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Expression, purification, crystallization and preliminary X-ray analysis of ribitol-5-phosphate cytidylyltransferase from *Bacillus subtilis*

TarI is a ribitol-5-phosphate cytidylyltransferase that catalyzes the formation of CDP-ribitol, which is involved in the biosynthesis of wall teichoic acids, from CTP and ribitol 5-phosphate. TarI from *Bacillus subtilis* (*BsTarI*) was purified and crystallized using the sitting-drop vapour-diffusion method. The crystals diffracted to a resolution of 1.78 Å and belonged to the monoclinic space group C2, with unit-cell parameters $a = 103.74$, $b = 60.97$, $c = 91.80$ Å, $\beta = 113.48^\circ$. The initial structural model indicated that the crystals of *BsTarI* contained a dimer in the asymmetric unit.

1. Introduction

Teichoic acids (TAs) are essential components of the cell wall in Gram-positive bacteria. There are two types of TAs: lipoteichoic acids (LTAs), which are anchored within the cytoplasmic membrane via a glycolipid, and wall teichoic acids (WTAs), which are covalently attached to the peptidoglycan layers of the cell wall. A wide range of physiological functions have been proposed for TAs, including morphogenesis, maintenance of cation homeostasis and biofilm formation. TAs also play crucial roles in the interaction of pathogenic bacteria with their host (Weidenmaier & Peschel, 2008; Neuhaus & Baddiley, 2003). In *Staphylococcus aureus*, WTAs are not essential for viability but are required for colonization and infection. Mutants of this organism lacking WTAs did not have the ability to colonize the nose of a rat model (Nahid & Sugii, 2006; Lynch *et al.*, 2004). In addition, a *Streptococcus pneumoniae* mutant lacking the capacity for choline modification of TAs demonstrated considerably decreased virulence and was rapidly eliminated from infected animals (Kharat & Tomasz, 2006).

Ribitol-5-phosphate cytidylyltransferase (TarI) is required for the synthesis of activated ribitol via the WTA-biosynthesis pathway. The enzyme catalyzes the formation of CDP-ribitol from CTP and ribitol 5-phosphate. TarI from *Bacillus subtilis* contains 237 amino acids and shares 48% sequence identity with a similar protein from *S. pneumoniae* (*SpTarI*). The crystal structure of *SpTarI* has been shown to consist of an α/β structure that resembles a Rossmann-fold domain and to exist as a dimer in its crystal form. The structure of *SpTarI* in complex with CDP revealed the residues responsible for the interaction of CDP with the enzyme (Baur *et al.*, 2009).

However, the structure of TarI in complex with the substrate ribitol 5-phosphate or the product CDP-ribitol has not been elucidated to date, but could be determined by crystallography. Here, we report the cloning, expression, purification, crystallization and preliminary crystallographic analysis of *BsTarI*, with the aim of identifying the substrate-binding residues and gaining insight into the mechanism of action of TarI.

2. Materials and methods

2.1. Cloning, expression and purification

The 711 bp open reading frame encoding TarI was PCR-amplified using *B. subtilis* genomic DNA as template and was then cloned into pET28a(+) vector (Invitrogen) to allow the expression of His₆-tagged *BsTarI* recombinant protein in *Escherichia coli* BL21 (DE3) cells. The

transformed cells were grown at 310 K in 1 l Terrific Broth medium containing 25 $\mu\text{g ml}^{-1}$ kanamycin until the OD at 600 nm reached 1.0. Protein expression was induced for 20 h by the addition of 1.0 mM IPTG at 288 K. Cultured cells were harvested by centrifugation at 1590g for 30 min at 277 K. The cell pellet was resuspended in buffer A (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM imidazole).

The cells were disrupted by sonication and the crude lysate was centrifuged at 22 300g for 90 min at 277 K. The supernatant was applied onto Ni-NTA His-bind resin (GE Healthcare) pre-equilibrated with buffer A. The protein was eluted with a 0–200 mM linear gradient of imidazole. Fractions that contained *BsTarI* were pooled for additional purification using a phenyl-Sepharose column (GE Healthcare). The column was developed with a linear gradient of ammonium sulfate in buffer B (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% glycerol). The protein eluted as a single peak at 0.8 M ammonium sulfate.

These fractions were concentrated and passed through a HiLoad 16/60 Superdex-200 size-exclusion column (GE Healthcare) equili-

Table 1

Data-collection statistics for the *BsTarI* crystal.

Values in parentheses are for the highest resolution shell.

X-ray wavelength (Å)	0.9762
Space group	C2
Unit-cell parameters (Å, °)	$a = 103.74$, $b = 90.97$, $c = 91.80$, $\beta = 113.48$
Resolution (Å)	30.0–1.78 (1.97–1.78)
No. of measured reflections	225855 (22023)
No. of unique reflections	49878 (4894)
Multiplicity	4.5 (4.5)
R_{merge} (%)	3.3 (45.8)
Data completeness (%)	97.9 (96.8)
$\langle I/\sigma(I) \rangle$	41.76 (3.40)

brated with buffer C (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% glycerol, 2 mM TCEP). The fractions containing *BsTarI* were pooled and concentrated to 25 mg ml^{-1} for crystallization screening.

2.2. Crystallization

Initial crystallization trials were performed using commercially available kits from Hampton Research (Index, PEG/Ion, PEGRx, Crystal Screen and Crystal Screen 2), Emerald BioSystems (Wizard I–IV) and Molecular Dimensions (PACTpremier, PGA Screen and Clear Strategy Screens I and II) by the sitting-drop vapour-diffusion method in 24-well VDX plates (Hampton Research). 1 μl protein solution (25 mg ml^{-1} in buffer C) and 1 μl reservoir solution were mixed and equilibrated against 400 μl reservoir solution at 277 K. Crystals were obtained after three months using condition B11 of the PACTpremier crystallization screen (Molecular Dimensions), which consists of 20% PEG 6K, 0.1 M MES-NaOH pH 6.0, 0.2 M CaCl_2 . However, *BsTarI* crystals tended to grow in a multiple-crystal form under this condition (Fig. 1). To resolve this difficulty, the multiple crystals were cleaved into crystal fragments and then mounted separately using a nylon loop for X-ray diffraction data collection.

Figure 1
BsTarI crystals. The crystal dimensions are approximately $0.4 \times 0.3 \times 0.1$ mm.

Figure 2
Diffraction pattern of *BsTarI* collected on NSRRC beamline 13C1 from a crystal flash-cooled in 25% glycerol.

Figure 3
 $\chi = 180^\circ$ section of the self-rotation function calculated by *MOLREP* (Vagin & Teplyakov, 2010) using data from 30 to 2.5 Å resolution.

Figure 4
Electron-density map ($2F_{\text{obs}} - F_{\text{calc}}$ contoured at 1.5σ) at 1.78 Å resolution in the region of the central β -sheet of *BsTarI* calculated from the initial phases after molecular replacement. The backbones of the initial target model (red) are shown.

2.3. Data collection

Crystals of *BsTarI* were soaked for 15 s in reservoir solution supplemented with 25% glycerol and flash-cooled in liquid nitrogen. Diffraction data were collected at 100 K on beamline 13C1 at the National Synchrotron Radiation Research Center (NSRRC), Taiwan. The exposure time and crystal oscillation were set to 30 s and 1° , respectively. The crystal-to-detector distance was maintained at 200 mm. A total of 210 images were recorded using an ADSC

Quantum 315R CCD detector. The diffraction data were indexed and integrated using the *HKL-2000* processing software (Otwinowski & Minor, 1997). Crystal parameters and data-collection statistics are summarized in Table 1.

3. Results and discussion

The *tarI* gene of *B. subtilis* consists of 711 bp encoding 237 amino-acid residues. A theoretical isoelectric point of 5.66 was calculated on the basis of the protein sequence. The purified *BsTarI* protein contains an extra octapeptide, LEH₆, at the C-terminal end. The polypeptide was represented by a single band of approximately 25 kDa on SDS-PAGE. Its molecular weight was estimated by gel filtration and the experiment suggested that *BsTarI* exists as a dimer in solution.

Crystals of *BsTarI* were obtained after three months using a buffer comprising 0.1 M MES monohydrate pH 6.0, 0.2 M CaCl₂, 20% PEG 6000. The crystal of *BsTarI* diffracted to 1.78 Å resolution and belonged to the monoclinic space group *C2*, with unit-cell parameters $a = 103.74$, $b = 60.97$, $c = 91.80$ Å, $\beta = 113.48^\circ$. A diffraction image of *BsTarI* is shown in Fig. 2. Assuming the presence of a dimer in the asymmetric unit, the Matthews coefficient was calculated to be $2.66 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 53%. We investigated the local symmetry connecting the units in the asymmetric unit using *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP4* package. Analysis of the self-rotation peaks revealed the presence of a noncrystallographic twofold symmetry on the *ab* plane (Fig. 3), which is compatible with the presence of a dimer in the asymmetric unit of the crystals.

The initial model of *BsTarI* was obtained by the molecular-replacement method using the program *Phaser* (McCoy *et al.*, 2005). The coordinates of monomeric *SpTarI* (PDB entry 2vsh; Baur *et al.*, 2009) were used as a search model. The initial *R* value after molecular replacement was 29.1%, with a rotation-function *Z*-score of 30.1 and a translation-function *Z*-score of 40.6. An electron-density map calculated from the initial phases is presented in Fig. 4.

Crystallization and structure determination of *BsTarI* is the first step towards identifying the substrate-binding residues of this enzyme and gaining insight into its mechanism of action. Model building and refinement of the model are currently in progress and the structural analysis will be described in a subsequent manuscript.

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