Protein expression, crystallization, and preliminary x-ray crystallographic analysis of the isolated *Shigella flexneri* VapC toxin

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Synopsis  Here we report purification and crystallization of the isolated toxin VapC (MvpT) from the VapBC toxin-antitoxin complex of the pathogen *Shigella flexneri*. The crystals belong to space group H3 and diffract to 1.9 Å resolution.

Abstract  Upon release from the stable complex formed with its antitoxin, VapB, the toxin VapC (MvpT) of the Gram-negative pathogen *Shigella flexneri* is capable of globally down-regulating translation by specifically cleaving initiator tRNA\(^{f\text{Met}}\) in the anticodon region. Recombinant *Shigella flexneri* VapCD7A harbouring an active site mutation was overexpressed in *Escherichia coli*, purified to homogeneity, and crystallized by the vapour diffusion technique. A preliminary x-ray crystallographic analysis shows that the crystals diffract to at least 1.9 Å at a synchrotron x-ray source and belong to the trigonal space group in hexagonal setting, H3, and unit cell parameters a=b=120.1 Å, c=52.5 Å, \(\alpha=\beta=90^\circ\), \(\gamma=120^\circ\). The Matthews' coefficient is 2.46 Å\(^3\) Da\(^{-1}\) suggesting two molecules per a.s.u. and corresponding to a solvent content of 50.0%.

Keywords: X-ray crystallography, toxin-antitoxin, ribonuclease, tRNA, PIN domain
1. Introduction

Toxin-antitoxin (TA) loci have been found in all prokaryotic genomes that have been sequenced to date and typically encode two proteins, a toxin and an antitoxin that associate to form a tight complex in which binding of the antitoxin inhibits the cellular action of the toxin (Makarova et al., 2009; Pandey & Gerdes, 2005). Upon changes in the cellular environment, such as during oxidative stress or nutrient deprivation, the antitoxin undergoes degradation and the more stable toxin is freed from the inhibitory TA complex. Once released, many toxins are active as nucleases that are able to cleave specific cellular RNAs and thus induce growth arrest by down-regulating the overall translational rate (Ahidjo et al., 2011; Yamaguchi & Inouye, 2009). The antitoxins, on the other hand, contain a DNA-binding domain conferring high affinity for the TA promoter, enabling auto-regulation of the level of transcription from the TA operon (Gerdes et al., 2005). TA gene pairs can be subdivided into several families, of which the vapBC (virulence associated proteins) loci are the most abundant (Pandey & Gerdes, 2005). Interestingly, vapBC loci are particularly common in pathogenic bacteria (Ramage et al., 2009) where they are involved in bacterial persister cell formation and thus highly relevant in disease control (Maisonneuve et al., 2011).

VapC toxins are characterized by an approximately 100 amino acid N-terminal ribonuclease motif belonging to the PilT N-terminus (PIN) domain type. This domain harbours four highly conserved acidic residues that have been shown to be essential for metal ion coordination in other PIN domain-containing ribonucleases, but the exact enzymatic mechanism used by the VapC toxins is yet unresolved (Backbro et al., 2004; Fatica et al., 2004). The VapB antitoxins are highly specific towards their cognate toxins and inhibit the ribonucleolytic activity through direct interaction (Ramage et al., 2009). Crystal structures have been determined of both VapBC-like complexes and isolated VapC-like toxins from archaea and pathogenic bacteria, which show a great diversity in both sequence, structure, as well as the cellular target of the toxin (Bunker et al., 2008; Mattison et al., 2006; Miallau et al., 2009). Like for most other TA systems, VapBC complexes bind to the operator sequences of their own promoter region and down-regulate transcription from the operon (Bodogai et al., 2006; Wilbur et al., 2005). A recent crystal structure of the intact VapBC complex from the Gram-negative pathogen, Shigella flexneri, revealed the DNA-binding complex is a compact hetero-octameric assembly with the two DNA-binding domains juxtaposed in a manner compatible with adjacent major groove binding (Dienemann et al., 2011). This structure also showed that VapB inhibits VapC through displacement of divalent metal ions at the active site.

Once activated, S. flexneri VapC (MvpT) has been found to specifically target the anticodon region of tRNA^Met through endoribonucleolytic cleavage by a mechanism involving Asp7 of VapC (Winther & Gerdes, 2011). In order to understand the basis of target RNA recognition and the enzymatic mechanism employed by the Shigella VapC toxin in degrading tRNA^Met, we introduced the D7A mutation with the aim of determining the structure of the VapC^{D7A} in isolation as well as bound to the
anticodon region of tRNA\textsuperscript{D Met}. Here we report the expression, purification and crystallization of the isolated VapC\textsuperscript{D7A} (MvpT) toxin as well as initial crystallographic analysis of diffraction data collected to 1.9 Å. The results provide a basis for determining the crystal structure of the isolated toxin and thus understanding both the principle of toxin activation as well as the mechanism of the ribonucleolytic activity of VapC.

2. Materials and methods

2.1. Cloning

The vapC\textsuperscript{D7A} gene with codon seven encoding aspartate substituted with an alanine codon was amplified from plasmid pKW254813 (Winther & Gerdes, 2011) using primers H6-VapC_SF_Down 5’-CCCCCGGTAC CGGATCCAAA ATAAGGAGGA AAAAAAATG CATCACCATC ACCATCACCT GAAGTTTATG CTC-3’ and VAPC#SF-UP 5’-CCCCCAAGCT TGAATTCGAT TTCTGATGAA CAGGTCAGC-3’. The resulting PCR product was digested with BamHI and HindIII restriction nucleases and ligated into pMG25 expression plasmid. This plasmid, pKW2583HN, expresses N-terminal polyhistidine-tagged VapC\textsuperscript{D7A} upon induction by IPTG.

2.2. Expression and purification

pKW2583HN was transformed into Escherichia coli C41 (DE3) for expression (Winther & Gerdes, 2011). The cells were grown in LB medium at 37 °C containing 100 mg/mL ampicillin until OD\textsubscript{600} reached 0.5 and were subsequently induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 25 °C with shaking at 120 rpm. Cells were harvested by centrifugation at 12,000 g for 15 min and resuspended in lysis buffer containing 50 mM Tris, pH 8.0, 500 mM KCl, 5 mM MgCl\textsubscript{2}, 5 mM 2-mercaptoethanol, 10 mM imidazole, and protease inhibitor tablets (Sigma). Cells were opened by a combination of sonication and high-pressure homogenization at 15,000 psi and the lysate cleared by centrifugation at 15,000 rpm for 45 min. The resulting supernatant was loaded onto a pre-packed 5 mL HiTrap column (GE Healthcare) charged with Ni\textsuperscript{2+} followed by extensive washing of the column (20 CV) in a buffer identical to the lysis buffer but including 35 mM imidazole. Elution was finally achieved with a very similar buffer only containing 250 mM imidazole. Next, the protein pool was concentrated to 3.37 mg/mL by ammonium sulphate precipitation (65% saturation) and resuspended in 25 mM Mes, pH 6.0, 5 mM MgCl\textsubscript{2}, and 5 mM 2-mercaptoethanol. As a final step, a monodisperse protein sample was obtained using a Superdex 75 10/300 HR size exclusion column (GE Healthcare) running in 25 mM Mes, pH 6.0, 50 mM KCl, 5 mM MgCl\textsubscript{2}, and 5 mM 2-mercaptoethanol.

2.3. Crystallization
Prior to crystallization, the monodisperse sample of VapC^{D7A} was concentrated to 4 mg/mL using a Vivaspin 6 filter (5 kDa cut-off, GE Healthcare), pre-washed in size exclusion buffer. Initial screening for crystallization conditions was carried out using an Oryx4 crystallization robot (Douglas Instruments) by mixing 250 nL protein solution with 250 nL reservoir solution (using the PEG/Ion HT and Index™ commercial screens, Molecular dimensions) for sitting-drop vapour diffusion drops placed in 96-well SwissCi plates (Hampton) at 19 °C. Optimization of the crystallization conditions was performed in standard 24-well Cryschem plates (Molecular dimensions) using a 1:1 protein: reservoir ratio.

### 2.4. X-ray data collection and processing

Single crystals were transferred into a solution containing initially 25% w/v followed by 30% w/v PEG 3350 in 0.05 M citric acid and 0.05 M Bis-Tris propane, pH 5.0 using a mounted CryoLoops (Hampton Research), and then flash frozen in liquid nitrogen. All data were collected at 100 K on beamline I911-2 (wavelength 1.04 Å) at MAX-Lab (Lund, Sweden) and processed with XDS (Kabsch, 2010) through Xia2 (Winter, 2010). Matthews' parameters were calculated using matthews_coef as implemented in CCP4 (Matthews, 1968; Winn et al., 2011).

### 3. Results and discussion

The D7A active site mutant of VapC, which has been shown to be inactive in vivo, can readily be expressed in *E. coli* and purified directly in the absence of the antitoxin VapB (Winther & Gerdes, 2011). Essentially pure VapC^{D7A} was obtained by Ni-NTA chromatography followed by size exclusion chromatography (Figure 1). The protein elutes after 13.5 mL from a standard 24 mL Superdex 75 10/300 HR column as a monodisperse peak corresponding to a molecular weight of 24.5 kDa (Figure 1), thus suggesting that the free toxin (theoretical MW 14.8 kDa) exists as a dimer in solution. The initial crystallization screening identified a condition containing 16% w/v PEG 3350, 0.05 M citric acid, 0.05 M Bis-Tris propane, pH 5.0 that yielded large, single crystals with a hexagonal morphology and did not require optimization (Figure 2). The crystals could readily be reproduced and appeared after 1 day at 19 °C and grew to maximum size (0.4 x 0.2 x 0.1 mm) within a week.

The flash-frozen VapC^{D7A} crystals diffract to beyond 1.9 Å at a synchrotron x-ray source and a full data set was collected at MAX-Lab, Lund and processed by XDS (Kabsch, 2010) through Xia2 (Winter, 2010). A summary of the data and processing statistics are shown in Table 1. The crystals belong to the trigonal space group in hexagonal setting, H3 (space group number 146), with unit cell parameters a=b=120.1Å, c=52.5 Å, α=β=90°, γ=120°. The Matthews' coefficient and solvent content were estimated using CCP4 (Winn et al., 2011) to 2.46 Å^3/Da and 50.0%, respectively, with an estimated 2 copies of the monomer per asymmetric unit suggesting that a single VapC dimer could form the crystallographic asymmetric unit. The self-rotation function, however, did not reveal any
non-trivial peaks suggestive of 2-fold NCS. Structure solution by molecular replacement and model building are in progress.

In summary, the purification, crystallization, and preliminary crystallographic analysis of the *S. flexneri* VapC<sup>Δ7Δ</sup> toxin reported here form the basis for determining the structure of the active conformation of the isolated toxin upon release of the anti-toxin, VapB. This structure will allow us to better understand the mechanism of toxin activation for the VapBC toxin-antitoxin family as well as the catalytic mechanism of the toxin. On the longer term, co-crystallization with tRNA<sup>Met</sup> will allow a full understanding of the toxin target specificity and the cleavage mechanism.

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**References**


Table 1  Crystallographic data statistics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
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<td>Space Group</td>
<td>H3 (146)</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>120.1, 120.1, 52.5</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90, 90, 120</td>
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<tr>
<td>Wavelength (Å)</td>
<td>1.041</td>
</tr>
<tr>
<td>Matthews’ coefficient (Å³/Da)</td>
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<tr>
<td>Likely number of protomer copies in the a.s.u.</td>
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</tr>
<tr>
<td>Solvent content (%)</td>
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<tr>
<td>Resolution (Å)</td>
<td>23.4 – 1.92 (1.97 – 1.92)³</td>
</tr>
<tr>
<td>No. of reflections (unique)</td>
<td>132,504 (21,158)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>6.4 (6.3)</td>
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<tr>
<td>Completeness (%)</td>
<td>98.1 (86.3)</td>
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<tr>
<td>Rmeas (%)²</td>
<td>3.9 (40.3)</td>
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<tr>
<td>CC1/2 (%)</td>
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</tr>
<tr>
<td>I/σ(I)</td>
<td>29.9 (5.3)</td>
</tr>
</tbody>
</table>

³Numbers in parentheses correspond to the outermost resolution shell, except for the number of reflections where it indicates the number of unique reflections. ²Redundancy-independent R factor calculated on intensities (Diederichs & Karplus, 1997).

Figure 1  Purification and oligomeric state of isolated *S. flexneri* VapC<sup>D7A</sup> toxin. A. Fractions of the purified protein were analysed on a 15% SDS-PAGE. Lane marked “MW”, molecular weight marker (numbers in kDa); lane marked “Ni-NTA”, elution from the Ni-NTA affinity column; lane marked “VapC”, final protein sample after size exclusion chromatography. The theoretical molecular mass of the VapC monomer is 14.8 kDa. B. Analysis of the oligomeric state of isolated VapC using size exclusion chromatography. The arrows at the top indicate the elution volumes of proteins of known mass. The elution volume of VapC<sup>D7A</sup> (13.5 mL) suggests that the molecule is present as a dimer in solution (app. 30 kDa).

Figure 2  Crystals of isolated *S. flexneri* VapC<sup>D7A</sup> toxin. Single crystals of hexagonal appearance, the scale bar indicates the approximate crystal size.

Figure 3  Synchrotron x-ray diffraction pattern collected from a single, cryo-cooled VapC<sup>D7A</sup> crystal. Diffraction was observed beyond 1.9 Å resolution. Inset shows close-up view of the reflections near the edge of the detector.
Figure 1. Purification and oligomeric state of isolated \textit{S. flexneri} VapC$^{D7A}$ toxin

(A) MW, Ni-NTA, VapC

(B) A$_{280}$ (mAU) vs. Elution volume (ml)

- 66 kDa
- 43 kDa
- 13.7 kDa

MW: Molecular Weight
Ni-NTA: Ni-Nitrotriacetic Acid
VapC: VapC protein

13.5 ml
Figure 2. Crystals of isolated *S. flexneri* Vap**C**<sup>D7A</sup> toxin
Figure 3. Synchrotron x-ray diffraction pattern collected from a single, cryo-cooled VapC^D7A crystal.