The VapBC1 toxin–antitoxin complex from *Mycobacterium tuberculosis*: purification, crystallization and X-ray diffraction analysis

Zuokun Lu,^{a*} Han Wang,^a Aili Zhang^a and Yusheng Tan^b

Received 17 March 2016

Accepted 6 May 2016

Edited by N. Sträter, University of Leipzig, Germany

Keywords: VapBC; toxin–antitoxin system; *Mycobacterium tuberculosis*; drug target.

^aCollege of Life Sciences, Nankai University, 94 Weijin Road, Tianjin City 300071, People's Republic of China, and

^bSchool of Life Sciences, Tianjin University, Tianjin City, People's Republic of China. *Correspondence e-mail: luzuokun@mail.nankai.edu.cn

Mycobacterium tuberculosis, a major human pathogen, encodes at least 88 toxin–antitoxin (TA) systems. Remarkably, more than half of these modules belong to the VapBC family. Under normal growth conditions, the toxicity of the toxin VapC is neutralized by the protein antitoxin VapB. When bacteria face an unfavourable environment, the antitoxin is degraded and the free toxin VapC targets important cellular processes in order to inhibit cell growth. TA systems function in many biological processes, such as in the stringent response, in biofilm formation and in drug tolerance. To explore the structure of the VapBC1 complex, the toxin VapC1 and the antitoxin VapB1 were separately cloned, co-expressed and crystallized. The best crystal was obtained using a crystallization solution consisting of optimized solution with commercial sparse-matrix screen solutions as additives. The crystal diffracted to a resolution of 2.7 Å and belonged to space group $P2_1$, with unit-cell parameters $a = 59.3$, $b = 106.7$, $c = 250.0$ Å, $\beta = 93.75^\circ$.

1. Introduction

One of the most striking features of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is its ability to survive within the macrophage, persisting for a long period of time, which is known as latent infection (Smith, 2003). This latency is characterized by the stationary phase, in which the bacteria enter into a dormant, antibiotic-tolerant state. The physiology of *M. tuberculosis* is markedly altered during latent infection (Dutta & Karakousis, 2014). However, little is known about the mechanisms that regulate the persistence of these bacilli in such unfavourable environments and how they are switched on and off.

A toxin–antitoxin (TA) system is a small genetic module typically organized as a bicistronic operon that is composed of a toxin and a labile antitoxin. The protein toxin inhibits cell growth or even kills the cell, and the antitoxin acts by neutralizing the cognate toxin (Pandey & Gerdes, 2005). Based on the nature of the antitoxin, TA systems have been further classified (Yamaguchi *et al.*, 2011). In a type I TA system, the antitoxin is a noncoding RNA which functions by binding the mRNA that encodes the cognate toxin, thus inhibiting the translation of the toxin mRNA. The protein toxin in a type II TA system is inactivated by forming a complex with another protein antitoxin. The type III antitoxin is a noncoding RNA that selectively binds the protein toxin (Gerdes *et al.*, 2005). In response to specific stress conditions, such as nutrition starvation, antibiotic exposure and hypoxia, the antitoxin is degraded and the free toxin is able to target important cellular processes, such as DNA replication and

Table 1
Macromolecule-production information.

	VapC1	VapB1
Source organism	<i>M. tuberculosis</i> H37Rv	<i>M. tuberculosis</i> H37Rv
DNA source	Genome	Genome
Forward primer	GGAGGATCCGGTGGATGAATGTGTAGTCGACGC†	CGTGGATCCATGGCTACCATTCAAGTTCGG†
Reverse primer	GCCAAGCTTTCACCGAACGAGTTTGATTTCGC‡	CTGCTCGAGTCATCCACCCCTGAGCTCCC§
Cloning vector	pRSFDuet-1	pGEX-6P-1
Expression vector	Same as the cloning vector	Same as cloning the vector
Expression host	Co-transformation and co-expression in <i>E. coli</i> BL21(DE3)	
Complete amino-acid sequence of the construct produced¶	MGSSHHHHHSQDPVDECVDVDAAVDALAGKGASAIVLRLKESISN- APHLLDAEVGHARRAVLSDEISEEQARAALDALPYLIDNRYPHSPR- LIEYTWQLRHNVTFYDALYVALATALDVPLLTGDSRLAAAPGLPCEI- KLVR	MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELG- LEFPNLPYYIDGDVKLTQSMATIRYIADKHNMLGGCPKERAISMLE- GAVLDIRYGVSRISAKDFETLKVDFLSKLPKMLKMFEDRLCHKTYL- NGDHVTHPDFMFLYDALDVLYMDPMCLDAFPKLVCFKKRIEAIPIQID- KYLKSSKYIAWPLQGWQATFGGQDHPKSLVLFQGP*LGSPMATIQ- VRDLPEDVAETYYRRRATAAGQSLQTYMRTKLIEGVRGRDKAEAEIL- EQALASTASPGISRETIASRRELRG††

† The BamHI restriction site is underlined. ‡ The HindIII site is underlined. § The XhoI site is underlined. ¶ Non-native residues produced by the construct are underlined. †† The residues before the asterisk are removed after HRV 3C protease digestion.

protein synthesis, to slow down cell growth. Importantly, this inhibition of growth is reversible when environmental conditions become favourable and the cognate antitoxin is synthesized (Sala *et al.*, 2014).

Remarkably, *M. tuberculosis*, a free-living slow-growing bacterium, possesses at least 88 TA loci (confirmed or putative), including members of the MazEF, RelBE, ParDE, HigBA and VapBC families, which may contribute to the long persistence and drug tolerance of this unique pathogen (Ramage *et al.*, 2009; Sala *et al.*, 2014). While it is not clear how the toxins are activated in *M. tuberculosis*, it has been shown in *Escherichia coli* that the protein antitoxins are degraded by the Lon protease pathway initiated by a stochastic high level of the stringent response alarmone (p)ppGpp (Maisonneuve *et al.*, 2011). Of the 88 TA loci in *M. tuberculosis*, 45 belong to the VapBC (virulence-associated protein) family, a type II TA system. The VapC toxin belongs to the PIN (pilT N-terminal) domain family, which are all small proteins (about 140 amino acids) that share ribonuclease activity (Arcus *et al.*, 2005). The translation of mRNA is inhibited by the VapC toxin via mRNA sequence-specific cleavage (Ramage *et al.*, 2009; Ahidjo *et al.*, 2011) or binding (Sharp *et al.*, 2012). Interestingly, VapC20 from *M. tuberculosis* inhibits translation in *E. coli* and *M. smegmatis* by cleavage of the sarcin–ricin loop of the 23S ribosome RNA, and the dormant state of the cells can be resuscitated by induction of the cognate antitoxin (Winther *et al.*, 2013). In *M. smegmatis*, only a single *vapBC* TA operon is found in the genome and the toxin VapC directly targets mRNAs related to carbon metabolism. A $\Delta vapBC$ mutant in *M. smegmatis* showed a 2.4-fold higher rate of glycerol consumption compared with the wild type (McKenzie *et al.*, 2012).

Presently, of the 45 VapBC systems in *M. tuberculosis*, the crystal structures of four VapBC complexes have been determined: VapBC3 (PDB entry 3h87; Min *et al.*, 2012), VapBC5 (PDB entry 3dbo; Miallau *et al.*, 2009), VapBC15 (PDB entry 4chg; Das *et al.*, 2013, 2014) and VapBC30 (PDB entry 4xgr; Lee *et al.*, 2015). Although no physiological experiments have been reported on VapBC1, VapC1 shows sequence-specific

ribonuclease activity *in vitro* (Ahidjo *et al.*, 2011). Furthermore, the toxicity of the toxins makes them attractive targets for antibiotic development. Several peptides have been designed to successfully disrupt the VapBC30 complex from *M. tuberculosis*, and the ribonuclease activity of VapC30 is thus activated (Lee *et al.*, 2015). Therefore, it is important to elucidate the functions of further VapBC systems from a structural point of view. Here, we describe the expression, purification and crystallization of the VapBC1 complex from *M. tuberculosis* H37Rv.

2. Materials and methods

2.1. Macromolecule production

The genes encoding VapC1 (Rv0065) and VapB1 (Rv0064A) were amplified by PCR using *M. tuberculosis* H37Rv chromosomal DNA as template. The gene encoding VapC1 was cloned into pRSFDuet-1 vector (Novagen) to encode a protein with an N-terminal hexahistidine tag. The gene encoding the labile antitoxin VapB1 was cloned into pGEX-6P-1 (GE Healthcare) to encode a protein with a GST fusion partner followed by a HRV 3C protease cleavage site and the antitoxin VapB1. *E. coli* BL21(DE3) competent cells were co-transformed with the two recombinant plasmids and the cells were cultured in LB medium containing 100 mg l⁻¹ ampicillin and 50 mg l⁻¹ kanamycin. For overexpression, 1 l LB medium was inoculated with 5 ml overnight culture at 310 K until the OD₆₀₀ reached 0.6. After induction with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), the cells were incubated at 289 K overnight. The cells were then collected by centrifugation at 5000 rev min⁻¹ (6238g) for 15 min and the pellet was resuspended in 25 ml ice-cold PBS buffer. The cells were sonicated on ice and the crude extracts were centrifuged at 18 000 rev min⁻¹ (39 191g) for 40 min to remove the cell debris. The supernatant containing the protein of interest was loaded onto an Ni²⁺-NTA column (Ni Sepharose 6 Fast Flow, GE Healthcare) pre-equilibrated with PBS buffer and the column was washed with the same buffer

Table 2
Crystallization.

Method	Vapour diffusion
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM Tris pH 8.0, 150 mM NaCl
Volume and ratio of drop	2 µl final volume, 1:1 ratio of protein:reservoir
Volume of reservoir (µl)	100

containing 30 mM imidazole. The VapBC1 complex was then eluted with PBS buffer containing 200 mM imidazole. The eluate was next loaded onto glutathione resin (Glutathione Sepharose 4B, GE Healthcare) and the resin was alternately washed with PBS buffer and PBS buffer containing 1 M NaCl. The column was finally equilibrated with PBS buffer and the complex was digested on-column with 0.1 mg ml⁻¹ HRV 3C protease at 277 K for 8 h.

The VapBC1 complex was then eluted with PBS buffer and buffer-exchanged into ion-exchange start buffer (20 mM Tris pH 8.0, 10 mM NaCl). The sample was loaded onto an anion-exchange column (HiTrap Q HP 1 ml, GE Healthcare) and eluted with a continuous salt gradient of 0.1–0.5 M NaCl in

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL17U, SSRF
Wavelength (Å)	0.97922
Temperature (K)	100
Detector	ADSC
Crystal-to-detector distance (mm)	400.0
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	0.3
Space group	<i>P</i> ₂ ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	59.3, 106.7, 250.0
α , β , γ (°)	90, 93.75, 90
Mosaicity (°)	0.41
Resolution range (Å)	49.1–2.7 (2.86–2.70)
Total No. of reflections	285832 (42574)
No. of unique reflections	83428 (13186)
Completeness (%)	97.5 (96.4)
Multiplicity	3.4 (3.2)
$\langle I/\sigma(I) \rangle$	8.8 (2.6)
<i>R</i> _{meas}	0.13 (0.61)
Overall <i>B</i> factor from Wilson plot (Å ²)	51.4†

† Sharp spikes occurred in the Wilson plot and ice rings were detected at 3.09, 3.67 and 3.44 Å, as shown by *phenix.triage* (Adams *et al.*, 2010).

Figure 1
Purification of the VapBC1 complex. (a) Elution profile of the VapBC1 complex by size-exclusion chromatography. (b) 15% SDS-PAGE showing the pooled fractions of the purified VapBC1 complex. The unstained protein MW marker (Pierce) is in the last lane and the molecular weights are labelled in kDa.

start buffer. The fractions containing the protein of interest were pooled and concentrated to 0.5 ml using an Amicon Ultra Centrifugal Filter Unit (Millipore, 10 kDa cutoff). The concentrated protein was further purified by size-exclusion chromatography using a Superdex 200 10/300 GL column (GE Healthcare), employing an elution buffer consisting of 20 mM Tris pH 8.0, 150 mM NaCl. The purity of the protein complex was assessed using 15% SDS-PAGE. Further macromolecule-production information is shown in Table 1.

2.2. Crystallization

The VapBC1 complex eluted from size-exclusion chromatography was concentrated to 25 mg ml⁻¹ using an Amicon centrifugal filter concentrator as described above. Crystallization screening was carried out using the sitting-drop vapour-diffusion method with the commercial kits Crystal Screen, Crystal Screen 2 and Index (Hampton Research, USA). Standard optimization was performed by varying the concentration or pH of the components of the crystallization solution. For extensive crystal optimization, reservoir solutions were prepared by mixing 80 µl of the optimized crystallization solution and 20 µl of each of the solutions from the commercial sparse-matrix screen kits. This optimization strategy was first described by Birtley & Curry (2005). Diffraction-quality crystals were finally obtained with reservoir solution consisting of 80 µl optimized solution [0.1 M imidazole pH 8.0, 20%(v/v) 2-methyl-2,4-pentanediol, 10%(w/v) PEG 3350, 0.2 M ammonium sulfate] and 20 µl SaltRx (Hampton Research, USA) condition No. 49 (1 M ammonium phosphate monobasic, 0.1 M sodium acetate trihydrate). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

A crystal of the VapBC1 complex was looped out using a Mounted CryoLoop (Hampton Research, USA) and immediately flash-cooled in liquid nitrogen. Diffraction data were collected on beamline BL17U at Shanghai Synchrotron Radiation Facility (SSRF) and were indexed, integrated and scaled using the *XDS* program package (Kabsch, 2010). The data quality was further assessed by using *POINTLESS* in the *CCP4i* GUI (Evans, 2011; Winn *et al.*, 2011). Data-collection and processing statistics are shown in Table 3.

3. Results and discussion

Size-exclusion chromatography showed that the VapBC1 complex eluted as a single homogeneous peak at 13.7 ml from a Superdex 200 10/300 GL column (Fig. 1*a*), which corresponds to a molecular weight of 40–60 kDa, indicating that the VapBC1 complex is a heterotetramer of two molecules of VapB1 (8.7 kDa each) and two molecules of VapC1 (14.3 kDa each). The same assembly is observed in other VapBC complexes. The purity was assessed by SDS–PAGE (Fig. 1*b*).

Tiny crystals were observed in several conditions during initial crystallization screening. Standard optimization was performed by varying each of the reservoir components. However, none of the crystals diffracted to a resolution higher than 3.2 Å. After applying the optimization method described by Birtley & Curry (2005), wedge-shaped crystals were

Figure 2
(*a*) A crystal of the VapBC1 complex obtained by sitting-drop vapour diffusion. (*b*) SDS–PAGE analysis of crystals of the VapBC1 complex. The first lane contains unstained protein molecular-weight marker (Pierce). The molecular weights are labelled in kDa.

Figure 3
X-ray diffraction image of a crystal of the VapBC1 complex. The crystal diffracted to a resolution of 2.7 Å.

obtained (Fig. 2*a*) and the best crystal diffracted to a resolution of 2.7 Å (Fig. 3). In order to verify the integrity of the VapBC1 complex after crystallization, several crystals were looped out and washed with the same reservoir. These crystals were then dissolved in PBS buffer and SDS–PAGE analysis with Coomassie Brilliant Blue staining showed two bands corresponding to VapC1 and VapB1 (Fig. 2*b*).

The crystals of the VapBC1 complex belonged to space group $P2_1$, with unit-cell parameters $a = 59.3$, $b = 106.7$, $c = 250.0$ Å, $\beta = 93.75^\circ$. Calculation of the Matthews coefficient (Matthews, 1968) indicated the most probable presence of six, seven or eight VapBC1 complexes in one asymmetric unit, with V_M values of 2.74, 2.35 and 2.06 Å³ Da^{−1} and solvent contents of 55, 48 and 40%, respectively. A sequence-similarity search of the VapC1 toxin using *Protein BLAST* (*BLASTP*) against Protein Data Bank proteins on the NCBI webpage showed 31% sequence identity to Pae0151 from *Pyrobaculum aerophilum* (PDB entry 2fe1; Bunker *et al.*, 2008); no other similar entries were found, including the four VapBC complexes from *M. tuberculosis* for which structures had been solved. With regard to VapB1, no similar sequence was found at a significant level. Molecular replacement with *Phaser* (McCoy *et al.*, 2007) using Pae0151 from *P. aerophilum* (PDB entry 2fe1; Bunker *et al.*, 2008) as the template failed to lead to a correct solution, probably owing to the lack of a suitable template and/or the presence of too many molecules in one asymmetric unit. Therefore, experimental phasing will be needed to determine the structure of the VapBC1 complex.

Acknowledgements

We are very grateful to the staff of BL17U, Shanghai Synchrotron Radiation Facility for their kind assistance and

suggestions. We are also grateful to Dr Chen Cheng for his fruitful suggestions on crystal optimization.

References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Ahidjo, B. A., Kuhnert, D., McKenzie, J. L., Machowski, E. E., Gordhan, B. G., Arcus, V., Abrahams, G. L. & Mizrahi, V. (2011). *PLoS One*, **6**, e21738.
- Arcus, V. L., Rainey, P. B. & Turner, S. J. (2005). *Trends Microbiol.* **13**, 360–365.
- Birtley, J. R. & Curry, S. (2005). *Acta Cryst.* **D61**, 646–650.
- Bunker, R. D., McKenzie, J. L., Baker, E. N. & Arcus, V. L. (2008). *Proteins*, **72**, 510–518.
- Das, U., Kumar, N., Gourinath, S. & Srinivasan, A. (2013). *Acta Cryst.* **F69**, 1242–1245.
- Das, U., Pogenberg, V., Subhramanyam, U. K. T., Wilmanns, M., Gourinath, S. & Srinivasan, A. (2014). *J. Struct. Biol.* **188**, 249–258.
- Dutta, N. K. & Karakousis, P. C. (2014). *Microbiol. Mol. Biol. Rev.* **78**, 343–371.
- Evans, P. R. (2011). *Acta Cryst.* **D67**, 282–292.
- Gerdes, K., Christensen, S. K. & Løbner-Olesen, A. (2005). *Nature Rev. Microbiol.* **3**, 371–382.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Lee, I.-G., Lee, S. J., Chae, S., Lee, K.-Y., Kim, J.-H. & Lee, B.-J. (2015). *Nucleic Acids Res.* **43**, 7624–7637.
- Maisonneuve, E., Shakespeare, L. J., Jørgensen, M. G. & Gerdes, K. (2011). *Proc. Natl Acad. Sci. USA*, **108**, 13206–13211.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- McKenzie, J. L., Robson, J., Berney, M., Smith, T. C., Ruthe, A., Gardner, P. P., Arcus, V. L. & Cook, G. M. (2012). *J. Bacteriol.* **194**, 2189–2204.
- Miallau, L., Faller, M., Chiang, J., Arbing, M., Guo, F., Cascio, D. & Eisenberg, D. (2009). *J. Biol. Chem.* **284**, 276–283.
- Min, A. B., Miallau, L., Sawaya, M. R., Habel, J., Cascio, D. & Eisenberg, D. (2012). *Protein Sci.* **21**, 1754–1767.
- Pandey, D. P. & Gerdes, K. (2005). *Nucleic Acids Res.* **33**, 966–976.
- Ramage, H. R., Connolly, L. E. & Cox, J. S. (2009). *PLoS Genet.* **5**, e1000767.
- Sala, A., Bordes, P. & Genevaux, P. (2014). *Toxins*, **6**, 1002–1020.
- Sharp, J. D., Cruz, J. W., Raman, S., Inouye, M., Husson, R. N. & Woychik, N. A. (2012). *J. Biol. Chem.* **287**, 12835–12847.
- Smith, I. (2003). *Clin. Microbiol. Rev.* **16**, 463–496.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Winther, K. S., Brodersen, D. E., Brown, A. K. & Gerdes, K. (2013). *Nature Commun.* **4**, 2796.
- Yamaguchi, Y., Park, J.-H. & Inouye, M. (2011). *Annu. Rev. Genet.* **45**, 61–79.