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Received 13 March 2013
Accepted 4 May 2013

PDB Reference: TssJ1, 3zhn

Structure of the T6SS lipoprotein TssJ1 from *Pseudomonas aeruginosa*

The type VI secretion system of *Pseudomonas aeruginosa* has been shown to be responsible for the translocation of bacteriolytic effectors into competing bacteria. A mechanistic understanding of this widely distributed secretion system is developing and structural studies of its components are ongoing. Two representative structures of one highly conserved component, TssJ, from *Escherichia coli* and *Serratia marcescens* have been published. Here, the X-ray crystal structure of TssJ1 from *P. aeruginosa* is presented at 1.4 Å resolution. The overall structure is conserved among the three proteins. This finding suggests that the homologues function in a similar manner and bolsters the understanding of the structure of this family of proteins.

1. Introduction

Pseudomonas aeruginosa is a ubiquitous environmental microbe as well as an opportunistic pathogen responsible for a range of diseases including pneumonia and septicaemia. Chronic *P. aeruginosa* infection in the lung of cystic fibrosis (CF) patients relies on the ability of *P. aeruginosa* to form biofilms and ultimately represents the main cause of morbidity in these patients. It is believed that the anti-bacterial action of one of the type VI secretion systems (T6SSs) of *P. aeruginosa* is involved in the colonization of the CF lung prior to the switch to a biofilm-producing state (Rao *et al.*, 2011). The T6SS is the most recently identified secretion system in Gram-negative bacteria. It is broadly represented in bacterial pathogens, where it usually acts as a virulence factor, and is also widely found in environmental bacteria, where it is used in ecological competition. Many of the major discoveries related to the T6SS have been made in *P. aeruginosa*, including the original description of the secretion system (Mougous *et al.*, 2006). The H1-T6SS in *P. aeruginosa* was the first to be shown to translocate bacteriolytic effector molecules to the periplasm of neighbouring bacterial cells and has been shown to be post-translationally regulated by phosphorylation (Mougous *et al.*, 2007; Hood *et al.*, 2010).

Structural characterization of the components of the T6SS from *P. aeruginosa* is only in its early stages, but representative structures of Hcp, VgrG and TssL from a variety of organisms have been determined (Mougous *et al.*, 2006; Leiman *et al.*, 2009; Durand *et al.*, 2012; Robb *et al.*, 2012). Structures of TssJ from *Serratia marcescens* and *Escherichia coli* have been reported (Felisberto-Rodrigues *et al.*, 2011; Rao *et al.*, 2011). These proteins were published using different names (Lip and SciN), but we have elected to follow the general nomenclature for T6SS proteins proposed by Shalom *et al.* (2007). This protein is an outer-membrane-associated lipoprotein that protrudes into the periplasm and it is essential for the function of the T6SS (Aschtgen *et al.*, 2008). TssJ is known to interact with TssM, an integral inner membrane protein that has large domains in both the periplasm and the cytoplasm. This interaction has been mapped to the $\beta 1$ – $\beta 2$ loop of TssJ and this loop has been shown to be necessary for the function of the T6SS (Felisberto-Rodrigues *et al.*, 2011).

Here, we present the X-ray crystal structure of TssJ1 from the H1-T6SS of *P. aeruginosa* (PaTssJ1). The structure was solved at a resolution of 1.4 Å by the single-wavelength anomalous dispersion

(SAD) method using an iodide derivative obtained by the halide-soak approach (Dauter *et al.*, 2000). The protein structure was refined in complex with 12 iodide ions and is very similar to the structures of TssJ homologues from *S. marcescens* and *E. coli*.

2. Materials and methods

2.1. Protein production, purification and crystallization of PaTssJ1

The gene fragment encoding the residues (17–154) that constitute the processed PaTssJ1 protein lacking the N-terminal lipid-attachment site (locus tag PA0080) was amplified by the polymerase chain reaction using *P. aeruginosa* PAO1 genomic DNA. Standard recombinant DNA-cloning procedures were used to clone the product into the *NheI* and *XhoI* sites of pET28b and the resulting recombinant clone was named pET28TssJ1. The primers used included an in-frame 5' *NheI* restriction site within the primer sequence AGGATGGC-TAGCTCGTCCAGCCCGCCGAAACCCC and a 3' *XhoI* site within the primer sequence GGCAGCCTCGAGTCAGGGCGCGG-

GACGCGCGACG to facilitate cloning. The vector created encodes a gene fusion of PaTssJ1 with a thrombin-cleavable N-terminal His tag. 2 l cultures of *E. coli* BL21 (DE3) transformed with pET28TssJ1 were grown to an OD₆₀₀ of ~1.0 in 2×YT medium supplemented with kanamycin; protein production was induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM. Cells were harvested and chemically lysed and the recombinant protein was purified by immobilized metal-affinity chromatography. The protein was concentrated to 19 mg ml⁻¹ (as determined from the absorbance at 280 nm using a calculated extinction coefficient of 0.914 l g⁻¹; Gasteiger *et al.*, 2003) in 20 mM Tris-HCl pH 8.0 without removing the affinity tag. A single crystal was obtained by the hanging-drop vapour-diffusion method at 291 K by mixing the protein solution in a 1:1 ratio with well solution consisting of 0.2 M sodium formate, 15% PEG 5K MME (Fig. 1*a*). The single crystal, which appeared after 2–3 months, was soaked for 10 min in crystallization solution supplemented with 30% ethylene glycol and 1 M sodium iodide and was then flash-cooled directly in an N₂ cryostream at 113 K.

Figure 1

(*a*) The crystal of PaTssJ1. The scale bar represents 200 μm. (*b*) The crystal structure of PaTssJ1 represented by its secondary structure. The strands of PaTssJ1 are numbered sequentially, as are the helices. The structure is colour-ramped from the N-terminus (blue) to the C-terminus (red). (*c*) Iodide coordination by PaTssJ1. The protein is shown as green sticks, a representative I atom is shown as a purple sphere and the electron-density map is shown as a blue mesh. The $2F_o - F_c$ map is displayed at 2.0σ ($1.01 \text{ e}^- \text{ \AA}^{-3}$).

2.2. X-ray data collection, structure determination and refinement

X-ray diffraction data were collected on a Rigaku R-Axis IV⁺⁺ area detector coupled to a MicroMax-002 X-ray generator with Osmic Blue optics and an Oxford Cryostream 700. A total of 275 1° images from 0–275° were collected with 4 min exposures. The data were scaled, averaged and integrated in space group $P2_12_1$ using *d*TREK* (Pflugrath, 1999). Data-processing statistics are given in Table 1.

Phases were generated with *SHELXC/D/E* using the single-wavelength anomalous dispersion (SAD) method (Sheldrick, 2008). The substructure of 11 I atoms determined by *SHELXC/D* had occupancies ranging from 1.0 to 0.13. The correlation coefficient for

finding sites was 45 for all sites and 28 for weak sites, and the Patterson figure of merit was 24.5. Phasing with *SHELXE* using both possible enantiomorphs resulted in final contrast values of 0.54 and 0.47 for the inverted and original hands, respectively. Visual inspection of the resulting maps revealed the inverted hand to be correct and that the maps were of sufficient quality to proceed directly to automated model building. Autobuilding with *Buccaneer* using the phases generated from *SHELXE* in the inverted hand resulted in a nearly complete model lacking iodide (Cowtan, 2006). Manual completion of the heavy-atom substructure with the help of an anomalous difference map resulted in the identification of 12 I atoms in *Coot* (Emsley *et al.*, 2010). The occupancies of these I atoms were

Figure 2

(a) Structural alignment of TssJ homologues. The structural alignment was generated using *SALIGN*, which takes into account both sequence and structural identity (Braberg *et al.*, 2012). The two homologues of PaTssJ1 shown are the two TssJ homologues for which structures have been solved. The secondary structure from the crystal structure of PaTssJ1 is shown above the sequence alignment. (b) Structural comparison of TssJ homologues. The side-by-side comparison of the three homologous structures highlights their similarities. The insertion of a helix between $\beta 6$ and $\beta 7$ in SmTssJ is the largest additional feature that is present among the three.

Table 1

Data-collection and structure-refinement statistics for PaTssJ.

Values in parentheses are for the highest resolution shell.

Data collection	
Resolution range (Å)	19.24–1.40 (1.45–1.40)
Space group	$P2_12_1$
Unit-cell parameters (Å)	$a = 38.3$, $b = 41.6$, $c = 76.9$
$R_{\text{merge}}^{\dagger}$	0.096 (0.460)
Completeness (%)	98.7 (87.1)
Multiplicity	9.27 (5.61)
$\langle I/\sigma(I) \rangle$	10.2 (2.7)
No. of reflections	228911
No. of unique reflections	24682
Mosaicity (°)	0.31
Refinement	
$R_{\text{work}}/R_{\text{free}}^{\ddagger}$ (%)	16/20
No. of atoms	
Protein	1011
Iodide	12
Water	147
B factors (Å ²)	
Overall	16.6
Protein	16.0
Iodide	48.4
Water	27.4
R.m.s.d.s	
Bond lengths (Å)	0.020
Bond angles (°)	2.017
Ramachandran statistics (%)	
Preferred	96.9
Allowed	3.1
Outliers	0.0

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the weighted average intensity for all observations i of reflection hkl . $\ddagger R_{\text{work}} = \sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively, and the statistic is calculated for all reflections except for the test set. R_{free} is calculated accordingly for reflections excluded from refinement (the test set).

refined and 99 water molecules were added automatically using *phenix.refine* (Adams *et al.*, 2010). Final model completion was carried out using manual building in *Coot* and anisotropic refinement in *REFMAC5* (Emsley *et al.*, 2010; Murshudov *et al.*, 2011). 5% of the observations were flagged as free and were used to monitor the progress of refinement. The structure was validated using *MolProbity* (Chen *et al.*, 2010) and was deposited in the PDB with accession code 3zhn.

3. Results and discussion

The asymmetric unit of the *P. aeruginosa* TssJ1 crystal contained a single monomer. The model resulting from refinement against diffraction data to 1.4 Å resolution included residues Pro24–Pro154 of PaTssJ1, 147 water molecules and 12 partially occupied iodide ions. The final refinement statistics R_{work} and R_{free} were 16 and 20%, respectively (Table 1). The protein possesses a compact β -sandwich fold comprising two four-stranded β -sheets. The β -sheets are composed of strands in the order 4–1–7–8 and 3–2–5–6 with a trio of α -helices inserted between $\beta 2$ and $\beta 3$ and packing against the second β -sheet. An especially long pair of loops extend out from one end of the structure between β -strands 1 and 2 and also between β -strands 5 and 6 (Fig. 1b).

The 12 iodide ions in the final structure were modelled using an anomalous difference map. The occupancies of these atoms displayed a range of occupancies from fully occupied to only partial occupancy. The I atoms with the highest occupancy were those bound by hydrogen-bond donors from backbone amides or the amine of tryptophan, as shown in Fig. 1(c). Other I atoms were coordinated by hydrogen-bond donors distributed over the protein surface or were

loosely associated with the solvent sphere surrounding the protein. Based on the similarity of PaTssJ to the other two TssJ structures, it does not appear that the binding of iodide ions to the protein caused significant conformational changes.

Overall, the three structures of TssJ share a significant degree of structural identity. The members of this protein family share a core fold, as demonstrated by comparing PaTssJ1 with TssJ from *E. coli* (EcTssJ) and *S. marcescens* (SmTssJ), which gives root-mean-square deviations of 1.67 and 1.60 Å², respectively (Fig. 2a). A detailed comparison of the structures reveals that the β -sandwich and the first two α -helices overlay easily in the three structures. However, the third α -helix of EcTssJ is rotated out of line compared with the other two structures (Fig. 2b). The conformation and size of the $\beta 1$ – $\beta 2$ loop and the neighbouring $\beta 5$ – $\beta 6$ loop are roughly conserved between the *E. coli*, *S. marcescens* and *P. aeruginosa* homologues, suggesting that these loops may be important for the function of all TssJ proteins. A small deletion inside the first loop in EcTssJ abrogated the observed binding to TssM and also disrupted the function of the T6SS (Felisberto-Rodrigues *et al.*, 2011). It may be that the interaction of this loop with TssM is conserved across all species and the observed structural conservation in the loop supports this hypothesis. The structures show some minor differences, the largest of which is an α -helix that is inserted in the $\beta 6$ – $\beta 7$ loop of SmTssJ but is positioned away from the putative binding site of TssJ and appears to be species-specific. The structure presented here expands upon the representative structures of the TssJ protein family.

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