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A preliminary X-ray study of D,D-heptose-1,7-bisphosphate phosphatase from *Burkholderia thailandensis* E264

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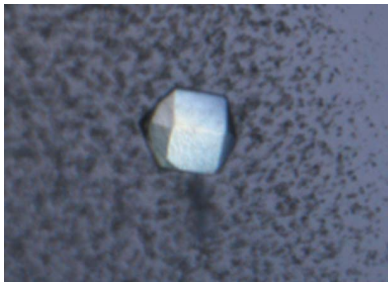
D,D-Heptose-1,7-bisphosphate phosphatase (GmhB), which is involved in the third step of the NDP-heptose biosynthesis pathway, converts D,D-heptose-1,7-bisphosphate to D,D-heptose-1-phosphate. This biosynthesis pathway is a target for new antibiotics or antibiotic adjuvants for Gram-negative pathogens. *Burkholderia thailandensis* is a useful surrogate organism for studying the pathogenicity of melioidosis owing to its extensive genomic similarity to *B. pseudomallei*. Melioidosis caused by *B. pseudomallei* is a serious invasive disease of animals and humans in tropical and subtropical areas. In this study, GmhB has been cloned, expressed, purified and crystallized. X-ray data have also been collected to 2.50 Å resolution using synchrotron radiation. The crystal belonged to space group *P*6, with unit-cell parameters a = 243.2, b = 243.2, c = 41.1 Å.

1. Introduction

The lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria is composed of a tripartite structure consisting of lipid A, a conserved core oligosaccharide region and a repeating saccharide O antigen (Raetz, 1996). LPS blocks the entry of toxic hydrophobic compounds by maintaining the structural integrity of the bacterial outer membrane (Nikaido & Vaara, 1985). The core oligosaccharide can be subdivided into an outer core comprising hexoses and hexosamines and an inner core made up of 3-deoxy-D-*manno*-oct-2-ulosonic acid, which are both essential for viability, and heptoses related to a pleiotropic phenotype. Deep rough mutants (Tamaki *et al.*, 1971) caused by defects in the biosynthesis of heptoses result in extreme susceptibility to antibiotics and a remarkable reduction in virulence. D,D-Heptose-1,7-bisphosphate phosphatase (GmhB), which is involved in the biosynthesis pathway of NDP-heptose, converts D,D-heptose-1,7-bisphosphate to D,D-heptose-1-phosphate (Kneidinger *et al.*, 2002). This biosynthesis pathway provides an alternative strategy for the development of new bactericidal agents that would weaken the outer membrane structure of Gram-negative pathogens or antibiotic adjuvants that synergize with known antibiotics. The Gram-negative soil saprophyte *Burkholderia pseudomallei* is notorious for its pathogenicity and causes melioidosis (Wiersinga *et al.*, 2006), which is an endemic disease throughout southeast Asia and northern Australia. Infection occurs through inhalation or transcutaneous inoculation and the most common manifestation is pneumonia. Since mortality from melioidosis septic shock remains high despite appropriate antimicrobial therapy, the development of more effective antibiotics or antibiotic adjuvants is required. *B. thailandensis* shares high genomic similarity and the majority of virulence factors with *B. pseudomallei*, although it is relatively avirulent. *B. thailandensis* is therefore a useful surrogate organism for studying the pathogenicity of melioidosis.

An open reading frame of *B. thailandensis* E264 codes for GmhB of 20.1 kDa with 61.9% amino-acid sequence identity to that of



B. pseudomallei. It shows 32% amino-acid sequence identity to the N-terminal domain of histidinol phosphate phosphatase (Rangarajan *et al.*, 2006) and 37% to GmhB from *Escherichia coli* (unpublished results). Both proteins belong to the haloacid dehalogenase-like hydrolase superfamily. Their crystal structures revealed a parallel five-stranded β-sheet flanked on both sides by several helices, with three conserved motifs being found in their active sites (Rangarajan *et al.*, 2006).

We have initiated the determination of the three-dimensional structure of GmhB from *B. thailandensis* E264 in order to provide a structural template for the development of a novel phosphatase inhibitor as an antimelioidosis agent. Here, we report the cloning, overexpression, purification, crystallization and preliminary X-ray study of this enzyme.

2. Experimental methods

2.1. Cloning of GmhB

The cloning primers (Genotech, Daejeon, Republic of Korea) prepared for ligation-independent cloning (LIC) were 5′-GGC-GGTGGTGGCGGCATGCCGACCAGTCCCAGCAAA-3′ for the forward strand and 5′-GTTCTTCTCCTTTGCGCCCCTACTCGT-GTTCTTTGGAAAGGAAATCGA-3′ for the reverse strand. The GmhB gene was amplified by PCR using 109 ng *B. thailandensis* E264 genomic DNA template and 0.25 µ*M* of primers. PrimeSTAR HS DNA polymerase with GC buffer (Takara Bio Inc., Shiga, Japan) designed for high-GC-content genomic DNA was used. The amplified LIC expression vector pB2 (Kim *et al.*, 2005), a derivative of the pET21a vector (Novagen, Madison, Wisconsin, USA), was incubated with T4 DNA polymerase (New England Biolabs, Beverley, Massachusetts, USA) in the presence of 1 m*M* dATP at 310 K for 30 min followed by incubation at 343 K for 20 min. The amplified PCR product was prepared for vector insertion using the same protocol except for the presence of 1 m*M* dTTP instead of 1 m*M* dATP. The prepared insert was annealed into the pB2 vector, which expresses the cloned gene fused to a noncleavable N-terminal His_6 tag, and transformed into DH5α competent cells to obtain fusion clones. Clones were screened by plasmid DNA analysis and transformed into *Escherichia coli* BL21 (DE3) for protein expression (Kim *et al.*, 1998).

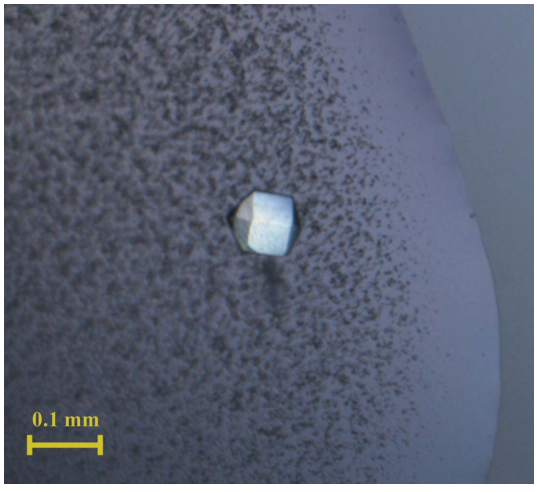


Figure 1
A hexagonal shaped crystal. The crystal appeared in the condition 1.3 *M* ammonium sulfate and 0.1 *M* sodium acetate pH 4.8. The crystal used to collect the X-ray data grew to approximate dimensions of 0.08 × 0.08 × 0.05 mm. GmhB crystals typically grew to dimensions of 0.05 × 0.05 × 0.025 mm.

2.2. Growth of cultures

E. coli BL21 (DE3) transformed with the cloned vector harbouring the GmhB gene was grown on Luria–Bertani (LB) agar plates containing 150 µg ml^{-1} ampicillin. Several colonies were picked and grown in capped test tubes with 10 ml LB broth containing 150 µg ml^{-1} ampicillin. A cell stock of 0.85 ml culture and 0.15 ml glycerol was prepared and frozen at 193 K for use in a larger culture. The frozen cell stock was grown in 5 ml LB medium and diluted into 500 ml fresh LB medium. The culture was incubated at 310 K with shaking until an OD_{600} of 0.6–0.8 was reached. At this point, GmhB was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 m*M*. The culture was further grown at 310 K for 3 h in a shaking incubator. Cells were harvested by centrifugation at 7650*g* (6500 rev min^{-1}) for 10 min in a high-speed refrigerated centrifuge at 277 K.

2.3. Protein purification

The cultured cell paste (1.5 g) was resuspended in 12.5 ml buffer consisting of 50 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 10 m*M* imidazole, 1 m*M* PMSF, 10 µg ml^{-1} DNase I and Roche protease-inhibitor cocktail (Roche Applied Science, Indianapolis, Indiana, USA). The cell suspension was disrupted using a Digital Sonifier 450 (Branson Ultrasonics Co., Danbury, Connecticut, USA). Cell debris was pelleted by centrifugation at 24 900*g* (15 000 rev min^{-1}) for 30 min in a high-speed refrigerated centrifuge at 277 K. The supernatant was affinity-purified using a HisTrap column on an ÄKTA Explorer system (GE Healthcare, Piscataway, New Jersey, USA). The column was equilibrated with a buffer consisting of 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl and 10 m*M* imidazole. The target protein was eluted with a buffer consisting of 50 m*M* Tris–HCl pH 8.0 and 100 m*M* NaCl with a gradient from 10 to 500 m*M* imidazole. GmhB was further purified by ion-exchange chromatography using a 5 ml Hi-Trap Q column (GE Healthcare, Piscataway, New Jersey, USA) equilibrated with buffer containing 20 m*M* Tris–HCl pH 8.5. The protein was eluted at 0.37 *M* NaCl using a linear NaCl gradient. SDS–PAGE showed one band around 21.3 kDa corresponding to the molecular weight of GmhB. The purified protein contained a noncleavable N-terminal His_6 tag followed by six glycine residues. The protein was concentrated to 20 mg ml^{-1} (using an extinction coefficient of 0.34 M^{-1} cm^{-1} at 280 nm) for crystallization in a buffer consisting of 0.4 *M* NaCl and 20 m*M* Tris–HCl pH 8.5. Dynamic light scattering (DynaPro 99, Proterion Corporation, Piscataway, New Jersey, USA) showed a single monodisperse peak, indicating homogeneity of the protein.

2.4. Crystallization

Screening for crystallization conditions was performed using the sparse-matrix method (Jancarik & Kim, 1991) with several screens from Hampton Research (Hampton Research, Laguna Niguel, California, USA). A Hydra Plus One crystallization robot (Matrix Technologies, Hudson, New Hampshire, USA) was used to set up the screens using the sitting-drop vapour-diffusion method at room temperature. A 96-well Intelli-Plate (Art Robbins Instrument, Salt Lake City, Utah, USA) was used; sitting drops were made by mixing 0.2 µl protein solution (20 mg ml^{-1}) and 0.2 µl reservoir solution and were equilibrated over 90 µl reservoir solution. A VDX48 plate (Hampton Research, Laguna Niguel, California, USA) was used to optimize the crystallization conditions using hanging drops made up by mixing 0.8 µl protein solution and 0.8 µl reservoir solution con-

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Pohang Light Source 4A
X-ray wavelength (Å)	1.000
Temperature (K)	100
Space group	*P*6
Unit-cell parameters	
a (Å)	243.2
b (Å)	243.2
c (Å)	41.1
Resolution range (Å)	50.0–2.50 (2.54–2.50)
Unique reflections	48851 (2256)
$R_{\rm merge}$† (%)	7.7 (47.0)
Data completeness (%)	99.2 (91.4)
Average $I/\sigma(I)$	21.7 (2.4)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

taining 1.3 *M* ammonium sulfate and 0.1 *M* sodium acetate pH 4.8 and equilibrated over 100 µl reservoir solution.

2.5. Data collection and reduction

A concentrated lithium sulfate solution was added to the well solution to a concentration of 1.0 *M* as a cryoprotectant. Before being flash-cooled in liquid nitrogen, crystals were soaked in the equilibrated well solution. X-ray diffraction data were collected at a single wavelength on beamline 4A at Pohang Light Source using an Area Detector System Co. (Poway, California, USA) Quantum 4 CCD detector placed 400 mm from the sample. The oscillation range per image was 0.5° with 15 s exposures. 200 oscillation images were collected with no overlap between two contiguous images. X-ray diffraction data were processed and scaled using *DENZO* and *SCALEPACK* from the *HKL*-2000 program suite (Otwinowski & Minor, 1997).

3. Results and discussion

The yield of the purified protein was ∼50 mg per litre of *E. coli* culture. After anion-exchange chromatography, GmhB appeared to be approximately 99% pure, with a prominent protein band at around 21.3 kDa on SDS–PAGE. Diffraction-quality crystals were obtained using a reservoir solution consisting of 1.3 *M* ammonium sulfate and 0.1 *M* sodium acetate pH 4.8. Crystals grew to dimensions of 0.05 × 0.05 × 0.025 mm within three weeks at 296 K (Fig. 1). The synchrotron data were collected to 2.5 Å resolution. The crystal belonged to the primitive hexagonal space group *P*6, with unit-cell parameters *a* = 243.2, *b* = 243.2, *c* = 41.1 Å. Based on the Matthews coefficient (Matthews, 1968), the asymmetric unit could contain between five and nine GmhB molecules ($V_{\rm M}$ of 1.83–3.29 Å^3 Da^{-1} with a solvent content of between 32.8 and 62.7%). A self-rotation peak search did not reveal clear evidence for noncrystallographic symmetry corresponding to twofold, threefold, fourfold, fivefold, sixfold, sevenfold, eightfold or ninefold symmetry and dynamic light-scattering analysis indicated that the protein forms a monomer in solution. Therefore, the number of molecules in the asymmetric unit and their NCS relationships remains unknown, but should be revealed later as structure determination progresses. The details of the data-collection statistics are presented in Table 1.

Molecular replacement was performed using histidinol phosphate phosphatase or GmhB from *E. coli* as search models. However, a molecular-replacement solution was not found. Therefore, selenomethionine-substituted GmhB crystals from *B. thailandensis* have been obtained using the same procedure as described above. Therefore, structure determination is being continued using the multiwavelength anomalous dispersion method.

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