The SecB-like chaperone Rv1957 from *Mycobacterium tuberculosis*: crystallization and X-ray crystallographic analysis

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Protein export is important in all bacteria, and bacteria have evolved specialized export machineries to fulfil this task. In *Mycobacterium tuberculosis*, the causative agent of tuberculosis, the general secretion pathway (Sec pathway) is conserved and is essential in performing the export of proteins. The bacterial Sec pathway post-translationally exports unfolded proteins out of the cytoplasm, and the core of the Sec pathway is composed of a heterotrimeric membrane-embedded channel, SecYEG, and two cytosolic components, SecA and SecB. SecB functions by stabilizing unfolded proteins, maintaining them in an export-competent state. Although SecB is mainly found in Proteobacteria, a SecB-like protein, Rv1957, that controls a stress-response toxin–antitoxin system, is found in *M. tuberculosis*. Rv1957 can also functionally replace the *Escherichia coli* SecB chaperone both *in vivo* and *in vitro*. In this work, the production, crystallization and X-ray crystallographic analysis of Rv1957 are reported. Notably, diffraction-quality crystals were obtained only at high concentrations of dimethyl sulfoxide, *i.e.* about 12% (*v/v*). The crystals of Rv1957 belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 64.5$, $b = 92.0$, $c = 115.4$ Å.

1. Introduction

It is estimated that one third of the world's population is infected with latent tuberculosis (TB) and that 9.6 million individuals have fallen sick (World Health Organization, 2015). TB now ranks alongside HIV/AIDS as a leading cause of death worldwide, killing 1.5 million people in 2014 (World Health Organization, 2015). With the emergence of multidrug-resistant TB and co-infection with HIV/AIDS (Wells *et al.*, 2007), there is an urgent need to develop new vaccines and drugs. In fact, the majority of patients are infected with latent TB; the pathogen can survive in the macrophage for a long period of time through inhibition of macrophage maturation (Smith, 2003). Therefore, it is important to understand the pathogen–host interactions in order to reveal how *Mycobacterium tuberculosis* escapes from being killed.

In all bacteria, a significant number of proteins are exported out of the cytoplasm through specific export systems (van Wely *et al.*, 2001; Costa *et al.*, 2015). In *M. tuberculosis*, about 62% of exported proteins are thought to be exported by the general secretory (Sec) pathway (Målen *et al.*, 2007). The bacterial Sec pathway is a post-translational process which delivers unfolded proteins across or into the cytoplasmic membrane. The core of the Sec system is composed of a heterotrimeric SecYEG complex channel embedded in the cytoplasmic membrane and two soluble cytoplasmic components, SecA and SecB (Natale *et al.*, 2008). SecA is an ATPase

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motor that drives preprotein movement into and across the SecYEG channel. SecB functions as a molecular chaperone, binding unfolded precursor proteins, preventing them from aggregation and maintaining them in a translocation-competent state (Sala *et al.*, 2014). This complex is then delivered to the SecA motor for translocation *via* specific interactions between SecA and SecB (Suo *et al.*, 2011; Patel *et al.*, 2006; Randall & Henzl, 2010).

The canonical SecB chaperone was considered to be unique to the phylum Proteobacteria (Driessen, 2001). However, phylogenetic analysis of 3813 bacterial SecB sequences showed that SecB evolved from a common ancestor (Yan & Wu, 2015), and SecB-like sequences can be found in genomes from Planctobacteria, Spirochaetae, Sphingobacteria, Eurybacteria and Endobacteria (Bohnsack & Schleiff, 2010). *M. tuberculosis* encodes a SecB-like protein, Rv1957, which shares 19% amino-acid sequence identity with *Escherichia coli* SecB. The functions of *E. coli* SecB can be substituted by Rv1957 both *in vivo* and *in vitro* (Bordes *et al.*, 2011). Interestingly, the gene encoding Rv1957 is clustered together with genes that are related to stress response (Fivian-Hughes & Davis, 2010; Bordes *et al.*, 2011). This locus, namely *higBA* (host inhibition of growth), encodes a type II toxin–antitoxin (TA) system that functions in stress response (Gupta, 2009; Ramage *et al.*, 2009). Type II TA systems generally consist of two elements: a stable toxin and a less stable antitoxin (Gerdes *et al.*, 2005). The toxin is usually inactivated by forming a tight complex with its cognate antitoxin under normal conditions. In response to specific stress conditions, such as nutrient starvation and hypoxia, the antitoxin is degraded and the free toxin subsequently targets important cellular processes to slow cell growth (Yamaguchi *et al.*, 2011). The *M. tuberculosis* *higBA* system is unusual given that Rv1957 is needed to ensure the normal function of the HigA antitoxin through direct interactions to protect HigA from aggregation and degradation (Fivian-Hughes & Davis, 2010; Bordes *et al.*, 2011). These data suggest that Rv1957 may be a dual-function chaperone that links protein export to the TA system. Further experiments are needed to confirm that Rv1957 can assist in protein export in *M. tuberculosis*.

To date, only structures of SecB from *Haemophilus influenzae* (PDB entry 1fx3; Xu *et al.*, 2000) and *E. coli* (PDB entry 1qyn; Dekker *et al.*, 2003) have been determined, and they share high sequence and structure similarity: 57% sequence identity and 0.57 Å root-mean-square deviation over 448 C α atoms, respectively. *E. coli* SecB is a homotetramer that assembles as a dimer of dimers. The structural details reveal that each monomer is composed of a four-stranded anti-parallel β -sheet and a long α -helix. While *H. influenzae* and *E. coli* both belong to the phylum Proteobacteria, it is interesting and meaningful to characterize SecB from other species. Here, we present the crystallization and preliminary X-ray crystallographic analysis of Rv1957 from *M. tuberculosis*. Molecular replacement using available templates failed, indicating large structural deviations from canonical SecB, which may be related to the special function of the *M. tuberculosis* SecB-like chaperone Rv1957.

Table 1
Macromolecule-production information.

Source organism	<i>M. tuberculosis</i> H37Rv
DNA source	Genome
Forward primer†	ATCAGGATCCCATGACTGACCGAACCAGC
Reverse primer‡	AGCTGAATTCCTTTGGTATCGCTTCCTTG
Cloning vector	pET-32M-3C derived from pET-32a
Expression vector	Same as the cloning vector
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced§	MSDKIIHLTDDSFDTVLKADGAILVDFWAEWCG-PCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKG-QLKEFLDANLAGSGSGHMHSHHSSGLEVLFGPGSMTDRDADDDLQRVGARLAARAQIRD-IRLLRTQAQVHRAPKPAQGLTYDLEFEPAVDA-DPATISAFVVRISCHLRINQAADDVKEGDT-KDETQDVATADFEFAALFDYHLQEGEDDPTEE-ELTAYAATTGRFALYPYIREYVYDLTGRLLALPLTLEILSRMPVSPGAQWPATRGTP

† The BamHI site is underlined. ‡ The EcoRI site is underlined. § The tag residues removed by the protease are shown in italics. The residues of native Rv1957 are underlined.

Figure 1
(a) 13% SDS-PAGE stained with Coomassie Brilliant Blue dye showing the purified Rv1957. Lane 5 contains molecular-weight marker (labelled in kDa). Lanes 1–4 and 6 contain the purified Rv1957 fractions eluted from anion-exchange chromatography. (b) Chromatogram representing the purification of Rv1957 by size-exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare, USA). UV absorption was recorded at 280 nm.

2. Materials and methods

2.1. Macromolecule production

The gene encoding Rv1957 was amplified by PCR using the *M. tuberculosis* H37Rv genome as a template and was cloned into the plasmid pET-32M-3C derived from pET-32a (Novagen). The resulting plasmid encodes a protein with an N-terminal thioredoxin fusion partner and a hexahistidine tag followed by a HRV 3C protease recognition site and Rv1957. *E. coli* BL21(DE3) competent cells were transformed with the plasmid and the cells were cultured in 2 l LB medium containing 100 mg l⁻¹ ampicillin. Protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 289 K for 18 h. Cloning and expression details are shown in Table 1. Cells were harvested by centrifugation at 5000 rev min⁻¹ (6238g) for 15 min and the pellet was resuspended in 80 ml PBS buffer (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl pH 7.4). After sonication on ice, the cell lysate was centrifuged (18 000 rev min⁻¹, 39 191g) for 40 min at 277 K and the supernatant was loaded onto 2 ml Ni²⁺-NTA resin (Ni Sepharose 6 Fast Flow, GE Healthcare, USA) packed into a gravity column (Econo-Pac chromatography column, Bio-Rad) and equilibrated with the same buffer. The column was washed with 50 ml PBS buffer containing 20 mM imidazole and the bound hexahistidine-tagged protein was digested on-column by adding 0.5 mg HRV 3C protease for 8 h at 277 K. The native Rv1957 was eluted

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion (screening), hanging-drop vapour diffusion (production)
Plate type	48-well plates (screening), 16-well plates (production)
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	5 mM Tris pH 8.0
Composition of reservoir solution	60 mM lithium sulfate, 100 mM Tris-HCl pH 8.0, 28.5%(w/v) PEG 4000, 12%(v/v) DMSO
Volume and ratio of drop	2 μ l; 1:1 ratio of protein:reservoir
Volume of reservoir (μ l)	100 (screening), 200 (production)

with PBS buffer and buffer-exchanged into ion-exchange start buffer (20 mM Tris pH 8.0) using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore; molecular-weight cutoff 30 kDa). The sample was further purified by anion-exchange chromatography using a HiTrap Q HP column (1 ml; GE Healthcare, USA). Protein purity and homogeneity were assessed by SDS-PAGE and size-exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare, USA) with elution buffer consisting of 20 mM Tris pH 8.0, 150 mM NaCl (Fig. 1). The purified protein was buffer-exchanged into crystallization buffer (5 mM Tris pH 8.0) by a dilution of about 400-fold and was concentrated as described above.

2.2. Crystallization

For crystallization, Rv1957 was concentrated to 10 mg ml⁻¹ as described above. The concentration of Rv1957 was measured from the absorption at 280 nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Initial crystallization trials were performed using Crystal Screen, Crystal Screen 2 and Index (Hampton Research, USA) in 48-well sitting-drop plates (XtalQuest, People's Republic of China). Small needle-like crystals (Fig. 2a) were observed under several conditions in 24 h. The crystals were further optimized in 16-well hanging-drop plates (Tianjin Xiangyushun Macromolecule Technology, People's Republic of China), and diffraction-quality crystals (Fig. 2b) were obtained in a condition consisting of 60 mM lithium sulfate, 100 mM Tris-HCl pH 8.0, 28.5%(w/v) PEG 4000, 12%(v/v) dimethyl sulfoxide (DMSO). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

The crystal was looped out and flash-cooled in liquid nitrogen at 100 K without cryoprotectant. X-ray diffraction data for Rv1957 were collected on beamline BL19U1 at Shanghai Synchrotron Radiation Facility (SSRF). Data were processed using the XDS program package (Kabsch, 2010). Data-collection and processing details are shown in Table 3.

Figure 2
Crystals of Rv1957 (a) before and (b) after optimization.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL19U1, SSRF
Wavelength (Å)	0.97855
Temperature (K)	100
Detector	Pilatus 6M
Crystal-to-detector distance (mm)	500
Rotation range per image (°)	0.5
Total rotation range (°)	360
Exposure time per image (s)	0.5
Space group	$P2_12_12_1$
a, b, c (Å)	64.5, 92.0, 115.4
α, β, γ (°)	90, 90, 90
Mosaicity (°)	0.19
Resolution range (Å)	50.0–3.09 (3.28–3.09)
Total No. of reflections	150884 (24322)
No. of unique reflections	13108 (2069)
Completeness (%)	99.8 (99.4)
Multiplicity	11.5 (11.8)
$\langle I/\sigma(I) \rangle$	31.75 (3.72)
R_{meas} (%)	5.3 (74.6)
Overall B factor from Wilson plot (Å ²)	102.1

3. Results and discussion

Rv1957 was purified *via* a two-step purification protocol. The protein purity and homogeneity were assessed by SDS–PAGE and size-exclusion chromatography. Although the theoretical molecular weight of the Rv1957 monomer is 20 kDa, the protein migrated on SDS–PAGE at an apparently higher molecular weight of about 27 kDa. The same observation was reported by Bordes *et al.* (2011). Rv1957 eluted from the size-exclusion chromatogram at 13.1 ml, which is slightly before BSA (67 kDa, eluting at 13.5 ml), indicating that Rv1957 is most probably a tetramer in solution (Fig. 1b). Initial crystals

were observed under several conditions in 1 d and all were tiny needle-like crystals (Fig. 2a). After extensive optimization, diffraction-quality crystals (Fig. 2b) were obtained in the condition 60 mM lithium sulfate, 100 mM Tris–HCl pH 8.0, 28.5% (w/v) PEG 4000, 12% (v/v) DMSO. It is noteworthy that diffraction-quality crystals could only be grown at such a high concentration of DMSO. DMSO is a powerful polar aprotic solvent which makes preferential interactions with aromatic and hydrophobic amino-acid side chains (Arakawa *et al.*, 2007). Whether the conformation of Rv1957 is affected by DMSO and whether DMSO is participating in crystal packing remains to be determined.

The best crystal diffracted to 3.1 Å resolution (Fig. 3) and belonged to space group $P2_12_12_1$. Data-collection and processing statistics are listed in Table 3. Calculation of the Matthews coefficient in the CCP4 suite (Winn *et al.*, 2011) indicated that there was probably one tetramer (80 kDa) in the asymmetric unit, with a solvent content of 42.7% and a Matthews coefficient of 2.15 Å³ Da^{−1} (Matthews, 1968). A sequence alignment using *Clustal Omega* (McWilliam *et al.*, 2013) showed 21% sequence identity between Rv1957 and *H. influenzae* SecB. Molecular replacement using monomer, dimer or tetramer models of *E. coli* SecB (PDB entry 1qyn; Dekker *et al.*, 2003) or *H. influenzae* SecB (PDB entry 1fx3; Xu *et al.*, 2000) as templates did not lead to correct phasing and no plausible solutions were found. It is likely that large structural differences between Rv1957 and the canonical SecB structure may cause this failure. Crystallization trials using selenomethionine-substituted Rv1957 failed to produce crystals.

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Figure 3
X-ray diffraction image of Rv1957.

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