunk to the Afp11–Afp12 bulk of the baseplate. Specifically, in the extended state, while the inner face of the Afp9 ring surrounds the tube initiator Afp7 and reaches to Afp5, its outer top side positions the first sheath ring formed by Afp4 and its lower external loops hold six Afp11–Afp12 modules in place (Fig. 5a,e). Comparison between the structures of the extended and contracted states reveals the crucial role of Afp9 as a power-stroke initiator. Indeed, when the Afp13 fibres transmit the contraction signal through Afp12 to the rest of the wedge, the Afp11–Afp12 module detaches from the needle and swings outwards (Fig. 5d and Supplementary Fig. 9a,b). This momentum is transmitted to Afp9, which, in the contracted state, ensures the main connection between the baseplate wedge and the sheath (Fig. 5d,f). The exact sequence of events and the

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**Figure 4 | Architecture of the apical cap of AFP in the extended state.**

**a.** Cryo-EM map of the apical cap of AFP in the extended state displaying the subunits Afp1, Afp2, Afp3 and Afp16 (left). Top view of fitted Afp2 (dark blue) and Afp16 (white to dark red) subunits (right). Note the central ring formed by a side-by-side stacking of the helix spanning residues 143-153 of the neighbouring Afp16 subunits (annotated by a dotted black circle). **b.** Interactions between Afp1, Afp2, Afp3 and Afp16 in the apical cap. The linker β-strand (residues 184-191) of Afp16 is involved in a handshake with Afp2. **c.** Density map of the central ring in the apical cap with fitted α-helices containing residues 143-153 of the neighbouring Afp16 subunits. The amino acids Q152 and W151 are labelled and shown as sticks. **d.** Density map displaying the interaction between Afp16 (red) and the N terminus of Afp1 in the apical cap (green). The Afp1 amino acids V10 and Y12, and Afp16 W78 and N80 are labelled and shown as sticks. An alignment of Afp1 in the sheath with apical Afp1 illustrates the rearrangements of the N-terminal residues V10 and Y12 following Afp16 binding.

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with a neighbouring Afp2 and even stretches down to an Afp3 subunit one layer below (Fig. 4a,b). The N-terminal domain of Afp16 possesses a short α-helical insertion which, following Afp16 hexamerization, organizes in a tight ring with a diameter of 9 Å that constricts the extremity of the particle and probably ensures the directionality of the toxin egress through the baseplate and the needle (Fig. 4c and Supplementary Fig. 7a). Noteworthy, no capping of the AFP or PVC sheath was observed in the contracted state.
cause-and-effect relationships require further investigation but examination of the protein interaction networks inside the structures leads us to speculate that the swinging motion of the Afp9 connector with the whole of the wedge may transfer the contraction signal to Afp4 through two routes. The C-terminal α-helix of Afp9 would act as a hook pulling on the adjacent α-helix of Afp4, thereby causing rotation of the Afp4 subunit and its detachment from the tube Afp5. In addition, because Afp9 is the sheath initiator and the receiver of the first handshake from Afp4, its reorientation and the resulting movement of Afp4 would activate the transmission belt of handshakes that would spread from Afp4 to Afp2 and further up to the Afp16 cap. This would in turn trigger the sheath contraction, leading to the release of the tube and the needle, perforation of the target membrane and injection of the toxin into its grass grub prey.

In conclusion, this work presents the atomic-resolution structures of an entire contractile injection nanomachine in both the extended and contracted states. It provides a molecular basis for understanding the PLTS mode of biological action and regulation, illustrates the degree of the universal conservation of CIS and offers a framework for their analysis with unprecedented detail. In addition, information offered by the AFP structures may be further used for biomedical design. One such example would be the rational modification of the AFP particle and its attachment fibres towards targeting specific mammalian cell surfaces for the injection of a tailored biocontrol agent.

Methods

**AFP expression and purification.** Recombinant AFP was obtained as previously described.1 The purified AFP sample was stored at 4 °C in TM buffer (20 mM Tris–HCl and 20 mM MgCl2, pH 7.4). Contracted AFP was generated by incubating purified AFP samples with guanidine hydrochloride at a final concentration of 3 M for 1 min at room temperature and then diluted in a large volume of TM buffer to stop the guanidine-hydrochloride action. The samples were then concentrated by ultracentrifugation for 1.5 h at 150,000 g and the pellet was resuspended in TM buffer. The cryo-EM specimens were then prepared immediately (see below).

**Mass spectrometry.** Briefly, protein samples were resolved by SDS–PAGE and bands were excised, destained, reduced with dithiothreitol, alkylated with iodoacetamide and then digested with sequencing-grade porcine trypsin (Promega) in a chilled microwave (CEM) at 45 °C for 1 h at 15 W. A 10 µl aliquot of each acidified digest was desalted on a 0.3 µm Reprosil C18 media (Dr Maisch). The peptides were then separated on a 100 × 300 mm picorific column (New Objective) packed in-house with 3 µm Reprosil C18 media using the following gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at 300 nl min⁻¹: 0 min, 5% B; 0.5 min, 5% B; 47 min, 30% B; 49 min, 98% B; 52 min, 98% B; 53 min, 5% B and 60 min, 5% B. The picorific spray was directed into a TripleTOF 6600 Quadrupole-Time-of-Flight mass spectrometer (Sciex) operating in positive-ion mode, scanning 350–1,200 m/z for 250 ms, followed by up to forty tandem mass spectrometry scans of 40 ms per cycle (80–1,600 m/z) on multiply charged species using Dynamic Collision Energy and Dynamic Accumulation.

The resulting tandem-mass-spectrometry data was then searched against an in-house protein sequence database containing selected entries from *S. entomophila* using ProteinPilot v5 (Sciex). The undetected proteins are indicated in Supplementary Table T. The relative amounts of each detected protein were

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**Fig. 5 | Molecular organization of the AFP baseplate in the extended and contracted states.**

Select subunits involved in interactions between the tube, sheath and baseplate, and within the baseplate itself are displayed as non-transparent density, whereas the transparent density contains the structures of the subunits Afp2, Afp4, Afp5, Afp7, Afp9, Afp11 and Afp12. a. Cryo-EM map of the AFP baseplate in the extended state. Select subunits involved in interactions between the tube, sheath and baseplate, and within the baseplate itself are displayed as non-transparent density, whereas the transparent density contains the structures of the subunits Afp2, Afp4, Afp5, Afp7, Afp9, Afp11 and Afp12. b. Density map of the AFP needle formed by three neighbouring Afp8 subunits (zoom of (i) in a). The hydrophobic residues in the threefold symmetric centre of the needle tip are shown as sticks. c. Pseudo-hexameric symmetry of the upper part of the AFP needle (zoom of (ii) in a). Note the alternating presence/absence of interactions between the H369 residue of Afp8 and a loop of Afp11 indicating the transition between sixfold to threefold symmetry in the AFP needle. d. Cryo-EM map of the AFP baseplate in the contracted state with fitted subunits Afp2, Afp4, Afp9, Afp11 and Afp12. e, f. Comparison between the structural organization of the extended (e) and contracted (f) baseplate. Note the α-helical conformation of the C terminus of Afp9 in the extended (e) AFP state (annotated with a dotted black oval), whereas it is unstructured and thus not present in the contracted (f) AFP map.
estimated using the total intensity of peptides corresponding to each protein divided by the number of peptides expected following trypsin digestion after cleaning the raw results by confidence limit (>80%), d mass (>0.03) and peptide molecular weight (1 000 – 3 300 Da). The relative numbers obtained were then normalized by considering the stoichiometry of Afp4 to be exactly six per AFP particle. This semi-quantification yielded relatively precise results, consistent with the cryo-EM map, particularly for proteins with a large number of expected peptides, such as Afp11 (estimated at 6.1 copies per AFP particle) and Afp12 (estimated at 5.09 copies per AFP particle). Only one Afp18 toxin molecule (estimated at 0.97 copies) would be present per AFP particle according to this quantification.

Cryo-EM sample preparation. The cryo-EM specimens were prepared using a Vitrobot (Mark IV) device. Typically, 2–3 µl of a purified AFP sample was applied on a R2/2 Quantifoil grid (Pro Sci Tec) freshly glow-discharged in an anil-ammine environment, blotted for 3 s and vitrified using a Vitrobot (Thermofisher) kept at 4°C and 100% humidity.

Cryo-EM data collection. The cryo-EM images of the extended AFP were collected at the EMBL Heidelberg on a Titan Krios, operated at 300 kV by W. Hagen, with a condenser and objective lens-aperture sizes of 70 µm, a nominal magnification of ×37,000. A summary of the cryo-EM data collection and processing (see below) parameters can be found in Supplementary Table 2.

Movie alignment, general processing strategy, particle picking and defocus estimation. The frame alignment for both datasets was performed with IMOD using 0.06 s frames, corresponding to approximately 2.1 e−Å−2 per frame and a total exposure time of 1.8 s. The calibrated pixel size at the specimen level was 1.35 Å at a nominal magnification of ×37,000.

The cryo-EM images of the contracted AFP were collected by J.H. at the Korea Basic Science Institute Ochang Center on a Titan Krios operated at 300 kV, equipped with a Cs corrector (measured Cs of 500 nm after correction), using condenser and objective lens-aperture sizes of 70 µm and 100 µm, respectively, a spot size of 3 and a beam diameter of 850 nm. A total of 2,847 movies of 30 frames were acquired on a Falcon III camera, with a dose rate of 35 e−Å−2 s−1, a total exposure time of 1.8 s, using 0.64 s frames, corresponding to approximately 2.1 e−Å−2 per frame and a total dose of 63 e−Å−2. The calibrated pixel size at the specimen level was 1.397 Å at a nominal magnification of ×47,000. A summary of the cryo-EM data collection and processing (see below) parameters can be found in Supplementary Table 2.

Extended AFP needle. Analogous to all known CIS structures, the trunk of the extended AFP particle can be considered as an assembly of six protofilaments forming a right-handed superhelix (Fig. 2a). Alternatively, one can adopt a historical description and define the inner tube as tightly stacked hexameric rings or layers that are related to each other by a 20.4° rotation and a translation of 39.3 Å along the particle axis, which is a helical symmetry similar to the other CIS. Due to Afp2/Af3p alternation, the helical geometry of the sheath is described by a rise of 78.64 Å and a rotation of 40.28°, exactly twice that of the enclosed tube of Afp1. To determine this symmetry, refinement was performed using the previous reconstruction of the AFP trunk with a 20 Å resolution as an initial model, with 20 cycles of IHRSR refinement implemented in Spider [9], wherein the helical rise was varied from 78 to 81 Å in steps of 1.5 Å and the rotation between subunits varied from 40.5 to 42.5° in 1° steps (Supplementary Fig. 1). For each initial symmetry choice, the symmetry parameters were refined with the hecsrch program using step sizes of 0.1 Å for the helical rise and 0.1° for the azimuthal rotation for iterations 2–13. The steps were decreased to 0.05 Å and 0.05°, respectively, for cycles 14–20 and a finer increment was used to project the reference volume. The average final values of 78.57 Å for the rise and 40.15° for the helical rotation were then used for a high-resolution refinement in SPRING, which yielded a reconstruction of the trunk at an overall estimated resolution of 2.9 Å at a Fourier shell correlation (FSC) cut-off of 0.143 and with an automatically determined b-factor of −105 Å−2.

A per-particle CTF determination with Gictr [22] and helical auto-refine procedure with symmetry refinement as implemented in RELION-3 (ref. [17]) improved the map further, resulting in the final reconstruction at overall resolution of 2.78 Å (FSC at a 0.143 cut-off) and with an automatically determined b-factor of −92 Å−2, obtained from 122,721 segments (after the removal of the outplane outliers) corresponding to 736,326 asymmetric units after C6 symmetrization.

Contracted AFP sheath. Using a hollow cylinder with an inner diameter of 70 Å and outer diameter of 230 Å as an initial model, a 3D refinement in RELION-2.1 (ref. [17]), imposing only C6 symmetry, resulted in a map with a resolution of 4.2 Å after automatic masking (FSC cut-off of 0.143). This map was then used to determine the helical symmetry as follows. Based on a first visual inspection of this C6-symmetrized map, helical rises between 30 and 36 Å (0.2 Å steps) and azimuthal rotations between 2 and 5° (0.1° steps) were tested by imposing each symmetry with the impose program and recording the variance of each symmetrized reconstruction. A finer search around the parameters corresponding to the highest variance (that is, reinforcement of features) was then performed with the rise ranging from 34.8 to 35.2 Å at 0.02 Å steps and the rotation ranging from 3.9 to 4.1° at 0.01° steps (Supplementary Fig. 2). The reconstruction with a helical rise of 34.97 Å and azimuthal rotation of 3.14° was used for further processing in SPRING. High-resolution 3D refinement gave a final map at an overall resolution of 3.8 Å at a FSC cut-off of 0.143 (Supplementary Fig. 4) obtained from 46,022 segments corresponding to 276,132 asymmetric units after helical and C6 symmetrization. This map was filtered to 3.8 Å and sharpened with a b-factor of −260 Å−2 for the model building and figures.

Contracted AFP baseplate. The extremities with and without a contracted baseplate were sorted by two-dimensional classification (Supplementary Fig. 2). The C6-symmetrized reconstruction of the entire contracted trunk was shifted and matched to the position of the extracted extremities, and an initial reference for a RELION-2.1 auto-refine procedure using C6 symmetry. The resulting map, with an average resolution of 4.6 Å (FSC at a 0.143 cut-off), showed a poorly resolved baseplate region. Masked 3D classification of this region with no further alignment enabled grouping of the particles by their baseplate conformation (more or less open). A final autounrefine of each group led to three contracted baseplate maps with an overall resolution of 5.4, 5.9 and 7.3 Å. The best map was used for refining the models of the proximal Afp2 and Afp4, and placing Afp9, Afp11 and Afp12 as rigid bodies in the lower-resolution baseplate wedge density.

Extended AFP baseplate. The previously published low-resolution AFP reconstruction [1] was appropriately masked to enclose the baseplate part and used as initial reference for a RELION-2.1 auto-refine procedure using C6 symmetry. This gave a reconstruction with a resolution of 3.2 Å (FSC at a 0.143 cut-off after automatic masking). Alignment and resolution-dependent weighting of the individual particle movie frames (particle polishing) was performed in RELION further improved the map with respect to the frame alignment and a running average of five frames during the movie expansion. A final refinement using these polished particles resulted in a reconstruction with an overall resolution of 3.1 Å (FSC at a 0.143 cut-off after automatic masking; 3.5 Å without masking) and with a determined overall b-factor of −105 Å−2.
as the inputs for further refinement of orientations using only local searches. This workflow led to a final C3-symmetrized map from the subtracted particles with an overall resolution of 3.7 Å (FSC at 0.143 cut-off). Inspection of this map revealed a poorly defined upper region of the needle, where the overlap with the rest of the projection of the baseplate would occur in the side views, suggesting artefacts due to the signal subtraction itself (Supplementary Fig. 1). Orientations of the subtracted images refined with a C3 symmetry were therefore applied to the original non-subtracted baseplate particles and a final local orientation refinement was performed with the C3 symmetry imposed. This gave a threefold symmetric map of the entire baseplate with no artefacts in the upper region of the needle and an overall resolution of 3.3 Å (FSC at 0.143 cut-off after automatic masking; 3.8 Å without masking), and with an automatically determined overall b-factor of −102 Å².

**Extended AFP cap.** The previously published AFP reconstruction was appropriately masked to enclose the cap region and used as initial reference for a RELION-2.1 auto-refine procedure using C6 symmetry. This gave a reconstruction with an overall resolution of 3.4 Å without masking, but with a poor definition of the cap end, and with sheath protrusions appearing at every sheath layer instead of every two layers (Supplementary Fig. 1). Using the orientations corresponding to this map, 3D classification into two classes (skipping alignment) showed that the terminal sheath ring was built by either Afp2 or Afp3. The particles from both ‘Afp2-terminating’ and ‘Afp3-terminating’ classes were further subjected to 3D classification into three classes each, still without alignment. One class of Afp2-terminating particles was shifted by approximately 40 Å (distance between Afp2 and Afp3 rings in the sheath) that ended with Afp3. This can be explained by the fact that the alignment in the first refinement was driven by the helical/sheath region, disregarding the cap region due to its much lower mass. Similarly, two classes from the Afp3-terminating particles were shifted by 40 Å, down and up the helical axis, and corresponded to the actual Afp2-terminating particles. These particles were not resolved by sheath-protruding protein according to this result and used for 3D refinement with a larger translational search. Two additional rounds of 3D classification and regrouping of particles according to the terminal sheath protein were performed, resulting in the separation of the dataset into 23,797 Afp2-terminating particles (77% of all particles) and 7,061 Afp3-terminating particles (23% of all particles). Finally, a 3D refinement of each dataset with an alignment parameter search and with the final 3D classes as references led to reconstructions of the cap with an overall resolution of 3.2 and 3.4 Å (FSC at 0.143 cut-off after automatic masking; 3.5 and 3.8 Å without masking), and a determined overall b-factor of −102 Å² and −82 Å² for the maps with the terminal Afp2–Afp16 and Afp3–Afp16 interactions, respectively. To assess the resolution of only the cap part, a tighter mask enclosing this region was created and combined with the automatic mask calculated by relion_postprocess; the cap resolution was estimated to be 3.3 and 3.4 Å for the terminal Afp2–Afp16 and Afp3–Afp16 maps, respectively (FSC at 0.143 cut-off).

**Model building and refinement.** Atomic models were built into their respective cryo-EM maps ab initio by first manually tracing each protein sequence into the map as a target. Several iterations of automatic real-space refinement followed by manual adjustments in Coot were performed until convergence. Symmetry-related copies and neighbouring subunits were taken into account by including all of the subunits present in each map during the final refinements. LocScale was used to apply local sharpening on the baseplate map for the final steps of the refinements of Afp11 and Afp12, which had regions with densities with poorer resolution. For Afp11 and Afp12, manual building in Coot was followed by a single round of molecular-dynamics flexible fitting using NAMDINATOR and by a final round of real-space refinement in Phenix. A summary of the model refinement and validation statistics can be found in Supplementary Table 3.

**Interaction energy calculations.** Calculations of the interaction energies between AFP subunits were performed using the PISA server, −0.50 kcal mol⁻¹ per hydrogen bond and −0.33 kcal mol⁻¹ per salt bridge were then added to the free energy to give the final values (Supplementary Tables 4–6).

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The cryo-EM maps and corresponding atomic coordinates have been deposited to the EMDB and PDB with the following accession codes: full extended AFP composite map, EMDB-4783; extended AFP baseplate C6 map, EMDB-4784 and 68RC; extended Afp baseplate C6 map, EMDB-4800 and 68RC; extended Afp cap ending in Afp2–Afp16, EMDB-4741 and 68AP; extended AFP cap ending in Afp3–Afp16, EMDB-4801; extended AFP shear map, EMDB-4802 and 68RN; extended AFP needle map from subtracted images, EMDB-4871; extended AFP shear map, EMDB-4805 and 68C; contracted AFP baseplate map, EMDB-4876 and 68GL; extended AFP and contracted AFP shear C6 map, EMDB-4859. All other data supporting the findings of this study are available from A.D. and I.G. on request.

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Author contributions

A.K.M. and M.R.H.H. designed and funded the project. A.D., H.V. and M.J. purified and initially characterized samples by negative staining and cryo-EM. A.D. analysed the mass-spectrometry data. A.D. performed the image analyses with significant input from I.G. and M.J. The mass-spectrometry data. A.D. performed the image analyses with significant input from I.G. and M.J. analysed the data. A.D., J.F. and M.J. prepared the figures and tables. A.K.M., M.R.H.H. and I.G. provided support and supervised the project at various stages. I.G. and A.K.M. wrote the manuscript with significant input from M.R.H.H., J.B.H., A.D., J.F. and M.J., and contributions from all of the authors.

Competing interests

The authors declare no competing interests.

Additional information

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