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Reassessment of MxiH subunit orientation and fold within native *Shigella* T3SS needles using surface labelling and solid-state NMR

Joeri Verasdonck [a,1], Da-Kang Shen [b,1], Alexander Treadgold [b,1], Christopher Arthur [c], Anja Böckmann [d,*], Beat H. Meier [a,*], Ariel J. Blocker [e,*]

[a] Physical Chemistry, ETH Zurich, 8093 Zurich, Switzerland
[b] School of Cellular & Molecular Medicine, University of Bristol, BS8 1TD Bristol, United Kingdom
[c] School of Chemistry, University of Bristol, BS8 1TS Bristol, United Kingdom
[d] Institut de Biologie et Chimie des Protéines, Bases Moléculaires et Structurales des Systèmes Infectieux, Labex Ecofect, UMR 5086 CNRS, Université de Lyon, Lyon, France
[e] Schools of Cellular & Molecular Medicine and Biochemistry, University of Bristol, BS8 1TD Bristol, United Kingdom

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ABSTRACT

T3SSs are essential virulence determinants of many Gram-negative bacteria, used to inject bacterial effectors of virulence into eukaryotic host cells. Their major extracellular portion, a ~50 nm hollow, needle-like structure, is essential to host cell sensing and the conduit for effector secretion. It is formed of a small, conserved subunit arranged as a helical polymer. The structure of the subunit has been studied by electron cryomicroscopy within native polymers and by solid-state NMR in recombinant polymers, yielding two incompatible atomic models. To resolve this controversy, we re-examined the native polymer used for electron cryomicroscopy via surface labelling and solid-state NMR. Our data show the orientation and overall fold of the subunit within this polymer is as established by solid-state NMR for recombinant polymers.

1. Introduction

Type III secretion systems (T3SSs) are found in many Gram-negative bacteria, forming injection devices to deliver bacterial proteins into eukaryotic host cells during infection. Hence, understanding their three-dimensional structure is important for design of new broad-spectrum strategies to combat bacterial pathogens (Kosarewicz et al., 2012). These macromolecular assemblies are composed of ~25 proteins and span the bacterial cytoplasm, membranes and the extracellular space to connect with the host cell plasma membrane. T3SSs are made up of three parts (Blocker et al., 1999): a cytoplasmic portion, a transmembrane region, and an extracellular 'needle'. The inner membrane region houses the export apparatus, which connects to the periplasmic end of the needle, itself held within the central cavity of an outer membrane secretin (Hodgkinson et al., 2009). The secreted proteins pass into the host cell via a translocation pore assembled from the tip of the needle into the host membrane (Blocker et al., 1999). The needle-like structure is ~50 nm long and 7 nm wide. It is hollowed by a central channel 15 Å in diameter (Fujii et al., 2012), which serves as a secretion conduit for effectors and is made by the helical assembly of a single, small, conserved protein (Blocker et al., 2001; Cordes et al., 2003).

Shigella flexneri, the agent of human bacillary dysentery, uses a T3SS for invasion of, and dissemination within, the gut epithelial lining (Schroeder and Hilbi, 2008). Within the *Shigella* needle, the MxiH subunit is arranged into a helical polymer that shares packing parameters with the extracellular portions of the bacterial flagellar filament (Cordes et al., 2003), to which T3SSs are evolutionarily related. The needle is topped by a "tip complex", which is the host-cell sensor and eventually forms the translocation pore (Cheung et al., 2015; Mueller et al., 2005; Veenendaal et al., 2007). Point mutations in the needle protein lead to deregulated secretion as well as functionally altered tips (Kenjale et al., 2005; Torruellas et al., 2005; Veenendaal et al., 2007), indicating it is directly involved in transducing the signal of host cell contact to the base of the apparatus (Kenjale et al., 2005; Torruellas et al., 2005). This and the fact that it is a major extracellular component of T3SSs justify the interest in understanding its structure.

Abbreviation: CR, Congo red.

* Corresponding authors.
E-mail addresses: a.bockmann@ibcp.fr (A. Böckmann), beme@ethz.ch (B.H. Meier), ariel.blocker@bristol.ac.uk (A.J. Blocker).

[1] Joint first authors.

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Following discovery that short C-terminal deletions prevent MxiH polymerization, partial crystal and NMR structures of monomers from several species revealed a helix-turn-helix hairpin fold (Blocker et al., 2008; Deane et al., 2006; Poyraz et al., 2010). In all these, the terminal half of the N-terminal α-helix is unstructured and invisible to various degrees. The *S. flexneri* pseudoatomic needle architecture was initially modelled by docking $MxiH_{C\Delta5}$ crystal monomers into a 16 Å-resolution electron density map of the natively polymerized needle obtained using negative stain electron microscopy (Cordes et al., 2003). Only one of the two crystallographic forms fitted into that map and it did so in only one orientation (Deane et al., 2006), leading to the N-terminus of MxiH facing the inner side of the channel in this initial model (Deane et al., 2006). This subunit orientation was also used in several subsequent models built using solution-state NMR structures (Chatterjee et al., 2011; Rathinavelan et al., 2010; Wang et al., 2010, 2007; Zhang et al., 2006, 2007) and in a refined model based on a 7.7-Å electron density map obtained using electron cryomicroscopy (cryoEM), where subunit orientation was also assessed using 1–3 amino acid N and C-terminal deletions (Fujii et al., 2012).

However, using solid-state NMR, an atomic-resolution model of the recombinantly generated *Salmonella enterica* serovar Typhimurium needle, composed of PrgI, which is a homologue of MxiH, was determined and the N-terminus was found on the outside face of the needle (Demers et al., 2014, 2013; Loquet et al., 2013a, 2012a). The outside orientation of the N-terminus was confirmed by immunogold labelling of both *Salmonella typhimurium* and *S. flexneri* needles generated by overexpression of their component subunits with 10–15 amino acid N-terminal tags. Subsequently, solid-state NMR measurements of wild-type *S. flexneri* serotype 6 needles, also polymerized *in vitro*, showed its MxiH subunit shared very similar secondary structure elements to those defined for PrgI using the same method (Demers et al., 2013). The N-terminus of the subunit in this polymer was also confirmed to lie on the outside using identical immunolabeling methods. Therefore, an atomic model of *S. flexneri* needles was built by homology to that calculated for *Salmonella* needles. However, the cryoEM and solid-state NMR models differ not only in subunit orientation but also in polypeptide chain location at their surface. Indeed a short "protrusion" is seen within the cryoEM map that is absent or different in each available solid-state NMR model (Demers et al., 2014, 2013; Loquet et al., 2012a). This is why the *Shigella* solid-state NMR model was then recalculated whilst simultaneously imposing constraints derived from the *Shigella* cryoEM map (Demers et al., 2014; Loquet et al., 2012b). However, even in this new model, the N-terminus of the subunit does not fit fully into the protrusion density. This led us to wonder if the native and recombinant polymers might not differ, even though they share similar numbers of subunits per helical turn (Loquet et al., 2013b). We here investigate *in vivo* assembled MxiH needles from *S. flexneri* serotype 5 shaved from the bacterial surface, as used in cryoEM (Fujii et al., 2012), using surface labelling and solid-state NMR and find that they form virtually identical structures to those established by solid-state NMR on recombinant *Shigella* needles.

2. Materials and methods

2.1. Bacterial strains and cell culture

All bacterial strains used in this study are listed in Supplementary Table S1. *S. flexneri* strains were maintained and selected on CR agar plates (Meitert et al., 1991), and grown at 37 °C in trypticase soy broth (Becton Dickinson) supplemented with antibiotics when necessary (100 μg of ampicillin ml^{-1}, 50 μg of kanamycin ml^{-1}, 20 μg of chloramphenicol ml^{-1}; Sigma).

2.2. Cloning and mutagenesis of mxiH

The DNA sequence of $mxiH_{L32C}$ was amplified by two-step PCR using the primers listed in Supplementary Table S2, with modification of T95G and G96T, whilst $mxiH_{V68C}$ was synthesized by Eurofins, with modification of G202T, T203G and G204T. Both constructs were cloned into vector pACT3 via NdeI and HindIII (Shen et al., 2010) and verified by commercial sequencing (Eurofins).

2.3. Analysis of protein secretion

Leakage of the Ipa proteins and CR-induced protein secretion were examined as previously described (Martinez-Argudo and Blocker, 2010).

2.4. Needle preparations for surface labelling

Needles were purified as described previously (Cordes et al., 2005), but using 200 μM IPTG (isopropyl-β-D-thiogalacto pyranoside, Sigma) to induce *mxiH* expression from pACT3*mxiH* (Shen et al., 2010). For wild-type and $MxiH_{L32C}$, 500 ml of trypticase soy broth culture were grown for needle preparation. However, due to the much lower yield of $MxiH_{V68C}$, 2–3 L of this strain were grown to obtain about 250 μg of needles.

2.5. Needle preparations for solid-state NMR

For this purpose, needles were obtained in batches from about 20 L total of bacteria growing in M9-type minimal media (Studier, 2005) supplemented with 2 g ml^{-1} ^{15}N labelled ammonium chloride and U–$^{13}C_6$ labelled D-glucose (Cambridge Isotope Laboratories/CK Gas). Overnight cultures of 5 ml M9 minimal Media with labelled glucose and NH_4Cl, were inoculated with Δ*mxiH* (pACT3*mxiH*; (Shen et al., 2010)), with kanamycin and chloramphenicol. The cultures were incubated at 37 °C with shaking at 180 rpm. After 16 hrs, the optical density at 600 nm (OD_{600}) was measured. If the OD_{600} was >1.00, all of the 5 ml culture was used to inoculate 1 L of M9 minimal media, labelled as above, in a 5 L sterile conical flask, with 200 μM IPTG and incubated at 37 °C with shaking at 180 rpm. After 16 hrs, the OD_{600} was measured again, if OD_{600} was >1.10, growth was halted and needles purified as previously described (Cordes et al., 2005; Fujii et al., 2012). The final pellet was resuspended in 0.01% of the initial culture volume in sterile 20 mM Tris–HCl pH 7.4, 100 mM NaCl, 10% w/v D-(+)-Trehalose (Sigma) and 0.02% w/v sodium azide, generally attaining 3–7 mg ml^{-1} in protein concentration, and then flash frozen in liquid nitrogen and stored at −80 °C until use. From 20 L of bacterial culture about 7 mg of labelled needles were obtained.

2.6. Electron microscopy

Appropriately diluted needles (about 0.1 μg ml^{-1}) were deposited onto 300-mesh, freshly glow-discharged, Formvar and carbon-coated copper grids, and subsequently stained for 1 min with 1% (w/v) phosphotungstic acid at pH 7. Needles were visualized in a Tecnai 12 transmission electron microscope (FEI) fitted with an FEI Eagle 4 k × 4 k CCD camera at ×20,000 magnification using FEI Tecnai Imaging Analysis software.

2.7. PEG maleimide-labelling and analysis

25 μl of needles of 1 mg ml^{-1} in PBS pH 6.6 were used for the cysteine modification by the thiol-specific reagent methoxypolyethylene glycol 5000 maleimide (PEG maleimide; Fluka) as previously described (Hara et al., 2012). Briefly, 2.5 μl of PEG maleimide at 100 mg ml^{-1} was added into needles solution, mixed