Raymond Andrew Prize 2015

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Structural determination of large assemblies at atomic resolution using solid-state NMR and electron microscopy: the Type-Three Secretion Needle of *Shigella* bacteria

The Type-Three Secretion System (T3SS) is a large supra-molecular assembly found in pathogenic Gram-negative bacteria. Bacteria use this system to deliver toxic proteins into a target host cell, such as human intestinal epithelial cells. The T3SS contains a needle which extends into the extracellular space. Upon contact of the needle tip with the host cell, translocator proteins form a pore through which effector proteins enter and subsequently alter host cell processes during infection[1-2]. The first molecular model of the T3SS needle was proposed in 2006 by docking the crystal structure[3] of a truncation mutant the *Shigella flexneri* needle subunit protein MxiH into a 16-Å negative stain EM density map[4]. Following improvements in the sample production protocols[5-6], the atomic structure of the *Salmonella typhimurium* T3SS needle has been determined by solid-state NMR (ssNMR)[7]. Shortly after, a cryo-electron microscopy (cryoEM) density map of the *Shigella flexneri* T3SS needle was determined with a resolution of 7.7 Å[8]. The two models of the *Shigella* needle, determined on the basis of cryoEM data, have the N-terminus of the needle subunit protein (MxiH) located on the inside of the assembly, pointing to the lumen of the needle. The model of the *Salmonella* needle, determined on the basis of solid-state NMR data and scanning transmission electron microscopy (STEM) data, has the N-terminus of the needle subunit protein (PrgI) located on the outside surface of the assembly, pointing towards the extra-cellular space. In addition, the secondary structure identified in the cryoEM model of MxiH contains an additional β-hairpin to account for a "protrusion" in the EM density map while the model of PrgI contains only α-helix—loop—α-helix as secondary structure elements. This created a controversy in the study field of the Type-III Secretion System: since the primary sequence of the homologous proteins PrgI and MxiH share 60%

identity, it was expected that the two needles would have similar structure and organization.

We thus aimed to answer the following questions:

- What is the secondary structure of MxiH in the assembled needle?
- What is the needle subunit orientation within the assembly?
- Is there a conserved architecture among bacterial needles?

We recorded ssNMR spectra on uniform and sparse labeled MxiH needles, enabling us to extract the secondary chemical shifts of backbone nuclei. This established that the MxiH protein contains a rigid segment (M1-T11), an N-terminal a-helix (L12-A38), a loop followed by the C-terminal α-helix (Q45-R83). The presence of a β-hairpin was ruled out by this analysis. Immuno-gold labeling experiments on *in vitro*-polymerized needles as well as needles present *in vivo* on Shigella bacteria revealed that the N-terminus of the MxiH subunit is present on the outside surface of the needle assembly, similar to the needle organization proposed for PrgI needles[7]. For the amino acids L12 to R83 of MxiH, we observed that the pattern of secondary chemical shift is highly similar between MxiH and PrgI. A homology model of the MxiH needle, produced for residues L12 to R83, could fit well (correlation of 0.66) in the 7.7-Å cryoEM density of MxiH needles[8], suggesting a single common architecture for all bacterial T3SS needles[9].

We then tackled the following questions:

- Do the two contradicting models of the *Shigella* T3SS needle reflect true differences in needle structure, for example due to the use of different sample production protocols?
- Is it possible to produce an atomic structure which satisfies all experimental data: ssNMR, cryoEM, and immuno-gold labeling?
- What is the conformation of the non-conserved N-terminal region from M1-T11? How can the “protrusion” in the cryoEM density map be explained?

To address these questions, we recorded an extensive set of ssNMR spectra for the purpose of collecting long-range distance restraints.

Using unambiguous long-range correlation, we were able to produce *de novo* a preliminary description of the MxiH protein fold and of the inter-molecular interfaces present in the MxiH needle. Using this description, he could complete the assignment of distance cross-peaks. In total, 12,350 cross-peaks were analyzed in long-range spectra and over 17,850 cross-peaks were analyzed for the full project. We classified the distance cross-peaks either as unambiguous correlations or as ambiguous correlation using a strategy based on the symmetric geometry of helical assemblies.

We developed with our collaborators a hybrid structure calculation approach based on Rosetta modeling to integrate both NMR and EM structural constraints[10]. In order to avoid the introduction of any bias by the initial NMR assignment, we introduced an automatic procedure for cross-validation of PDB models using NMR constraints prepared for the purpose of validation. This procedure is similar to the use of R_{free} used in crystallography. The structure calculations include the 7.7-Å cryoEM density map as restraint. The final structural models are determined to a precision of 0.4 Å backbone RMSD. The final models produced by this hybrid approach (Figure 1) are compatible with all experimental data available: both with the ssNMR constraints, with few NMR constraint violations (4.5%) for the validation set, and with the cryoEM density map, with correlation in the range 0.62–0.67. The MxiH structural model produced by the fit to the cryoEM density map alone[8] however does not satisfy the constraints of the independent NMR validation data set. Using STEM images independently recorded on the *in vitro* polymerized MxiH needles, we confirmed that the polymerized needles and the hybrid models have the same helical symmetry and compatible axial subunit displacement along the helical axis.

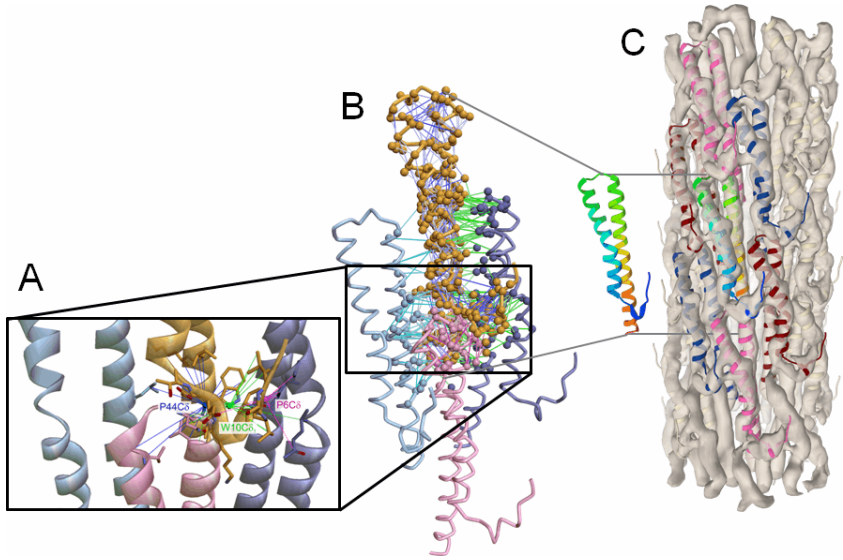

Figure 1: Atomic structure of the T3SS needle of *Shigella flexneri*. A) Detailed view of the N-terminal region (M1-T11) occupying the "protrusion" in the EM density. B) Four adjacent MxiH subunits with intra-molecular and inter-molecular distance restraints represented as colored lines. C) Six adjacent MxiH subunits fitted in the cryoEM density map.

This work resolves the controversy in the field regarding the architecture of bacterial T3SS needles: it is now accepted by the community that T3SS needles adopt an α-helix–loop–α-helix motif with the N-terminus located at the outside surface of the assembly. The final models clearly define the position and conformation of the rigid N-terminus of the MxiH subunit, occupying the protrusion present in the cryoEM density map. This collaborative work has led to the development of a new hybrid structural determination approach which integrates ssNMR and EM data. The final models of the T3SS needle reveal an electrostatically balanced surface, suggesting a mechanism for the transport of toxic substrates through the T3SS needle.

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