Expression, crystallization and preliminary X-ray crystallographic analysis of D-alanine-D-alanine ligase from OXA-23-producing *Acinetobacter baumannii* K0420859

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Acinetobacter baumannii causes bacteraemia, pneumonia, other respiratory-tract and urinary-tract infections in humans. OXA-23 carbapenemase-producing *A. baumannii* K0420859 (*A. baumannii* OXA-23) is resistant to carbapenem, a common antibacterial drug. To develop an efficient and novel antibacterial drug against *A. baumannii* OXA-23, D-alanine-D-alanine ligase, which is essential in bacterial cell-wall synthesis, is of interest. Here, the D-alanine-D-alanine ligase (*AbDdl*) gene from *A. baumannii* OXA-23 was cloned and expressed, and the *AbDdl* protein was purified and crystallized; this enzyme can be used as a novel target for an antibacterial drug against *A. baumannii* OXA-23. The *AbDdl* crystal diffracted to a resolution of 2.8 Å and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 113.4$, $b = 116.7$, $c = 176.5$ Å, a corresponding V_M of $2.8 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 56.3%, and six protomers in the asymmetric unit.

1. Introduction

Acinetobacter baumannii is a Gram-negative short, round or rod-shaped bacterium. *A. baumannii* causes bacteraemia, pneumonia, other respiratory-tract and urinary-tract infections in humans, and has become an important causative agent of nosocomial infections. The ability of *A. baumannii* to improve its resistance mechanisms and survival time makes it difficult to eradicate *A. baumannii* infections from the clinical system (Liakopoulos *et al.*, 2012; Espinal *et al.*, 2013). Carbapenems have been widely used to treat *A. baumannii* infections, but a worldwide trend of increasing resistance to these antibiotics associated with the production of acquired carbapenem-hydrolyzing OXA-type class D β -lactamases has been reported. Until recently, *A. baumannii* strains resistant to carbapenem have been identified and grouped into four main groups according to the β -lactamase produced by them: OXA-23, OXA-24, OXA-51 and OXA-58 (Lee *et al.*, 2011).

We analyzed OXA-23-producing *A. baumannii* K0420859 (*A. baumannii* OXA-23) strain carrying the *bla* OXA-23 gene primarily located on plasmids or intergrons (Bogaerts *et al.*, 2008). The *A. baumannii* OXA-23 strain was first identified in Spain in 2010 (Espinal *et al.*, 2013). In the case of other carbapenem-resistant *A. baumannii* strains, no drugs that can completely inhibit *A. baumannii* OXA-23 have been found. D-Alanine-D-alanine ligase catalyzes the formation of the precursor of peptidoglycan, an essential component of the bacterial cell wall, which has been a novel target for antibacterial drug development (Doan *et al.*, 2008). Recently, several antibiotics directed at inhibiting bacterial cell-wall synthesis have been developed.

Here, we report the cloning of the D-alanine-D-alanine ligase (*AbDdl*) gene of *A. baumannii* OXA-23, the expression, purification and crystallization of *AbDdl* and the preliminary X-ray crystallographic analysis of its crystals. The atomic resolution structure of *AbDdl* will be helpful in developing a novel antibacterial drug against *A. baumannii* OXA-23.

Table 1

Data-collection statistics.

Values in parentheses are for the outer shell.

Source	Beamline 7A, PAL
Wavelength (Å)	0.97935
Detector	ADSC Q270 CCD
Temperature of data collection (K)	100
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	5
Resolution range (Å)	50.0–2.8 (2.85–2.80)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 113.4, b = 116.7, c = 176.5$
Total No. of reflections	413163
No. of unique reflections	55982
Completeness (%)	96 (92)
Molecules per asymmetric unit	6
V_M (Å ³ Da ^{−1})	2.8
Solvent content (%)	56.3
Average $\langle I/\sigma(I) \rangle$	12.9 (1.6)
$R_{\text{merge}}^{\dagger}$ (%)	9.1 (42.0)
Multiplicity	7.4 (3.7)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum of the overall reflections and $\sum_i$ is the sum over the i measurements of reflection hkl .

2. Methods

2.1. Cloning

The gene encoding the Ddl (*AbDdl*) protein from *A. baumannii* OXA-23 was cloned by polymerase chain reaction (PCR). The sequences of the oligonucleotide primers were designed based on the data on the genome sequences of other *A. baumannii* strains from the NCBI website. The forward and reverse primers 5'-CCC CCC **ATA TGT** CAA ATG CTA CAA AAT TCG GC-3' and 5'-CCC CCG **GAT** CCT TAA GCC GTA CCT TCC AAT GT-3', respectively, contain *NdeI* and *Bam*HI restriction sites denoted by the bold letters. After double-digestion with *NdeI* and *Bam*HI enzymes, the PCR product was inserted into a modified pET11a vector; the pET11a vector (Novagen) was engineered to have additional residues of a

7×His tag and a *Tobacco etch virus* (TEV) protease-cleavage site before the *NdeI* site.

2.2. Overexpression and purification

The recombinant vector pET11a-*AbDdl* was transformed into *Escherichia coli* strain BL21 (DE3) pLysS and the *E. coli* cells were cultured in Luria–Bertani medium containing 50 µg ml^{−1} ampicillin. Overexpression of *AbDdl* was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture at 310 K until an OD₆₀₀ of 0.6 was observed. After induction, the cells were cultured for an additional 4 h. The cultured cells were harvested by centrifugation for 20 min at 6000g (Supra 30K A1000S-4 rotors, Hanil, Seoul, Republic of Korea) at 277 K. The cell pellets were then resuspended in ice-cold lysis buffer (25 mM Tris–HCl pH 7.5, 300 mM NaCl, 15 mM imidazole, 3 mM β-mercaptoethanol) and homogenized by ultrasonication on ice (Sonomasher, S & T Science, Republic of Korea). The lysate was then centrifuged for 40 min at 21 000g (Vision VS24-SMTi V508A rotor) at 277 K. About 40% of the total expressed *AbDdl* was observed to be soluble (data not shown). The supernatant containing soluble *AbDdl* was loaded onto an Ni²⁺-charged resin (Ni-NTA His-Bind Resin, Bio-Rad) previously equilibrated with the lysis buffer. Affinity purification was performed according to the manufacturer's protocol at 277 K. The lysis buffer was used to wash out the nonspecifically bound proteins. *AbDdl* was eluted using an elution buffer consisting of 25 mM Tris–HCl pH 7.5, 300 mM NaCl, 250 mM imidazole, 3 mM β-mercaptoethanol. The resulting protein solution was dialyzed for 12 h at 277 K in buffer A (25 mM Tris–HCl pH 7.5, 3 mM β-mercaptoethanol). The His tag was cleaved using TEV protease at 277 K at a ratio of 20:1 (*AbDdl*:TEV) by weight in an overnight reaction. Almost 80% of the total *AbDdl* was cleaved and was then purified again using Ni²⁺-charged resin. After that, the *AbDdl* without the His tag was further purified by anion-exchange chromatography using a Hi-Trap Q FF column (GE Healthcare) (Fig. 1). The protein was then concentrated to 11 mg ml^{−1} in crystallization buffer (25 mM Tris–HCl pH 7.5, 5 mM NaCl, 3 mM β-mercaptoethanol) by using Vivaspin 20 (10000 MWCO, Satorius).

2.3. Crystallization and X-ray diffraction data collection

The crystallization trials were initially conducted at 287 K by the sitting-drop vapour-diffusion method in a 96-well Intelli-Plate (Art Robbins) using a Hydra II e-drop automated pipetting system

Figure 1

Purified *AbDdl* on 12% SDS–PAGE. Lane *M* contains molecular-mass marker.

Figure 2

A single *AbDdl* crystal.

(Matrix) and the Crystal Screen Lite, Crystal Screen Cryo, PEGRx (Hampton Research), Wizard Precipitant Synergy (Emerald Bio) and Morpheus (Molecular Dimensions) screening kits. We developed several different crystallization conditions, and a condition consisting of 0.06 M MgCl_2 , CaCl_2 , 0.1 M imidazole, MES-HCl at pH 6.5 and 30% of precipitant EDO-P8K containing 40%(v/v) ethylene glycol and 20%(w/v) polyethylene glycol 8000 was chosen as the best for crystal growth, while maintaining the protein concentration at

10 mg ml⁻¹. Crystals grew in the abovementioned condition after 2 d (Fig. 2). The fully grown crystals were flash-cooled in liquid nitrogen using a cryoprotectant consisting of the reservoir solution plus 20%(v/v) glycerol. X-ray diffraction data for the crystals were collected using an ADSC Quantum 270 CCD detector on beamline 7A at the Pohang Accelerator Laboratory, Pohang University of Science and Technology, Republic of Korea. The statistics of the data collection are summarized in Table 1.

Figure 3
Self-rotation function of the *AbDdl* crystal.

Table 2

Noncrystallographic rotation axes between the protomers in the asymmetric unit.

The protein chain IDs of six protomers of *AbDdl* are depicted as *A*, *B*, *C*, *D*, *E* and *F*.

	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>	<i>F</i>
<i>A</i>		Twofold	Threefold	Twofold	Threefold	Twofold
<i>B</i>			Twofold	Fourfold	Twofold	Threefold
<i>C</i>				Twofold	Fourfold	Twofold
<i>D</i>					Twofold	Threefold
<i>E</i>						Threefold
<i>F</i>						

3. Results

AbDdl was successfully overexpressed and purified by affinity chromatography and anion-exchange chromatography. The homogeneity of the purified protein was examined by SDS–PAGE (Fig. 1). Only one band was visible on SDS–PAGE after a two-step purification, indicating that the purified protein has a molecular weight of about 35 kDa, which is in agreement with the predicted molecular weight of 34.4 kDa. X-ray diffraction data were collected to a resolution of 2.8 Å. The crystallographic orthorhombic space group $P2_12_12_1$ with unit-cell parameters $a = 113.4$, $b = 116.7$, $c = 176.5$ Å was determined by auto-indexing (Otwinowski & Minor, 1997). According to the Matthews coefficient calculation, there are six molecules in the asymmetric unit with a solvent content of 56.3% (Matthews, 1968). Analysis of the self-rotation peaks revealed the presence of three twofold, two threefold and one fourfold rotation axes, in addition to three crystallographic twofold axes (Fig. 3). The crystal structure was determined *via* molecular replacement (MR) using *AutoMR* from the *PHENIX* package of crystallographic programs (Adams *et al.*, 2010; McCoy *et al.*, 2007). The search model was generated using *CHAINS*AW in the *CCP4* package (Stein, 2008; Winn *et al.*, 2011) and D-alanine-D-alanine ligase from *Yersinia pestis* (PDB entry 3v4z; 53% sequence identity; Center for Structural Genomics of Infectious Diseases, unpublished work). The MR was successful and indicated the presence of six protomers in the asymmetric unit. The MR solution model was inputted into *AutoBuild* (Terwilliger, 2002, 2004; Terwilliger *et al.*, 2008; Zwart *et al.*, 2005; Afonine *et al.*, 2012; Adams *et al.*, 2010) from the *PHENIX* package. The noncrystallographic rotation axes were confirmed with the MR solution model (Table 2). The current model, with an *R* factor of 26.0% and an R_{free} of 30.0%,

showed good crystal packing. The *AbDdl* structure thus determined will be useful in developing an antibacterial drug against *A. baumannii*.

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