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Preliminary crystallographic analysis of recombinant VapBC-15 toxin–antitoxin complex from *Mycobacterium tuberculosis*

The *Mycobacterium tuberculosis* *vapBC15* locus encodes a toxin–antitoxin complex. VapC-15 is a toxin and possesses ribonuclease activity and VapB-15 is an antitoxin which both binds and inhibits the VapC-15 toxin. In this study, *vapBC15* genes were cloned and co-expressed in *Escherichia coli*. The complex was purified to homogeneity by affinity and size-exclusion chromatography. The VapBC-15 complex was crystallized using the sitting-drop vapour-diffusion technique. The crystals diffracted to 2.6 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 85.63$, $b = 139.09$, $c = 148.86$ Å. The self-rotation function combined with Matthews coefficient and solvent-content calculations suggests the presence of either six or eight molecules of the complex in the asymmetric unit.

1. Introduction

Toxin–antitoxin (TA) loci were first discovered as factors that protect low-copy-number plasmids in bacteria from segregational loss (Jaffé *et al.*, 1985). The TA loci are arranged in bicistronic operons (Anantharaman & Aravind, 2003). The operons code for a stable toxin which targets essential cellular machinery and a labile antitoxin which both binds to and inhibits the toxin. The antitoxin also binds to DNA *via* its N-terminus and interacts with specific sequences within the promoter of the TA operon (Gerdes *et al.*, 1986). Under stress conditions the antitoxin is degraded by cellular proteases, leaving the free toxin to slow the growth of and/or kill the cell (Hayes, 2003).

TA systems are ubiquitous in a wide range of microbial genomes and are usually present in abundance in pathogens such as *Mycobacterium tuberculosis* (*Mtb*), the causative agent of one of the world's deadliest diseases, tuberculosis (TB). The genome of *M. tuberculosis* codes for 88 putative TA loci, of which 47 belong to the virulence-associated protein (Vap) family (Ramage *et al.*, 2009). VapBC is the largest family of TA loci in *M. tuberculosis*. The *vap* gene loci are bicistronic operons coding for two proteins, namely VapB and VapC. The *vapB* gene codes for an antitoxin and the *vapC* gene codes for a toxin. The toxic component, VapC, contains a PIN (homologues of the PilT N-terminal) domain (Wall & Kaiser, 1999), a motif thought to have ribonuclease activity (Arcus *et al.*, 2005). A bioinformatics analysis led to the conclusion that the PIN-domain proteins are Mg^{2+} -dependent ribonucleases and have conserved active-site residues consistent with the architecture of phage Flap endonucleases and T4 RNase H (Clissold & Ponting, 2000).

Presently, the crystal structures of only two VapBC complexes have been determined from *M. tuberculosis* (Miallau *et al.*, 2009; Min *et al.*, 2012) out of 47 putative VapBC family members. The function of these Vaps is still unknown in *M. tuberculosis* and therefore structural and functional elucidations of these protein complexes are necessary in order to understand the pathogenicity of tuberculosis. The VapBC-15 protein complex was found to be induced in hypoxic conditions, suggesting a role in the adaptation of *M. tuberculosis* to a low-oxygen environment (Ramage *et al.*, 2009). Therefore, the structure of the VapBC-15 complex might help in understanding the biochemistry enabling the adaptation of *M. tuberculosis* to a hypoxic environment. Here, we report the cloning of the *vapB15* and *vapC15* genes from *M. tuberculosis* in a pET vector, their co-expression in *Escherichia*

Figure 1

The schematic figure shows the toxin in MCS I (blue) and the antitoxin in MCS II (pink) under the influence of T7 promoters (green) followed by ribosome binding sites (red). The black portion at the end of the antitoxin depicts the C-terminal (His)₆ tag.

coli, the purification and crystallization of the VapBC-15 complex and the preliminary X-ray data analysis. The VapB-15 and VapC-15 proteins in this study have 18 and 20% sequence homology to the previously reported VapB-3 and VapC-3 proteins (Rv0300–Rv0301), respectively, and 16 and 25% sequence homology to the VapB-5 and VapC-5 proteins (Rv0626–Rv0627), respectively, from *M. tuberculosis*.

2. Materials and methods

2.1. Cloning of *vapC15* and *vapB15* from *M. tuberculosis*

The cloning strategy for the co-expression of Rv2009, which encodes VapB-15 (antitoxin), and Rv2010, which encodes VapC-15 (toxin), was designed for the pET-28b vector (Novagen). The vector was engineered with two multiple cloning sites (MCSs) taken from the pETDuet1 vector, each under the influence of a T7 promoter. The open reading frame (ORF) encoding the *vapB15* gene (243 base pairs) was amplified from the genomic DNA of *M. tuberculosis* (H37Rv) using Phusion high-fidelity DNA polymerase (Finnzymes) with the forward primer 5'-GGAATCCCCATATGTATAGTGGTGTGTGTCA-3' and the reverse primer 5'-CTCCCGCTCGAGCGACTCGTCGGCG-3' possessing *Nde*I and *Xho*I restriction-enzyme sites, respectively. The Rv2010 ORF encoding *vapC15* (399 base pairs) gene was amplified in a similar manner using the forward primer 5'-CGCGGATCCGATGATCGTCGACACCTCG-3' and the reverse primer 5'-CCCAAGCTTTCAGAACACGGCTCGGT-3' possessing *Bam*HI and *Hind*III restriction-enzyme sites, respectively. The amplified Rv2010 and Rv2009 genes were digested with the respective enzymes as mentioned above and inserted into the corresponding MCS I and MCS II sites of the pETDuet-1 vector. Thereafter, the entire MCS region of the pETDuet-1 construct possessing Rv2010 at MCS I and Rv2009 at MCS II was re-amplified using the forward primer 5'-CATGCCATGGGCATGATCGTCGACACCTCG-3' and the reverse primer 5'-CTCCCGCTCGAGCGACTCGTCGGCG-3' possessing *Nco*I and *Xho*I restriction-enzyme sites, respectively. The PCR product was digested with *Nco*I and *Xho*I and ligated between the *Nco*I and *Xho*I sites of the pET-28b vector, with the *vapB15* antitoxin gene terminating with a restriction site coding for LE and a C-terminal His₆ tag. A schematic diagram of the arrangement of the toxin and antitoxin genes in MCS I and II, respectively, of the modified pET-28b vector is represented in Fig. 1.

2.2. Expression and co-purification of the VapBC-15 complex

The pET-28b-*vapBC15* construct was transformed into *E. coli* BL21(DE3) cells and the cells were cultured in LB medium supplemented with 50 µg ml⁻¹ kanamycin and grown to an OD₆₀₀ of 0.6 at 310 K. The culture was cooled to 293 K and protein expression was induced by the addition of a 1 mM final concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 h at the same temperature. The cells were harvested by centrifugation at 5000g for 10 min at 277 K and stored at 193 K until further processing.

All chromatographic steps were carried out at 277 K. The cell pellet was resuspended in binding buffer consisting of 25 mM Tris–

HCl pH 7.4, 500 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol to which cOmplete EDTA-free Protease-Inhibitor Cocktail (Roche) was added. The cells were lysed by lysozyme treatment (0.1 mg ml⁻¹ for 30 min on ice) followed by ultrasonication on ice for 6 × 1 min with 25% maximum output power (Bendelin SONO-PULS). The cell lysate was centrifuged at 30 000g for 45 min at 277 K. The clarified supernatant was filtered through 0.45 µm filters and passed through a Nickel–NTA Sepharose (GE Healthcare) column pre-equilibrated with binding buffer. The column was washed with 50 column volumes of washing buffer (25 mM Tris–HCl pH 7.4, 500 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol) to remove non-specifically bound protein. The protein complex was eluted with elution buffer consisting of 25 mM Tris–HCl pH 7.4, 300 mM NaCl, 300 mM imidazole, 10 mM β-mercaptoethanol. Protein purity was checked by 13% SDS–PAGE and protein identity was confirmed by MALDI–TOF. The pooled fractions were concentrated to 2 ml using Amicon Ultra 10 kDa cutoff filters (Millipore) and further purification was performed by size-exclusion chromatography using a HiLoad 16/60 Superdex 75 column (GE Healthcare) pre-equilibrated with buffer consisting of 25 mM Tris–HCl pH 7.4, 100 mM NaCl, 10 mM β-mercaptoethanol. The peak fractions were collected and analysed by 13% SDS–PAGE and the protein concentration was determined spectroscopically (OD₂₈₀). The VapBC-15 complex with a combined molecular mass of 22 500 Da (14 000 Da toxin and 8500 Da antitoxin) has a molar extinction coefficient (ε) of 16 960 M⁻¹ cm⁻¹. These values were used to determine the concentration of the protein complex.

Figure 2

Diffraction image of an *M. tuberculosis* VapBC-15 complex crystal.

2.3. Crystallization

The protein complex was desalted and concentrated to 5 mg ml⁻¹ in 25 mM Tris-HCl buffer pH 7.4 for crystallization trials. The initial crystallization condition was determined using various commercial screens from Hampton Research and Molecular Dimensions. Sitting-drop vapour diffusion was carried out by mixing 1 µl each of protein and precipitant solutions and equilibrating against 50 µl reservoir buffer at 289 K in 96-well sitting-drop plates (Corning). Optimization of the crystallization conditions was performed in standard 24-well crystallization plates at 289 K using drops consisting of 2 µl each of protein and precipitant solutions equilibrated against 1 ml reservoir buffer.

2.4. Data collection and processing

For diffraction data collection, a single crystal was soaked in a cryoprotectant solution containing 22% PEG 400 in addition to the mother liquor and flash-cooled in liquid nitrogen. Diffraction data were collected at 100 K on beamline BM14 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France using a MAR CCD detector. The wavelength of the radiation source was set to 0.9788 Å and a MAR CCD detector was used to record X-ray diffraction intensities as 424 images with an oscillation range of 0.5° per image (Fig. 2). Diffraction data were processed and scaled using *HKL-2000* (Otwinowski & Minor, 1997). The scaled reflections were converted to MTZ files using *SCALEPACK2MTZ* from the *CCP4* suite (Winn *et al.*, 2011) before using these reflections for molecular-replacement trials. Data statistics are reported in Table 1.

3. Results and discussion

The VapB-15 and VapC-15 proteins formed a complex and this was purified to homogeneity by size-exclusion chromatography. Approximately 5 mg protein was obtained from 1 l cell culture. SDS-PAGE analysis showed two proteins with apparent molecular weights of 14 and 16 kDa (Fig. 3). The theoretical molecular weight of the toxin VapC-15 is 14 kDa, corresponding to the 14 kDa band in SDS-PAGE, and this assignment was confirmed by MALDI-TOF (results not shown). The expected molecular weight of VapB-15 with a six-histidine tag is 8.5 kDa, but it appeared at 16 kDa in SDS-PAGE both from the complex as well as when subjected to SDS-PAGE alone, and this assignment was also confirmed by MALDI-TOF (results not shown).

The protein complex crystallized from various conditions, but the best diffracting crystal (Fig. 4) was obtained from the condition 100 mM HEPES pH 7.0, 8% PEG 8000. The resulting crystals grew to maximum dimensions of 0.1 × 0.2 × 0.05 mm within three weeks. The VapBC-15 complex diffracted to 2.6 Å resolution and belonged to space group *P*2₁2₁2₁, with unit-cell parameters *a* = 85.63, *b* = 139.09, *c* = 148.86 Å.

The result of a self-rotation function calculation with *MOLREP* (Vagin & Teplyakov, 2010) using data between 10 and 3 Å resolution is shown in Fig. 5. The $\kappa = 180^\circ$ section shows evidence of only one noncrystallographic twofold axis (and its symmetry-related equivalents). Varying the radius of integration or the resolution of the data input to the calculation did not reveal any additional noncrystallographic symmetry elements. The native Patterson function did not show any significant pseudo-translation. The calculated Matthews coefficient (*V_M*) and solvent content for six molecules were 3.16 Å³ Da⁻¹ and 61.16%, respectively, and those calculated for eight molecules were 2.37 Å³ Da⁻¹ and 48%, respectively, suggesting the presence of either six or eight molecules of the complex in the

Table 1

Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

X-ray source	BM14, ESRF
Wavelength (Å)	0.9788
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 85.63, <i>b</i> = 139.09, <i>c</i> = 148.86, $\alpha = \beta = \gamma = 90$
Matthews coefficient <i>V_M</i> (Å ³ Da ⁻¹)	3.16/2.37†
Resolution range (Å)	50–2.6 (2.64–2.60)
<i>R_{merge}</i> ‡ (%)	7.5 (35.9)
Completeness (%)	99.6 (99.7)
Total No. of reflections	391597
No. of unique reflections	52927 (2601)
Multiplicity	7.4 (7.2)
Average <i>I</i> / σ (<i>I</i>)	34.12 (4.1)
Crystal mosaicity (°)	0.6

† The Matthews coefficient (*V_M*) was 3.16 Å³ Da⁻¹ for six molecules in the asymmetric unit and 2.37 Å³ Da⁻¹ for eight molecules. ‡ *R_{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 intensity of the *i*th measurement of reflection *hkl* and $\langle I(hkl) \rangle$ is the average intensity of reflection *hkl*.

Figure 3

SDS-PAGE profile of the purified toxin-antitoxin complex after size-exclusion chromatography. Lane *M* contains molecular-weight markers (labelled in kDa), and lanes 1 and 2 contain the purified TA complex. Approximately 20 and 10 µg protein was loaded in lanes 1 and 2, respectively.

Figure 4

Crystals of the *M. tuberculosis* VapBC-15 complex.

Figure 5

Stereographic projection of the self-rotation function of the $P2_12_12_1$ reduced data set for $\kappa = 180^\circ$ calculated for data between 10 and 3 Å resolution at a radius of integration of 30 Å using *MOLREP* from the *CCP4* suite. The four minor noncrystallographic peaks are indicated by arrows. The x , y and z axes of the plot align with the a , b and c^* crystallographic axes, respectively.

asymmetric unit (Matthews, 1968). The *M. tuberculosis* VapBC-3 (Rv0300–Rv0301) TA complex (Min *et al.*, 2012; PDB entry 3h87), which shares 39% sequence identity with *M. tuberculosis* VapBC-15,

was used as a template for molecular-replacement studies. PDB entry 3h87 has two heterodimers of toxin (chains *A* and *B*) and antitoxin (chains *C* and *D*) in the asymmetric unit. The antitoxin in this structure is very flexible and the two molecules in the same asymmetric unit had two different conformations. Therefore, we used one toxin molecule (chain *A* alone) and two toxin molecules as a dimer (chains *A* and *B*) for structural solution, without success. Selenomethionine-labelled protein expression is in progress in order to obtain phases.

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