Three-dimensional Structure of the Toxin-delivery Particle Antifeeding Prophage of *Serratia entomophila* \(^*\)\(^1\) \(^2\)

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**Background:** Antifeeding prophage (Afp) is a toxin-delivery bacteriophage tail-like particle.

**Results:** The syringe-like three-dimensional structure, composed of a helical sheath formed by 10 disks, a baseplate, and a central tube displays 6-fold symmetry.

**Conclusion:** Although similar to other type VI secretion systems, Afp possesses unique features.

**Significance:** This is the first insight into the three-dimensional structure of a tailocin.

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The *Serratia entomophila* antifeeding prophage (Afp) is a bullet-shaped toxin-delivery apparatus similar to the R-pyocins of *Pseudomonas aeruginosa*. Morphologically it resembles the sheathed tail of bacteriophages such as T4, including a baseplate at one end. It also shares features with the type VI secretion systems. Cryo-electron micrographs of tilted Afp specimens (up to 60 degrees) were analyzed to determine the correct cyclic symmetry to overcome the limitation imposed by exclusively side views in nominally untilted specimens. An asymmetric reconstruction shows clear 6-fold cyclic symmetry contrary to a previous conclusion of 4-fold symmetry based on analysis of only the preferred side views (Sen, A., Rybakova, D., Hurst, M. R., and Mitra, A. K. (2010) *J. Bacteriol*. 192, 4522–4525).

Electron tomography of negatively stained Afp revealed right-handed helical striations in many of the particles, establishing the correct hand. Higher quality micrographs of untilted specimens were processed to produce a reconstruction at 2.0-nm resolution with imposed 6-fold symmetry. The helical parameters of the sheath were determined to be 8.14 nm for the subunit rise along and 40.5° for the rotation angle around the helix. The sheath is similar to that of the T4 phage tail but with a different arrangement of the subdomain of the polymerizing sheath protein(s). The central tube is similar to the diameter and axial width of the Hcp1 hexamer of *P. aeruginosa* type VI secretion system. The tube extends through the baseplate into a needle resembling the “puncture device” of the T4 tail. The tube contains density that may be the toxin and/or a length-determining protein.

A large plasmid of *Serratia entomophila*, pADAP (2, 3), codes for a phage-tail like particle, the antifeeding prophage (Afp)\(^3\) that is thought to be a delivery vehicle for an insecticidal toxin (4). pADAP also encodes the *S. entomophila* pathogenicity determinants SepABC insect-active toxin complex (5). The toxin carried by Afp causes cessation of feeding activity in the New Zealand grass grub, *Costelytra zealandica*, whereas the SepABC toxin complex causes clearing of the gut and an amber coloration (5).

The Afp particle has a central tube surrounded by a contractile sheath and a baseplate with associated fibers (6). Similar particles with toxin-delivery functions are encoded by the *Photorhabdus* virulence cassettes (4, 7), which in turn resemble several R-type pyocins (8, 9). In the broader picture, the Afp particles resemble the tails of contractile bacteriophages and bacterial type VI secretion systems (T6SS) (10, 11).

Eighteen ORFs in pADAP plasmid are associated with the activity of Afp (4). No less than three (Afp2–4) of these ORFs that have significant similarity to proteins of the phage sheath 1 superfamily (COG3497) (4) are thought to compose the contractile sheath. This superfamily includes the sheath proteins from an R-type pyocin (12) and the tail of bacteriophage T4 (13, 14) (Table 1). In addition, Afp8 resembles the T4 gp5-gp27 “puncturing device” (15) and the related T6SS VgrG protein (16). Afp9 is likely a baseplate protein similar to gp25 in T4, and Afp13 may be a component of the fiber (4). Finally, the molecular masses of homologous proteins Afp1 and Afp5 are similar to that of Hcp1, the tube protein of a T6SS (17) and gp19 of the T4 tail tube (16).

Of the 18 ORFs, only 16, Afp1 to Afp16, are required to produce mature Afp particles (18), with Afp17 and 18 considered to be candidates for the toxin (4). The omission of Afp16 results in a sheathless particle featuring the baseplate and fibers with a tube of highly variable length, termed the tube-baseplate complex (TBC). It was concluded that Afp16 is required for stable assembly of the sheath and regulating the length of the central tube (18).

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**This article contains supplemental Figs. 1–3 and Results.**

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\(^3\) The abbreviations used are: Afp, anti-feeding prophage; TBC, tube-baseplate complex; T6SS, type VI secretion system; C4, 4-fold cyclic symmetry; C6, 6-fold cyclic symmetry.
As a first step in understanding the mechanistic basis for the structural roles of the Afp proteins, we recorded electron micrographs of tilted and untilted frozen-hydrated specimens of Afp. Analysis of the rotational symmetry of an asymmetric reconstruction generated from only images of tilted specimens indicated that Afp has C6 symmetry, contrary to the earlier conclusion of C4 symmetry for the helical sheath (1). A reconstruction from the untilted specimens with imposed C6 symmetry provided a detailed protein-domain level density map of the particle, delineating the helical sheath surrounding the central tube, the baseplate, and fibers linking the baseplate and the sheath. We carried out tomography of negatively stained Afp particles to establish the hand of the sheath. We also recorded images of TBC particles, allowing us to distinguish the sheath from density associated with the baseplate. Finally we relate regions of density maps of Afp and TBC to known structures of analogous proteins from bacteriophage T4 and the T6SS.

EXPERIMENTAL PROCEDURES

Sequence Comparisons—Previous searches of the Databases at the National Center for Biotechnology Information revealed three candidate Afp sheath proteins, Afp2, Afp3, and Afp4 (4). Using BlastP (19), the corresponding sequences of the sheath proteins of the Enterobacteria phage T4 (gp18) and R-type pyocin (PA0662) sheath proteins were identified (Table 1). Significant BlastP or domain hits of Afp1, -5, -8, and -9 to sequences in the current database are also listed in Table 1.

Sample Preparation—Details of constructs to generate the sheathless TBC as an expression product are given elsewhere (18). Afp and TBC extracts were prepared as described by Hurst et al. (6); however 0.4× Luria broth (LB) media was used for induction instead of 0.5× LB. Sedimentation of Afp/TBC particles was undertaken using a modified procedure of Nguyen et al. (20). Polyethylene glycol 6000 and NaCl were added to the supernatant to a final concentration of 8% (w/v) and 0.5 M, respectively. The mixture was gently stirred overnight on ice. Afp/TBC particles were sedimented by centrifugation at 8000 × g for 30 min using Eppendorf centrifuge 5810R, Rotor F34, and suspended at 4 °C in 16 ml of 20 mM Tris, 20 mM MgCl2 (TM) and incubated on a Labnet minilab roller at ambient temperature for 2 h. The sample was centrifuged at 13,000 × g for 10 min at 4 °C, and the clear supernatant was divided into 8-ml aliquots and then centrifuged further at 151,139 × g for 2 h at 4 °C in a Beckman coulter OptimaTM L-100K ultracentrifuge. After removing most of the supernatant, the pellet was resuspended in the residual 500-ml buffer in the tube, filtered through a 0.2-μm pore-size filter, and applied onto a Sepharcl S-400 HR (GE Healthcare) gel filtration column (1.5 × 46-cm bed volume). The column was washed with eluant buffer (25 mM TBS with 130 mM NaCl, pH 7.5) at a flow rate of 1 ml/min at room temperature. The combined column fractions I, II, and III were separately concentrated in an Ultracel-50K device (Amicon). The retenate, composed of 56% Afp particles, was resuspended in ~20 μl of TM buffer with 0.02% NaN3.

Cryo-electron Microscopy—An aliquot of 5 μl of a purified preparation of Afp particles was applied to a Quantifoil grid (Pro SciTeC) rendered hydrophilic by glow-discharging in an atmosphere of amyl amine, blotted, and plunge-frozen in liquid ethane using a manual plunge device operated at 90% ambient humidity or an FEI Vitrobot at 100% humidity, both operated at 4 °C. The vitrified specimens were imaged at a nominal 42,000× (41423×, calibrated using beef liver catalase two-dimensional crystals) magnification using a Tecnai 12 electron microscope (FEI, Eindhoven, The Netherlands) fitted with a LaB6 filament and a GATAN 626 cryoholder and operating at an accelerating voltage of 120 kV. Micrographs were recorded on SO163 Eastman Kodak Co. film at ~1500 e−/nm2 per exposure and 1.6–2.8 μm underfocus. The specimen was imaged both untilted and tilted by 45° or 60°.

Negative Stain Electron Microscopy—An aliquot of 4 μl of a purified TBC preparation was applied to a glow-discharged copper EM grid overlaid with a carbon film and allowed to settle for 1 min. The grid was then washed 3 times with deionized water and stained with 2% uranyl acetate; excess solution was wicked off with a filter paper (Whatman #5) and finally air-dried. The specimens were imaged at a nominal 52,000× (50,364×, calibrated using beef liver catalase two-dimensional crystals) magnification using a Tecnai T12 microscope with a LaB6 filament (FEI) and operating at an accelerating voltage of 120 kV. Micrographs were recorded on SO163 Kodak film.

Negative Stain Electron Tomography—An aliquot of 4 μl of a purified Afp preparation was applied to a glow-discharged copper EM grid overlaid with a carbon film and allowed to settle for 1 min. The grid was then washed 3 times with deionized water followed by application of 4 μl of 20 nm colloidal gold fiducial markers (BBI Solutions) for 90 s. After tapping the grid to remove excess solution, the grid was stained with 2% uranyl acetate, and excess solution was wicked off with a filter paper (Whatman #5) and air-dried. The specimens were imaged on a Tecnai T12 microscope with a LaB6 filament (FEI) equipped with an energy filter (GIF 2002, Gatan) operating in zero-loss mode with an energy slit width of 20 eV. Tilt series of images (from ~60° to +60° at 2° intervals) at a target defocus of 4 μm were acquired on a 2048×2048-pixel CCD camera (GATAN™) at an effective magnification of 38,500× (0.78 nm/pixel) using the SerialEM package (21).

Single Particle Reconstruction—All micrographs were digitized using a Nikon 9000ED scanner at 0.307-nm pixel size for the Afp particles and 0.252 nm for the TBC particles. All further image processing for both Afp and TBC particles was done in BsoT (22). The contrast transfer function parameters were automatically determined for each micrograph from the averaged power spectra of 256 × 256 pixel-non-overlapping tiles and manually checked for accuracy. Particles were picked, extracted, and then contrast transfer function-corrected by phase flipping and baseline correction. A grid-search projection-matching algorithm was used for aligning the particles. For this purpose, an initial synthetic reference map in the form of a cylinder with a small bulb at one extremity was used. The orientations were refined to an accuracy of 0.3° by reciprocal space central section matching. The resolution was estimated by Fourier shell correlation and differential phase residual of appropriately masked reconstructions from two halves of the data set using cutoffs of 0.3 (FSC0.3). To prevent overfitting, the matching coefficients were calculated only over a resolution band where the FSC curve for the reference map exceeds 0.7.

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We also repeated the processing using the “golden standard” approach, where two independent sets of particles are processed and their maps compared for resolution estimation (23). This yielded essentially the same maps for both Afp and TBC as before. The maps shown here are from these runs.

Tomographic Reconstruction—The tomographic tilt series was aligned using 20-nm colloidal gold fiducial markers, and reconstruction was carried out using Bsoft (24). The correct hand of the Afp particles was established by processing in parallel a specimen of vitrified coated vesicles, for which the handedness is known (25) using the same microscope conditions and the same software (26).

RESULTS

Asymmetric Reconstruction of Afp to Determine Cyclic Symmetry—In both negatively stained and frozen hydrated nominally untilted specimens, only images that are exclusively projections of side views normal to the long axis were observed (Fig. 1, A and D). This was true even for regions of thick ice in cryo specimens where alternate orientations could be expected and is certainly due to the long, bullet shape of the Afp particle. One of the problems has been the lack of top views, key in determining the symmetry of the T4 sheath (27). This propensity to assume preferred orientation compromises unambiguous determination of the cyclic symmetry using only untilted projections. In TBC preparations we did observe some end-on views of baseplates showing distinct 6-fold symmetry and typically 4–6 fibers extending radially from them (Fig. 1C).

To overcome this limitation of exclusive side views of the Afp particles, cryo-electron images tilted up to 60° (Fig. 1B) were processed to generate a reconstruction without any imposed symmetry, which was then analyzed for unbiased determination of the cyclic symmetry. As an initial reference map, we used a simple cylindrical rod with a small bulb at one end. This resulted in an arbitrary assignment of the rotation around the long axis of the particle but with distinction of the two extremities. Multiple rounds of local refinement of these orientations using 455 highly tilted particle images led to an asymmetric reconstruction at a resolution of 6 nm (Fig. 2A).
The cyclic symmetry of this map was established as follows. First, the map was low pass-filtered to 6 nm and masked with a soft-edged cylindrical mask to minimize noise. It was then rotated around the $z$ axis (coinciding with the long axis of the particle) and compared with the original by calculating a real space correlation coefficient. This analysis displayed correlation coefficient peaks at intervals of $\frac{\pi}{H} = 60^\circ$ clearly showing the presence of C6 symmetry (Fig. 2B). The low resolution of the map with the needle-like extension protruding from the baseplate showing very weak density did not allow observation of the symmetry directly (Fig. 2C). However, the use of the information in the whole map complemented by the rotational correlation analysis rules out other cyclic symmetries, including the previously deduced C4 symmetry (1), which would lead to correlation peaks spaced $90^\circ$ apart.

### C6 Reconstruction of Afp and Determination of the Helical Symmetry of the Sheath

Having established the C6 symmetry, particle images from untilted specimens were next processed by imposing C6 symmetry. The alignment process was started by using the asymmetric reconstruction as an initial reference map now 6-fold-symmetrized. After multiple iterations of the refinement of alignment using 1848 particle images, a reconstruction with resolution of 2.0 nm was achieved, revealing subunit details of the particle at the protein domain level (Fig. 3, A and B). The helical nature of the sheath was obvious in this C6-symmetrized reconstruction, and the parameters for the helix were next determined ab initio directly from the reconstruction.

To further examine the helical nature of the map, it was unwrapped into cylindrical projections that were generated by integrating along radial lines at $2^\circ$ increments around the helical axis and autocorrelated within resolution limits of 3.5–20 nm. This exercise yielded an initial estimate of the helical parameters: a rise of 8.0 nm for the subunit and a rotation angle of $40^\circ$ for the azimuthal location corresponding to the rise. The evaluated subunit rise is consistent with observations from the power spectrum of the map that displays meridional layer lines at 8.16, 4.08, 2.72, and 2.04 nm, indicating a subunit repeat of 8.16 nm (data not shown).

The helix parameters were refined using a grid search whereby the map was compared in real space with a copy that...
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was produced by rotation around and shifting along the axial direction. The range searched for subunit rise was 7–9 nm with a 0.1 nm step, and that for the helical subunit rotation angle was 30–50° with a 1° step. These were subsequently refined to an accuracy of 0.01 nm and 0.1°, respectively, yielding a subunit rise of 8.14 nm and a rotation angle of 40.5°.

A second method was used to refine the helix parameters wherein the map was rotated and compared with the original by cross-correlation, with the rise calculated from the shift along the z axis from the position of the correlation peak. The subunit rotation angle was again varied in the range of 30–50°, starting with a 1° step and refined to a 0.1° step. From this procedure, subunit rise of 8.13 nm and a rotation angle of 40.5° were obtained.

**Symmetry of the Central Tube**—The central tube in the Afp map was excised (diameter 7.5 nm) to remove the strong density contributed by the sheath. The power spectrum calculated from this map showed meridional layer lines at 4.08 and 2.04 nm (data not shown). The helix parameters of the tube were determined using the two methods described above. Whereas the sheath has very strong continuous features, the tube has a thin wall with periodic openings or fenestrations. The analysis for helix parameters for maps from different iterations in the processing yielded slightly different values. These ranged from 4.04 to 4.11 nm for the helical rise per subunit and 19.7° to 20.4° for the rotation angle per subunit. The tube in the final, refined Afp map had a rise of 4.06 nm and a rotation angle of 20.1° per subunit. The rise is about half that of the sheath subunit, indicating possible correspondence between the packing in the two helical structures.

**Reconstruction of the TBC**—The length of the tubes in TBC particles varied significantly and often showed some curvature (Fig. 1D). Two independent reconstructions were generated. A density map of the TBC with C6 symmetry was reconstructed by using 1146 images wherein only a short section of the tube attached to the baseplate was included in the boxed images. This yielded a reconstruction with a resolution of 1.9 nm (Fig. 3, E and G). The long, extended tubes in the micrographs offered an opportunity to examine the tube without the baseplate. A second map was calculated from 7222 segments of only the tubes, also yielding a resolution of 1.9 nm (Fig. 3, D and F).

In both maps the tube displays two cylindrical shells, the outer one with clear helical fenestrations (Fig. 3, D–G and I). In both cases the power spectra show meridional layer lines at 4.14 and 2.08 nm. The helix parameters for the tube were determined using the real space method, with a similar uncertainty as those observed for the tube extracted from the Afp map. For the tube associated with the baseplate in the TBC map, we found a subunit rise of ~4.05 nm and a rotation angle of ~22.1°, whereas for the tube-only map, the subunit rise was ~4.13 nm, and the rotation angle was ~21.6°.

The tube reconstructed from the TBC specimen appears to be wider than that extracted from the Afp particle (compare Fig. 3, D–G with C). Here also the walls appear thicker, with a double-shell appearance, as opposed to the single shell appearance in the Afp particle. The likely explanation is that the TBC swells in the negative stain, separating the two shells in the wall or that it reflects small structural change in the absence of the protein sheath in the TBC. This may also contribute to the differences in the measured helical parameters.

**Layers in the Helical Sheath**—The most striking feature of the sheath is the repeated protrusions, delineating 8.14-nm-thick layers in this section of the Afp particle. Comparing the Afp map (Fig. 3A) with the TBC map (Fig. 3E), the sheath starts just above the density indicated by the arrow in Fig. 3E. From this location, six protofilaments wrap around the central tube, forming hexameric rings arranged in 10 layers (blue numbers in Fig. 3A). The repeating subunit in each protofilament is composed of three distinguishable domains (red numbers in Fig. 3A). Moving up along a protofilament, the first prominent density is attributed to the first sheath domain, the next density volume to the second, and the protrusion to the third.

To provide a measure of how similar each layer is to the overall structure of the helix, the density map was helically symmetrized (supplemental Fig. S3), and each 8.14-nm-thick segment was masked and correlated with the corresponding segment in the non-helically symmetrized map. The first 4 layers have lower correlation coefficients of 0.84–0.92, likely due to the presence of the fibers. In contrast, the top 6 layers have correlation coefficients of 0.94–0.99. The cross-correlation of sheath layers also included an estimate of the offset of each layer with respect to the average. This was less than 0.02 nm, indicating a highly regular helix formed by the 10 layers.

**Estimation of the Masses of the Sheath Domains**—The density map of the Afp particle was segmented in Chimera (28) at thresholds of 1.0 and 1.3σ to estimate the volumes of the three domains in the asymmetric unit of the sheath. The first threshold was chosen at a level where some background noise is evident, whereas the second threshold is clearly above the noise. The analysis with these two thresholds should give a realistic indication of the volumes of components of the particle. The differences in calculated protein volumes between the two thresholds are small enough to allow a reasonable comparison with the sizes of known sheath-like proteins. The molecular weight for a domain was calculated from the estimated volume using a protein density of 0.81 kDa/nm³. The first two domains are very similar in size and shape, with the two estimates for each domain being domain 1 (53 and 47 kDa) and domain 2 (40 and 36 kDa) based on the chosen thresholds. The protruding domain is much smaller in size, corresponding to 15 and 13 kDa. Using these estimates, the asymmetric units attributed to the sheath possess a protein mass of ~100–108 kDa. This value is larger than the mass for any one of the putative sheath proteins (Afp2, 39 kDa; Afp3, 49 kDa; Afp4, 46 kDa) and, therefore, may represent the combined mass of at least two.

**The Baseplate and Fibers**—The baseplate is a cone-shaped structure with a needle-like mass protruding axially (Fig. 3). The bottom view (Fig. 3,J and K) shows the needle in the middle surrounded by six small densities and six larger densities at the periphery. The fibers are connected to these peripheral densities. In the side view in Fig. 3G, the inner part of the baseplate (blue) is composed of the needle and the bottom part of the central tube. Surrounding this is a cylindrical shell (yellow) located below the sheath; in Fig. 3B this shell appears to be continuous with the sheath.
The fibers connected to the outer baseplate ring extend ~40 nm, each adhering to one peripheral baseplate density at one end and to the protrusion (domain 3) on sheath layer number 4 at the other end (Fig. 3A). The inclination of the fibers with respect to the particle axis is the same for both Afp and TBC maps (Fig. 3, A and F). However, in the latter case, the only point of connection is the protrusion on the baseplate, and therefore, many fibers are detached or with variable orientation and do not contribute to the map, resulting in their weak density in Fig. 3, E and G.

Density in the Tube Cavity—Fig. 3B shows significant density (blue) inside the central tube. The cavity is ~97 nm long and 4 nm wide, giving a total volume of ~1200 nm³. This could easily accommodate more than one molecule of the putative toxin (Afp17 (~39 kDa) and/or Afp18 (~260 kDa)). Alternatively, the density could also represent a ruler protein determining the length of the Afp particle.

Handedness from Negative Stain Tomography—To clearly resolve the hand in the three-dimensional structure, we carried out electron tomography of negatively stained Afp particles. The tomograms show the characteristic bullet-shaped particles with tail fibers, most of which are rotated away from the sheath (Fig. 4A). The stain preferentially accumulated along the long edges of the particle and less so on the top and the bottom, which represent the faces away and in contact with the carbon film, respectively. The stain on the top and bottom faces fill in the crevices and grooves of the particle, yielding a topographic signature of the Afp particle. In the z direction (parallel to the electron beam), the reconstruction appears elongated with poor resolution and does not directly yield information about the azimuthal symmetry. In addition to the effects of the uneven stain distribution and dehydration, this elongation is due to the limited tilt of ±60°, resulting in a part of the three-dimensional information missing from the tomogram (the missing wedge effect). Attempts to align and average multiple particles were unsuccessful, mainly due to the dominance of unevenly distributed stain.

Despite these limitations, the top and bottom faces of the particles can be readily distinguished. Unfortunately, we did not find any particle where the directions of the top and bottom striations were clear within the same particle. Fig. 4, B and C, shows selected z-slices through six different particles, with those in panel B indicating striations in the opposite direction to those in panel C. Also evident are tail fibers in panel B but not in panel C. Thus the z-slices selected for the particles in panel B are close to the carbon film to which the tail fibers adsorb, whereas the z-slices in panel C are further away from the carbon. This observation means that the former shows the bottom views of the particles, and the latter shows the top views. At many places along the long axis of the particle, a line drawn across the width of the particle crosses three strands of the striations, consistent with C6 symmetry. The striations indicate that the main grooves in the sheath have right-handed helical twists. These strands likely follow the protofilaments in the helical sheath (Fig. 3A).

DISCUSSION

The Afp particle is a toxin-delivery device with a contractile sheath mechanism also found in other tailocins, bacteriophages, and T6SSs. The features we address first are the basic structure of the particle, including symmetry and handedness, and its definable components, such as the inner tube, the sheath, the baseplate, and the fibers. We further examine the relationships of the Afp proteins with available structures from other contractile sheath assemblies.

The Cyclic Symmetry of the Afp Particle—We recorded cryo-electron micrographs of tilted Afp particles (Fig. 1B) to carry out an asymmetric reconstruction with no imposed symmetry (Fig. 2). Although the reconstructed map is of low resolution, it displays unambiguous C6 symmetry. This symmetry is in contrast to that arrived in a previous study where only side views from nominally untilted specimens were analyzed, and it was concluded that the helical sheath has C4 symmetry (1) (the reasons for this ambiguity are addressed in the supplemental Results and Figs. S1 and S2). Tomography of negatively stained Afp particles is also consistent with the C6 symmetry (Fig. 4).

The Structure of the Afp Particle—The Afp is an ~110-nm-long bullet-shaped particle with a tapered end at one extremity and a baseplate of ~30-nm diameter at the other with a global 6-fold cross-sectional symmetry (Fig. 3A). A central tube of inner diameter ~4 nm and outer diameter ~7.5 nm ran ~97 nm along the length of the particle. The sheath surrounding the
central tube is composed of subunits, each with two domains similar in size and shape and a third, protruding domain attached to the second domain. The estimated sizes of these domains are ~50 kDa for the first, ~38 kDa for the second, and ~14 kDa for the third. The whole subunit is about 100 kDa in size, too large for only one of the putative sheath proteins listed in Table 1. The sheath subunits are arranged in 10 layers, each with a ring of six subunits. At the tapered end, an additional ring is present with significantly different shape and no protruding domain but still following the helical pattern. This is capped by a smaller 6-fold ring, potentially the Afp16 protein required for proper sheath assembly (18).

At the other end, the structure widens into the baseplate, displaying six prominent protrusions and a needle-like structure emerging from the center. Six thin (~1.2–1.8 nm in diameter) volumes of density in the form of straight fibers are attached to the baseplate protrusions, folded back with a left-handed twist onto the sheath, and connected to sheath protrusions. The averaged length of the fibers measured from the tomograms (as in Fig. 4A) was 40 nm (S.E. = 0.7 nm). The length of the fiber in the map (Fig. 3A) was 40 nm as well, stretching from the baseplate to sheath layer 4. In the negatively stained specimens, many fibers were dislodged from the host particles or rotated away from the sheath (Fig. 4A). In the absence of sheath in the TBC particle, the fiber density was less defined (Fig. 3E), and many of the fibers were rotated away from the central tube. The attachment of the fibers to the sheath was, therefore, weaker than to that to the baseplate but still strong enough to be preserved in the frozen-hydrated specimens. The fibers may dissociate more readily in stained samples, either mechanically or due to the chemical environment (such as the lower pH of the stain).

The Relationship of Putative Sheath Proteins of Afp with Those in Other Similar Sheathed Structures—The sheath-like proteins of Afp previously identified, Afp2, Afp3, and Afp4, are closely related to the Photorhabdus virulence cassettes (>40% identity) (4). However, the closest relatives with available structural information are the sheath proteins of the R-type pyocin and the tail of phage T4 (Table 1). These sheath proteins show overall identities of around ~25% and similarities of ~40% only in the C-terminal regions. In the sheath protein of T4, gp18, the C terminus is involved in the polymerization of the sheath and is the only part for which an atomic resolution structure is not yet available (14). This suggests that there may be some conservation in a polymerization domain, whereas the rest of the protein may adopt different conformations in the various cases.

Both the R-type pyocin and T4 sheaths show similarities to the Afp sheath as well as important differences. All three have 6-fold disks stacked on top of each other in a helical arrangement, 10 for Afp, 35 for R pyocin (29), and 23 for T4 (30). The heights of the disks are 3.7 nm for R pyocin (29), 4.06 nm for T4 (30), and 8.14 nm for Afp (Fig. 3A). The Afp subunit appears to have two similar segments along the protofilament, giving a subdivision of the disk into two parts, each ~4 nm high. For gp18, the polymerization domain is about 150 residues in size (14) and similar in diameter (~4 nm) to one of the domains of the Afp subunit. Although there is amino acid similarity between parts of the Afp sheath proteins (Table 1), the low sequence similarity suggests that the atomic structures may differ significantly.

The rotation angle around the helix axis for each subunit of the R pyocin sheath was given as 100.8° (31). Given the C6 symmetry, an equivalent angle is ±19.2°, with the sign depending on the hand (unknown for R pyocin). The corresponding rotation angle for the T4 sheath is 17.2° with a right-handed twist of the protofilament (30). The Afp sheath also has a right-handed twist but with a rotation angle of 40.5° per subunit.

The T4 tail has six long tail fibers attached to the sheath and span from the baseplate to the collar (30). The fibers in the Afp particle also lie against the sheath, but each only spans a distance of 40 nm up the sheath (Fig. 3A). As with the T4 long tail fibers, the Afp fibers can rotate away from the sheath (Fig. 4A).
The Repeating Motif in the Central Tube Resembles the Type VI Secretion System Protein Hcp1—The Hcp1 protein (17 kDa) of the T6SS of *P. aeruginosa* is a ring-like hexamer with internal and external diameters of ~4 and ~8.5 nm, respectively (17). The hexamers can be stacked to form a tube (32). The diameter and wall thickness of the Hcp1 hexamer is about the same as the central tube in the Afp particle and in the maps for the TBC. Counting the fenestrations in the central tube indicates a maximum of 24 repeating units, each approximately the height of Hcp1. A tube with 24 Hcp1 hexamers repeated in helical fashion with a 4.06-nm rise and 20.1° rotation fits the inner tube of the Afp particle (orange). The puncture device and Hcp1 hexamers (blue) are packed closely but with little or no overlap of the loops. Scale bar, 10 nm.

The Afp Needle May Be a Puncture Device—The tip of the bacteriophage T4 tail features a puncture device composed of three copies each of gp5 and gp27 (15). The head of this assembly is homologous to VgrG, a T6SS protein (16) with some amino acid sequence similarity to Afp8 (4). The gp5-gp27 structure of T4 with the lysozyme domains removed fits well into the needle-tip of the Afp particle or that in the TBC (Fig. 5). Part of VgrG also shows homology to Hcp1, suggesting that it forms the base of the tube (16). In the Afp particle, we find that the X-ray models of Hcp1 and gp5-gp27 can be placed in a manner that suggests plausible interactions between the corresponding Afp orthologues (Fig. 5).

Density in the Tube May Be Toxin and/or Ruler Protein—The Afp particle has density inside the length of the tube cavity (Fig. 3B). This is absent from the TBC particle (Fig. 3, F and G), which lacks the proteins Afp16–18. One possibility is that one or more molecules of the toxin proteins (Afp17/18) was packaged in the tube. A second possibility is that some of the density is for a ruler protein that determines the length of the Afp particle as has been described in bacteriophages (33, 34). The TBC particles have variable length tubes, indicating that any length-determining factor is absent. Afp16 has been shown to form small 6-fold rings that are approximately the size of the cap at the top of the Afp particle (Fig. 3A). It plays some role in determining the length of the Afp particle and supporting the sheath (18) but is likely to be similar to the proposed cap protein of bacteriophage T4, gp3 (35).

**Conclusion**—This study adds valuable insight into the structure and function of a growing class of needle-type injection systems that include the T6SSs and contractile bacteriophage tails. We have presented the three-dimensional structure of the resting state of Afp particle, a new member of T6SS, and a sheathless Afp mutant, the TBC-based on analyses of single particle images. This is the first reported structure of a toxin-delivery “tailocin” composed of components familiar in tailed bacteriophages and R-pyocins, namely a central tube surrounded by a helical sheath attached to a baseplate at one end and fibers attached to the baseplate. The various components in the particle are well defined, as demonstrated by the quality of the reconstruction.

T6SS are encoded by a single gene cluster present in ~25% of sequenced Gram-negative bacterial genomes (36). It was suggested based on structural similarities of the protein components that T6SS mimic a bacteriophage machinery to puncture target cell membranes and translocate their protein cargo (10). In this study we observe that the central tube shows remarkable similarity to the structure of the putative tube of the T6SS, and the baseplate needle corresponds to the size and shape of the puncture device of bacteriophage T4. This strongly validates the notion that the putative puncturing device in Afp and those in other protein-delivering T6SSs are structurally conserved, sharing a common mechanism for cell rupture (37). Although the “inner core” shows conservation, clearly there exists divergence in the sheath structure among T6SS members as discussed above. The sheath is composed of a trilobed subunit structure that may include two or more of the can-
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didate proteins, Afp2, Afp3, and Afp4, unlike only one in other T6SS members, e.g. T4 tail and R-pyocin. This could reflect on the sheath organization and particular mechanism controlling the contractile apparatus in Afp for which our current three-dimensional structure provides a platform for further investigations.

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