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Cloning, purification and preliminary X-ray crystallographic analysis of the OmpA-like domain of peptidoglycan-associated lipoprotein from *Acinetobacter baumannii*

Peptidoglycan-associated lipoprotein (Pal) is one component of the Tol–Pal system that is involved in maintaining the integrity and stability of the outer membrane. The C-terminal OmpA-like domain of Pal interacts noncovalently with peptidoglycan. In this study, the OmpA-like domain of Pal from *Acinetobacter baumannii* was overexpressed in *Escherichia coli* strain BL21 (DE3), purified and crystallized using the vapour-diffusion method. A native crystal diffracted to 1.4 Å resolution and belonged to space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 72.58$, $c = 44.65$ Å, a calculated Matthews coefficient of $2.64 \text{ Å}^3 \text{ Da}^{-1}$ and one molecule per asymmetric unit.

1. Introduction

Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria is one component of the Tol–Pal system, which plays an important role in the integrity of the outer membrane (OM; Gerding *et al.*, 2007; Godlewska *et al.*, 2009; Yeh *et al.*, 2010). The Tol–Pal system is composed of five proteins encoded by two operons: TolQ, TolR and TolA from the *orf1-tolQ-tolR-tolA* operon form a protein complex in the inner membrane, while TolB and Pal from the *tolB-pal-orf2* operon form an OM-associated complex in the periplasmic space (Dubuisson *et al.*, 2005; Godlewska *et al.*, 2009; Muller & Webster, 1997; Vianney *et al.*, 1996). *Escherichia coli* Pal (EcPal) is released during sepsis-induced inflammation and plays a significant role in bacterial infections (Hellman *et al.*, 2002).

Acinetobacter baumannii is an opportunistic pathogen that induces cytotoxicity in host cells by nosocomial infection of compromised patients (Bergogne-Bérézin & Towner, 1996; Choi *et al.*, 2005; Dent *et al.*, 2010). Full-length *A. baumannii* Pal (AbPal) shares 38% amino-acid sequence identity with EcPal. AbPal is composed of an N-terminal motif that is required for delivery and anchoring to the OM and a C-terminal OmpA-like domain. AbPal is found in the outer membrane vesicle (OMV) used to deliver virulence effectors into host cells (Jin *et al.*, 2011; Yun *et al.*, 2011). However, the direct role of AbPal in pathogenesis has not yet been investigated.

Generally, OmpA-like domain proteins interact noncovalently with peptidoglycan in the periplasm of Gram-negative bacteria, as shown in the structure of the OmpA-like domain from *A. baumannii* OmpA (AbOmpA-PD; Park *et al.*, 2012). Although a solution structure of the OmpA-like domain of Pal protein in complex with a peptide fragment of peptidoglycan has been determined (Parsons *et al.*, 2006), its detailed atomic structure in complex with peptidoglycan components will be helpful in understanding the structural role played by Pal in OM integrity. Here, we report the crystallization and preliminary X-ray crystallographic analysis of the OmpA-like domain of AbPal.

2. Materials and methods

2.1. Cloning and recombinant protein purification

The DNA sequence encoding the OmpA-like domain of Pal (AbPal-71; residues 71–181; molecular mass 12.5 kDa) was amplified via polymerase chain reaction (PCR) using genomic DNA from

A. baumannii 19606 with the primers 5'-ACTCATATGGAGTTA-ACTGAAGACCTTAACATGG-3' and 5'-ACTCTCGAGTTAAC-GGCTACCAGTGATTGTGCG-3'. To generate an N-terminally His₆-tagged fusion protein, the PCR product was cloned into the *Nde*I/*Xho*I-digested expression vector pET-28a(+) (Novagen). The resulting plasmid was transformed into the expression host *E. coli* BL21 (DE3) and bacterial cells were grown at 310 K until the OD₆₀₀ reached 0.5–0.6 in Luria–Bertani (LB) medium containing 50 µg ml⁻¹ kanamycin. The expression of recombinant AbPal-71 was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside at 291 K for 18 h. Bacterial cells were harvested by centrifugation at 5000 rev min⁻¹ for 20 min at 277 K.

AbPal-71 protein was prepared as described previously (Park *et al.*, 2011). The protein was initially isolated using a nickel-affinity column. After thrombin digestion, AbPal-71 was further purified using gel-filtration chromatography (Superdex 75 HiLoad 16/60 prep grade, GE Healthcare). The purified protein was concentrated to a final concentration of 20 mg ml⁻¹ in 50 mM bis-Tris pH 6.5, 100 mM glycine by ultrafiltration using an Amicon Ultra 10 kDa cutoff filter (Millipore) and stored at 193 K until use in crystallization experiments.

2.2. Crystallization

Initial crystallization screening by the sitting-drop vapour-diffusion method was performed using various screening kits (Crystal Screen HT and Index HT from Hampton Research and Wizard Classic 1 and 2 from Emerald BioSystems) at 293 K. A Mosquito crystallization robot (TTP LabTech) was used for initial screening trials. From initial screening, it was found that formulations with salt precipitants such as sodium formate, sodium chloride, ammonium phosphate or sodium/potassium phosphate prompted the crystallization of AbPal-71. After several hits were obtained from the initial screening, crystallization-condition optimization experiments were carried out by the hanging-drop vapour-diffusion method using condition No. 19 from Wizard Classic 1 and yielded crystals suitable for X-ray diffraction study. In the final optimization, 1 µl protein solution consisting of 20 mg ml⁻¹ protein in 50 mM bis-Tris pH 6.5, 100 mM glycine was mixed with 1 µl reservoir buffer (0.1 M phosphate–citrate pH 4.2, 0.4 M K₂HPO₄, 1.0–1.4 M NaH₂PO₄) and equilibrated by vapour diffusion against the reservoir solution. Clusters of rod-shaped crystals grew within one week to dimensions of 0.05 × 0.05 × 0.5 mm and were suitable for X-ray analysis.

Figure 1
Native crystals of the OmpA-like domain of AbPal.

2.3. X-ray data collection

The crystals grew as clusters of rod-shaped crystals and a single crystal had to be separated prior to data collection. A detached single crystal of suitable size was transferred into reservoir buffer supplemented with 20% (v/v) glycerol as a cryoprotectant and mounted on a CryoLoop (Hampton Research) for diffraction studies under cryogenic conditions. X-ray diffraction tests to screen crystals for the synchrotron trip were performed using a MicroMax-007 HF rotating-anode X-ray generator and an R-Axis IV⁺⁺ imaging-plate area detector (Rigaku, Japan). Final diffraction data were collected on the BL-17A beamline at the Photon Factory, Tsukuba, Japan. A total of 90 frames of images with an oscillation angle of 1° were collected on an ADSC Quantum 315r detector (Area Detector Systems Corporation, USA). The beam slit size was 0.05 × 0.05 mm. The diffraction images were integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997).

3. Results and discussion

The OmpA-like domain of AbPal (AbPal-71; molecular weight 12.5 kDa) was cloned into pET28a vector containing an N-terminal hexahistidine tag followed by a thrombin protease cleavage site and overexpressed in *E. coli* BL21 (DE3) cells. After purification using a nickel-affinity column and subsequent size-exclusion chromatography, the homogeneity of the purified protein was verified by SDS-PAGE, showing >95% purity. The results of gel-filtration chromatography suggested that AbPal-71 is monomeric in solution (data not shown). Initial screening for crystallization conditions was performed by the sitting-drop vapour-diffusion method using commercially available screening kits. Based on hits from the initial screening, crystallization conditions were manually optimized and clusters of

Figure 2
An oscillation diffraction image of a native crystal of the OmpA-like domain of AbPal. The image was recorded on an ADSC Quantum 315r detector (315 × 315 mm) on BL-17A at the Photon Factory with a crystal-to-detector distance of 181.3 mm and an oscillation angle of 1.0°.

Table 1

X-ray data-collection statistics for AbPal.

Values in parentheses are for the highest resolution bin.

Beamline	BL-17A, Photon Factory
Wavelength (Å)	0.9800
Space group	$P6_1$ or $P6_5$
Unit-cell parameters (Å)	$a = b = 72.58$, $c = 44.65$
Resolution range (Å)	50–1.4 (1.45–1.40)
No. of observations	139887
No. of unique reflections	26445
$R_{\text{merge}}^{\dagger}$	0.058 (0.429)
Multiplicity	5.3 (5.1)
Completeness (%)	99.6 (99.2)
Mean $I/\sigma(I)$	35.8 (3.8)

$$^{\dagger} R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

rod-shaped crystals were obtained (Fig. 1). A native AbPal-71 crystal diffracted to beyond 1.4 Å resolution using synchrotron radiation (Fig. 2). Upon processing the X-ray diffraction data, the native AbPal-71 crystal was found to belong to space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 72.58$, $c = 44.65$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ (Table 1). The Matthews coefficient was $2.64 \text{ Å}^3 \text{ Da}^{-1}$ and the solvent content was 53.4%, assuming that the asymmetric unit contained one molecule of AbPal (Matthews, 1968).

The OmpA-like domain of AbPal shares ~50% amino-acid sequence identity with EcPal, for which a crystal structure has been determined (PDB entry 1oap; C. Abergel, A. Walburger, E. Bouveret & J.-M. Claverie, unpublished work), in contrast to only ~26% sequence identity with AbOmpA-PD. Given their similar role in peptidoglycan binding, comparing the structures of AbPal and AbOmpA-PD will reveal valuable structural insights into the distinct functional aspects of each protein. In conclusion, our preliminary X-ray data will be useful for further structural characterization of the OmpA-like domain of AbPal. Structure determination by molecular replacement using the structure of EcPal and cocrystallization experiments with peptidoglycan fragments are currently under way.

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