Purification, crystallization and preliminary X-ray crystallographic analysis of TssL from *Vibrio cholerae*

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The type VI secretion system (T6SS) is a macromolecular complex that is conserved in Gram-negative bacteria. The T6SS secretes effector proteins into recipient cells in a contact-dependent manner in order to accomplish cooperative and competitive interactions with the cells. Although the composition and mechanism of the T6SS have been intensively investigated across many Gram-negative bacteria, to date structural information on T6SS components from the important pathogen *Vibrio cholerae* has been rare. Here, the cloning, purification, crystallization and preliminary X-ray crystallographic analysis of the cytoplasmic domain of TssL, an inner membrane protein of the T6SS, from *V. cholerae* are reported. Diffraction data were collected to 1.5 Å resolution using synchrotron radiation. The crystal belonged to the hexagonal space group $P6_1$, with unit-cell parameters $a = 78.4$, $b = 78.4$, $c = 49.5$ Å. The successful structural characterization of TssL from *V. cholerae* will contribute to understanding the role of the membrane-associated subunits of the T6SS in more detail.

1. Introduction

The type VI secretion system (T6SS) complex found in many species of Gram-negative bacteria has been reported to be involved in cooperative or competitive interactions with both prokaryotic and eukaryotic cells (Russell *et al.*, 2014; Schwarz *et al.*, 2010; Jani & Cotter, 2010). To accomplish such roles, the T6SS apparatus delivers effector proteins into recipient cells in a contact-dependent manner (Russell *et al.*, 2011). The essential components of the T6SS can be categorized into three groups based on their putative functions: bacteriophage tail-related subunits, membrane-associated subunits and unknown proteins (Silverman *et al.*, 2012). The membrane-associated subunits are known to play critical roles in the function of the T6SS by forming the membrane complex composed of TssL, TssM, TssJ and TagL (Felisberto-Rodrigues *et al.*, 2011; Aschtgen *et al.*, 2010). The membrane complex might be involved in the assembly of the T6SS apparatus across the bacterial cell envelope.

Recent structural studies have revealed crystal structures of the N-terminal cytoplasmic domain of TssL from *Escherichia coli* (EcTssL) and *Francisella novicida* (FnTssL) (Robb *et al.*, 2012; Durand *et al.*, 2012). Interestingly, charge-inversion mutations on the $\alpha 3$ – $\alpha 4$ loop and the $\alpha 7$ – $\alpha 8$ loop, which are not conserved among the TssL proteins, significantly attenuated the function of the T6SS in *E. coli* (Durand *et al.*, 2012). TssL from *Vibrio cholerae* shares relatively low sequence identity (less than 20%) with EcTssL and FnTssL. These results suggest that the function of TssL largely depends on unique structural elements which are variable among bacterial species. Although *V. cholerae* is pathogen causing cholera, a life-threatening diarrhoeal disease, to date structural information on T6SS components from this significant pathogen has been rare. To provide insights into the functional mechanism of TssL from *V. cholerae*, we have initiated its structural determination. Here, we

Table 1
Macromolecule-production information.

Source organism	<i>V. cholerae</i>
DNA source	Genomic DNA
Forward primer†	AGATATACATATGTCACAGAGTAAAAAGAG
Reverse primer†	CAAGCTTGTGACAGGATCTGTCTGCTTAG
Expression vector	pET-30a
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced‡	MSQSKKETPLASLLFDDVEKINHDDYWFQLRGDNPVNLIDA-ATPLFGLSLRVRTLTECDNIEQIYRQTIEEIKAEIELTE-QGYEHAIMAYRYILCAFLDESVMGTGWGASSLWAEHSM-LSRFHNETWGGEKVFTILSRLEGEPHRYQALLAFIYHCLIL-GFEGKYRVMGGAEREKVISRLHQLLSLESEPDQLTR-PTDHVVRKYTLRQMPLEHHHHHH

† The *Nde*I and *Xho*I restriction sites are underlined. ‡ The His₆ tag and additional residues are underlined.

report the cloning, purification, crystallization and preliminary X-ray crystallographic analysis of this protein.

2. Materials and methods

2.1. Macromolecule production

The amplified *tssL* gene (UniProt ID Q9KN50) was inserted into the pET-30a vector (Invitrogen, USA) via *Nde*I and *Xho*I restriction sites. The gene insertion was confirmed by DNA sequencing. The resulting expression vector pET-30a:*tssL* was transformed into *E. coli* BL21(DE3) cells, which were then grown at 310 K in Luria–Bertani medium containing 50 µg ml⁻¹ kanamycin until the OD₆₀₀ reached

Table 2
Crystallization.

Method	Vapour diffusion
Plate type for screening	96-well sitting-drop crystallization plate, Axygen
Plate type for optimization	24-well VDX plate, Hampton Research
Temperature (K)	295
Protein concentration (mg ml ⁻¹)	15
Buffer composition of protein solution	10 mM Tris–HCl pH 8.0, 100 mM NaCl
Composition of reservoir solution	1.8 M sodium chloride, 0.1 M sodium potassium phosphate pH 6.4
Volume and ratio of drop	2 µl, 1:1
Volume of reservoir (µl)	500

~0.8. After induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside at 291 K for a further 12 h, the cells were harvested by centrifugation at 5000g at 277 K. All subsequent steps were conducted at 277 K. The cell pellet was resuspended in ice-cold buffer A (30 mM Tris–HCl pH 8.0, 300 mM NaCl) and lysed by sonication. The lysate was centrifuged at 15 000g for 30 min and the supernatant was loaded onto Ni–NTA resin (Qiagen, USA) equilibrated with buffer A. After washing with buffer A containing 20 mM imidazole, the bound protein was eluted in one step using 30 mM Tris–HCl pH 8.0, 50 mM NaCl, 200 mM imidazole. The target protein was bound to a 5 ml HiTrap Q column (GE Healthcare) and eluted with a 20-column-volume linear gradient from 50 to 500 mM NaCl in a buffer consisting of 30 mM Tris–HCl pH 8.0. Finally, the protein was further purified using a Superdex 75 26/60 prep-grade (GE Healthcare) column equilibrated in 10 mM Tris–HCl pH 8.0, 100 mM NaCl. Fractions containing TssL were pooled and concentrated to 20 mg ml⁻¹. The protein concentration was determined by UV absorption spectroscopy with a calculated extinction coefficient $\epsilon_{280} = 35\,410\text{ M}^{-1}\text{ cm}^{-1}$. Aliquots were flash-frozen in liquid nitrogen and stored at 193 K. Macromolecule production is summarized in Table 1.

2.2. Crystallization

In order to facilitate the crystallization of TssL, the purified protein was subjected to limited proteolysis with trypsin (Calbiochem). The trypsin was dissolved in water to a concentration of 0.2 µg µl⁻¹. For each 100 µl of TssL protein at approximately 20 mg ml⁻¹ in buffer consisting of 10 mM Tris–HCl pH 8.0, 100 mM NaCl, 10 mM CaCl₂, 1 µl of trypsin (0.2 µg) was added. The digestion was left for 4 h at 297 K and was stopped by the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). The sample was then used directly for crystallization without further purification. Digested TssL was crystallized by the sitting-drop vapour-diffusion method in 96-well sitting-drop crystallization plates (Axygen). Initial crystallization conditions were screened by the sparse-matrix method (Jancarik *et al.*, 1991) using the commercial kits Index HT, PEG/Ion HT, Crystal Screen HT, SaltRX HT (Hampton Research, USA), Wizard I, II, Cryo I and II (Emerald Bio, USA). All crystallization trials were carried out at 295 K. For screening, 0.4 µl protein solution was mixed with 0.4 µl reservoir solution and equilibrated against 70 µl reservoir solution. Initial crystals were obtained from Wizard I condition No. 16 consisting of 2.5 M sodium chloride, 100 mM sodium potassium phosphate pH 6.2. To obtain crystals suitable for X-ray diffraction, the initial crystallization conditions were further optimized by varying the concentration of protein, pH and salts using the hanging-drop vapour-diffusion method. The same volumes of protein and reservoir solution (2 µl) were mixed and equilibrated against 500 µl reservoir solution in 24-well trays (Hampton Research, USA). Crystallization is summarized in Table 2.

Figure 1
Preparation of the protein sample. (a) Gel-filtration chromatography of *V. cholerae* TssL. The apparent molecular weight of TssL was analyzed with a Superdex 75 column. The blue dots indicate the elution positions of standard marker proteins: conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease (13 kDa) and aprotinin (6.5 kDa). (b) Limited proteolysis of TssL with trypsin. Truncated TssL appeared as a single form (~20 kDa) after trypsin digestion for 360 min.

2.3. Data collection and processing

For X-ray data collection, a single crystal was immersed briefly into reservoir solution containing 20% glycerol as a cryoprotectant and was immediately flash-cooled in a 100 K nitrogen stream. Native X-ray diffraction data were collected using an ADSC Q315r CCD detector on beamline 5C at Pohang Accelerator Laboratory (PAL; Republic of Korea) using 1° oscillations with a crystal-to-detector distance of 150 mm. The crystal was exposed for 1 s per image. A data set was collected to 1.5 Å resolution from a single crystal. The data were indexed and scaled with the *HKL-2000* software package (Otwinowski & Minor, 1997).

3. Results and discussion

TssL is one of the essential components of the T6SS and is a membrane protein that is anchored through a C-terminal transmembrane helix. To facilitate purification and crystallization, the C-terminal 38 residues forming the transmembrane helix were not included in the expression construct. The resulting construct is predicted to contain a similar structure as TssL (DotU) orthologues, which contain only seven α -helices based on an *InterProScan* analysis (Zdobnov & Apweiler, 2001). Recombinant TssL protein (residues 1–219) was successfully overexpressed and purified using sequential chromatographic steps after nickel-affinity chromatography. The final yield of purified protein was ~ 20 mg per litre of *E. coli* culture. The calculated monomeric molecular weight of TssL including a C-terminal His tag was 26 435 Da and the protein eluted at approximately 30 kDa on size-exclusion chromatography, suggesting that it exists as a monomer in solution, as observed previously (Durand *et al.*, 2012; Fig. 1*a*). The homogeneity was assured by SDS-PAGE, on which a single band with 98% purity was observed (data not shown).

The first crystallization attempt was carried out using purified TssL under various conditions. However, all of the crystallization trials using purified TssL were not successful, indicating that flexible loops in the protein might hinder the crystal packing of the protein. As the next trial to facilitate crystallization, the purified protein was subjected to limited trypsin digestion. Aliquots of TssL were treated with trypsin at a ratio of 1:10 000(*w:w*) at 297 K for 4 h and the reaction was then stopped by adding 1 mM PMSF. The tryptic

proteolysis of purified TssL produced a fragment of approximately 20 kDa (Fig. 1*b*). N-terminal amino-acid sequencing of the trypsinized TssL revealed the trypsin cleavage site to be at lysine residue 6 (data not shown), which could not explain the large mobility shift of trypsinized TssL on SDS-PAGE (Fig. 1*b*). Therefore, there should be another cleavage site in the C-terminal region. The susceptibility of the N-terminal and C-terminal regions to proteolytic cleavage suggests that intrinsic flexibility of the terminal regions probably hindered the first crystallization attempt. The initial crystals of trypsinized TssL were obtained from Wizard I condition No. 16 consisting of 2.5 M sodium chloride, 0.1 M sodium potassium phosphate pH 6.2. The quality of the crystals was improved by decreasing the protein concentration to 15 mg ml⁻¹ and the sodium chloride

Figure 3
(*a*) Representative X-ray diffraction image of a TssL crystal from *V. cholerae*. The crystal was exposed for 1 s over a 1° oscillation range. In the magnified inset, diffraction spots extending to 1.5 Å resolution can be observed. (*b*) A schematic drawing of the crystallographic packing of TssL. The C α trace of one TssL molecule is coloured yellow and those of symmetry-related molecules are coloured grey. The hexagonal unit cell is shown in a projection along the *c* axis and is coloured grey.

Figure 2
Crystal of TssL from *V. cholerae*. The crystal grew within one week at 295 K to maximum dimensions of approximately $80 \times 80 \times 120$ μm .

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline 5C, PAL
Wavelength (Å)	0.9795
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	150
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	<i>P</i> 6 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	78.4, 78.4, 49.5
α , β , γ (°)	90, 90, 120
Mosaicity (°)	0.263
Resolution range (Å)	30.00–1.50 (1.53–1.50)
Total No. of reflections	307105
No. of unique reflections	27818
Completeness (%)	99.76 (99.89)
Multiplicity	11.0 (8.5)
$\langle I/\sigma(I) \rangle$	59 (6.63)
$R_{\text{r.i.m.}}$ (%)	6.92 (36.09)
Overall <i>B</i> factor from Wilson plot (Å ²)	14.8

concentration to 1.8 *M*. After optimizing the crystallization conditions, hexagonal trapezohedron crystals were obtained with dimensions of approximately 80 × 80 × 120 µm in a week (Fig. 2). Crystals were grown in sitting drops by mixing equal volumes of protein (15 mg ml^{−1}) and reservoir solution consisting of 1.8 *M* sodium chloride, 0.1 *M* sodium potassium phosphate pH 6.4.

A selected TssL crystal diffracted to a resolution of 1.5 Å and belonged to space group *P*6₁, with unit-cell parameters *a* = 78.4, *b* = 78.4, *c* = 49.5 Å (Fig. 3*a*). The diffraction data set had a completeness of 99.9% with an $R_{\text{r.i.m.}}$ of 6.92% (Table 3). The asymmetric unit contained one TssL molecule of about 20 kDa, resulting in a crystal volume per protein weight of 2.58 Å³ Da^{−1} and a solvent content of 52.3% (Matthews, 1968).

Phase determination by molecular replacement was attempted using the programs *MOLREP* (Vagin & Teplyakov, 2010) and *Phaser* (McCoy *et al.*, 2007). An initial search using the structure of TssL from *E. coli* (PDB entry 3u66; Durand *et al.*, 2012) as a starting model

failed despite a sequence identity of 26% with the starting model. However, we found that the correct solution could be obtained using both of the programs when we used a truncated model lacking a C-terminal loop region (residues 159–178). This result indicates that the C-terminal region could lead to clashes in the crystal lattice during the molecular-replacement search. Therefore, the corresponding region seems to be missing from the crystallized protein or may have a different conformation in the crystal. The best MR model give *Z*-scores of RFZ = 4.0 and TFZ = 3.7, a log-likelihood gain of 32.7 and an R_{free} of 47.8% in the resolution range 30–1.5 Å (Fig. 3*b*). Further structural refinement is now in progress.

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