

Ultrastructural Analysis of IpaD at the Tip of the Nascent MxiH Type III Secretion Apparatus of *Shigella flexneri*

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Shigella flexneri is a Gram-negative bacterium that is a significant cause of dysentery after ingestion of only 10-200 bacteria. *Shigella* uses its type III secretion system (T3SS) to deliver proteins into target cells, hijacking normal cellular functions. The type III secretion apparatus (T3SA) consists of a basal body and an extracellular needle. Nascent T3SA needles have at their tip the Invasion plasmid antigen D (IpaD). The presence of IpaD controls tip secretion and acts as an environmental sensor for triggering recruitment of the first translocator protein to the bacterial surface. Our hypothesis was that IpaD is situated at the needle tip alone and thereby blocks subsequent translocators as well as secretion. To test this hypothesis, we set out to determine the structure of the nascent T3SA needle tip, and verify its components.

The available IpaD crystal structure [1] could not be modeled at the tip of the T3SA such that it allowed passage of translocators and effectors. Here we use transmission electron microscopy (TEM) and EMAN2 (Electron Microscopy ANalysis Software) to produce the first three-dimensional (3D) reconstruction of wild type IpaD, IpaD^{Δ192-267}, and an IpaD-null needle tip from *S. flexneri*. We show that wild type IpaD sits atop the T3SA needle tip as an elongated homopentamer [2]. The central coiled coil of IpaD is situated 20° from the needle axis, the distal domain is elongated, and the N-terminal domain is proximal to the central coiled coil. Furthermore, we show that the distal domain, which is necessary to prevent hyper-secretion of effector proteins, is positioned to block the central channel of the T3SA needle.

Bacterial cultures: *S. flexneri* were grown to late-log phase in TSB complemented with 0.2% arabinose, harvested and resuspended in 2% glutaraldehyde. **Needle shearing:** Bacterial suspension was pushed through a 21G needle to allow the removal of T3SA needles by shear force. **Grid Prep:** Needles are laid on carbon-coated 300 mesh copper grids. Samples are washed with Tris-EDTA buffer and stained with 1% uranyl acetate. **Transmission Electron Microscopy (TEM):** Grids were imaged onto film using a Philips transmission electron microscope at an electron acceleration voltage of 100kv and a magnification of 60,000x. **Tobacco Etch Virus (TEV) cleavage:** TEV was added to an unfixed bacteria resuspended in PBS and allowed to incubate for 30 min at 32°C followed by 30 min at 37°C. Culture supernatant was collected and proteins were precipitated with TCA and analyzed by western blot.

References

- [1] Johnson, S., Roversi, P., Espina, M., Olive, A., Deane, J. E., Birket, S. et al. (2007). Self-chaperoning of the type III secretion system needle tip proteins IpaD and BipD. *J. Biol. Chem.* **282**, 4035–4044.
- [2] Epler, C. R., Dickenson, N. E., Bullitt, E. & Picking, W. L. (2012). Ultrastructural analysis of IpaD at the tip of the nascent MxiH type III secretion apparatus of *Shigella flexneri*. *J Mol Biol* **420**, 29-39.

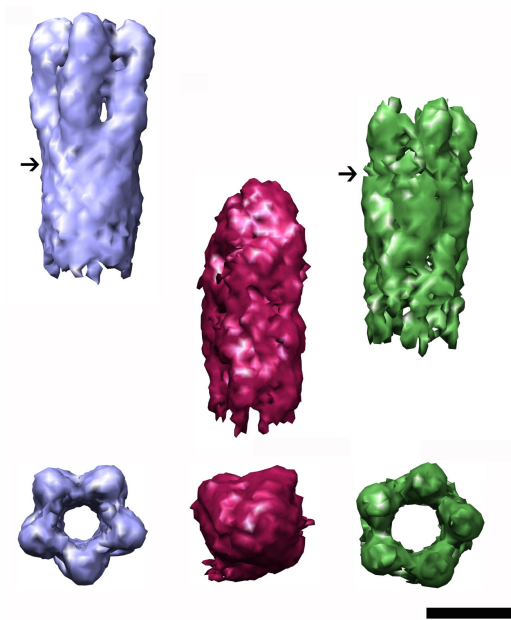


Figure 1. *Type III Secretion Apparatus Tips.* Electron density maps of wild-type IpaD (blue), *ipaD*-null (magenta), and IpaD Δ 192-267 (green) are shown at 20Å, 24Å and 22Å resolution, respectively. The lower panels show the reconstructions tilted 90° in the positive *z* plane to show the constricted opening of wild-type IpaD and *ipaD*-null needle ends. The black arrows indicate the collar region and the scale bar is 50Å.

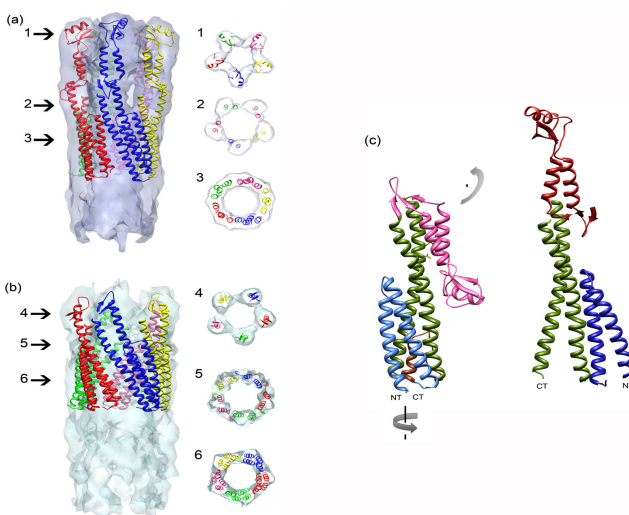


Figure 2. *Modeling the IpaD Crystal Structure into Electron Density Maps.* To find a satisfactory fit the crystal structure of IpaD had to be elongated by shifting the distal domain upward (c, pink to red). The central coiled coil fits at a 20° tilt from the needle axis while the N-terminal domain pivots proximal to the central coiled coil (c, light blue to blue). The numbers and arrows are highlighting 15-Å-slices to show the fit. Wild-type IpaD (a) was modeled with full length IpaD while IpaD Δ 192-267 (b) was modeled with IpaD minus residues 192-267.