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Purification, crystallization and preliminary X-ray crystallographic analysis of Lmo0540 from *Listeria monocytogenes*

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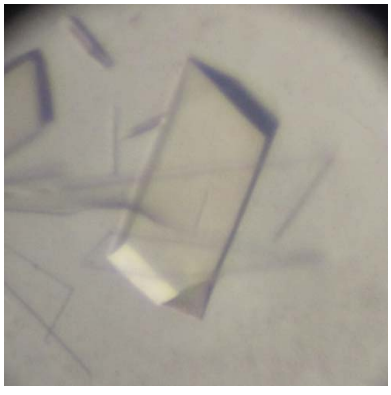
Penicillin-binding proteins catalyze the biosynthesis of the peptidoglycan chains of the bacterial cell wall, which protects cells from osmotic pressure. Although Lmo0540 has been identified as a putative penicillin-binding protein that contributes to the virulence of *Listeria monocytogenes*, the biochemical role of Lmo0540 remains unclear. To provide insights into its biochemical function, Lmo0540 was overexpressed, purified and crystallized by the sitting-drop vapour-diffusion method. Diffraction data were collected to 1.5 Å resolution using synchrotron radiation. The crystal belonged to the *C*-centred monoclinic space group *C*2, with unit-cell parameters $a = 82.5$, $b = 75.7$, $c = 75.9$ Å, $\alpha = \gamma = 90$, $\beta = 121.8^\circ$. A full structural determination is under way in order to elucidate the structure–function relationship of this protein.

1. Introduction

The bacterial cell wall is mainly composed of peptidoglycan chains, which play critical roles in controlling the shape of cells and in protecting them from osmotic pressure (Young, 2003; Popham & Young, 2003). These peptidoglycan chains are constantly remodelled and new chains are incorporated at specific places as the bacteria grow and divide (Macheboeuf *et al.*, 2006). Penicillin-binding proteins (PBPs) catalyze the assembly of peptidoglycan chains through the transglycosylation and transpeptidation of lipid II, which is composed of an undecaprenyl-linked disaccharide-pentapeptide containing β-linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) (Sauvage *et al.*, 2008). The five-residue peptide stem is attached to the *N*-acetylmuramic acid. The transglycosylation reaction catalyzes the polymerization of the NAG-NAM disaccharide subunit of bacterial peptidoglycan (Halliday *et al.*, 2006; Lovering *et al.*, 2007), whereas the transpeptidation reaction catalyzes the cross-linking of peptidoglycan chains by a peptide bridge (Born *et al.*, 2006). In addition, the endopeptidase activity of some PBPs is responsible for breaking peptide cross-links to allow new peptidoglycan chains to be inserted and the peptidoglycan to be recycled (Meberg *et al.*, 2004). At present, many proteins from newly released genomic databases are annotated as putative PBPs. However, their biochemical and/or physiological roles remain unclear despite extensive studies of several representative PBPs.

Listeria monocytogenes is a Gram-positive intracellular pathogen that causes listeriosis. It is sensitive to penicillins, but has a relatively high natural resistance to cephalosporins and fosfomycin (Conter *et al.*, 2009; Espaze & Reynaud, 1988). Recently, nine PBPs expressed by *L. monocytogenes*, including Lmo0540, were shown to bind the fluorescent antibiotic BOCILLIN FL (Korsak *et al.*, 2010). However, only a few PBPs from *L. monocytogenes* have been studied in order to elucidate their biochemical and/or physiological properties (Zawadzka-Skomial *et al.*, 2006; Vicente *et al.*, 1990; Korsak *et al.*, 2005). Interestingly, disruption of Lmo0540 produced no defects in cell morphology but did lead to attenuated virulence, as determined from colonization of the spleen in mice (Guinane *et al.*, 2006). Lmo0540 from *L. monocytogenes* was initially annotated as a low-molecular-mass (LMM) PBP homologue with variations in the classical PBP motifs. *BLAST* and domain analysis of Lmo0540 showed that it contained the class C β-lactamase domain. In addition, the



overall fold of Lmo0540 may be most similar to the structure of the DD-peptidase (PDB entry 3pte; Kelly & Kuzin, 1995) from *Streptomyces* sp. R61, which shows the highest sequence identity to Lmo0540 (27%) among solved PBP structures. However, the biochemical and enzymatic properties of Lmo0540 have not been reported.

We successfully purified and crystallized the putative penicillin-binding protein Lmo0540. The resulting monoclinic crystal diffracted to 1.5 Å resolution using a synchrotron source. The crystal structure of Lmo0540 will provide insights into the biochemical function of the protein.

2. Materials and methods

2.1. Cloning, expression and purification of Lmo0540

The gene encoding the putative penicillin-binding protein Lmo0540 (UniProt accession No. Q8Y9I8) was amplified by the polymerase chain reaction (PCR) with *Pfu* DNA polymerase from chromosomal DNA of *L. monocytogenes* EGD. The primers were 5′-GAATTC**CATATG**GATCCTGAACCAGTAGAAGAAAAGC-3′ for the forward primer and 5′-CCCGG**CTCGAG**TTAATTTAAGA-AACCATAAATATCATTA-3′ for the reverse primer and contained *Nde*I and *Xho*I sites, respectively (bold). The PCR product was subcloned into the pProExHTa vector (Invitrogen, USA) to generate an expression plasmid encoding residues 72–396 of Lmo0540 with an N-terminal His_6 tag. The *lmo0540* gene insertion was confirmed by DNA sequencing. The resulting expression vector pProExHTa:*lmo0540* was transformed into *Escherichia coli* BL21 (DE3) RIL cells, which were then grown at 310 K in Luria–Bertani medium containing 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol until the OD_{600} reached ∼0.8. After induction with 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside at 291 K for a further 12 h, the cells were harvested by centrifugation at 5000*g* at 277 K. The cell pellet was resuspended in ice-cold buffer *A* (30 m*M* Tris–HCl pH 8.0, 0.3 *M* NaCl) and lysed by sonication. The lysate was centrifuged at 15 000*g* for 30 min and the supernatant was loaded onto Ni–NTA resin (Qiagen, USA) equilibrated with buffer *A*. After washing with buffer *A* containing 20 m*M* imidazole, the bound protein was eluted using a solution consisting of 30 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl and 200 m*M* imidazole. The eluted sample was digested overnight with TEV protease (Invitrogen, USA) at a ratio of 1:100(*w*:*w*) at 277 K to remove the hexahistidine tag. The target protein was bound to a 5 ml HiTrap Q column (GE Healthcare, Sweden) and was eluted with a 20-column-volume linear gradient from 50 to 500 m*M* NaCl in a buffer consisting of 30 m*M* Tris–HCl pH 8.0 and 1 m*M* dithiothreitol. Finally, the protein was further purified using a Superdex 200 (GE Healthcare) column equilibrated in 10 m*M* Tris–HCl pH 8.0 and 100 m*M* NaCl. The fractions of Lmo0540 that eluted as a monomer were pooled and concentrated to 25 mg ml^{-1}. Aliquots were flash-frozen in liquid nitrogen and stored at 193 K for subsequent use in crystallization. The purity of the protein was greater than 95% based on an SDS–PAGE analysis (data not shown). The removal of the hexahistidine tag leaves three residues (GHM) encoded by the expression vector at the N-terminus of the recombinant protein.

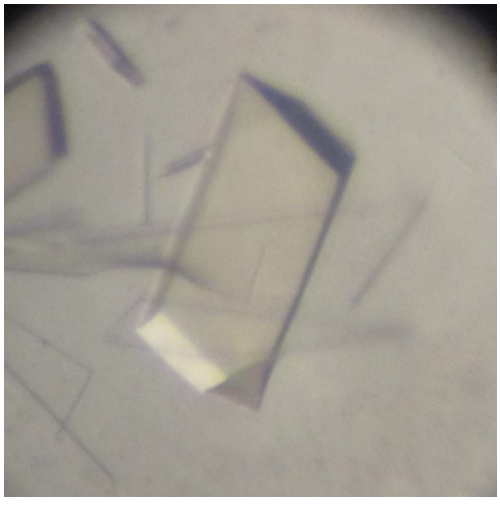


Figure 1
Crystals of Lmo0540. The crystals grew within one week at 295 K to maximum dimensions of approximately 0.2 × 0.2 × 0.4 mm.

Table 1
Diffraction statistics.

Values in parentheses are for the highest resolution shell. A 0σ cutoff filter was applied during the scaling process.

X-ray source	Beamline 4A, PAL
Wavelength (Å)	1.000
Resolution (Å)	30.0–1.5 (1.53–1.50)
Space group	*C*2
Unit-cell parameters (Å, °)	a = 82.5, b = 75.7, c = 75.9, $\alpha = \gamma = 90$, β = 121.8
Completeness (%)	98.5 (95.1)
R_{merge}†	5.0 (26.0)
Multiplicity	5.2 (3.4)
Average $I/\sigma(I)$	34.6 (3.2)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the observed intensity and the mean intensity of related reflections, respectively.

2.2. Crystallization

Truncated Lmo0540 (325 amino acids) was crystallized using the sitting-drop vapour-diffusion method in 96-well Intelli-Plates (Hampton Research, USA). Initial crystallization conditions were obtained by sparse-matrix screening (Jancarik *et al.*, 1991) using commercial kits from Hampton Research (California, USA) and Emerald BioSystems (Washington, USA) by manually mixing 0.4 µl protein solution and 0.4 µl reservoir solution and equilibrating against 100 µl reservoir solution at 295 K. Hits from initial crystallization screens were further optimized by varying the concentration of PEG 8000 and monobasic potassium phosphate using a VDX plate (Hampton Research, USA) to obtain crystals that were suitable for X-ray diffraction.

2.3. X-ray data collection

For X-ray data collection, a single crystal was immersed briefly into reservoir solution containing 15% glycerol as a cryoprotectant and immediately flash-cooled in a 100 K nitrogen stream. Native X-ray diffraction data were collected using an ADSC Q315r CCD detector on beamline 4A at Pohang Accelerator Laboratory (PAL; Republic of Korea) using 1° oscillations with a crystal-to-detector distance of 250 mm. The crystal was exposed for 5 s per image. A data set was collected to 1.5 Å resolution from a single crystal. The data were indexed and scaled with the *HKL*-2000 software package (Otwinowski & Minor, 1997). The data-collection statistics are summarized in Table 1.

3. Results and discussion

To facilitate purification and crystallization, the N-terminal 71 residues of Lmo0540, which contain the signal sequence and a putative

transmembrane helix, were truncated in the expression construct. Recombinant Lmo0540 protein was expressed and purified to homogeneity using sequential chromatographic steps. The yield of purified protein was ∼10 mg per litre of *E. coli* culture. After optimizing the crystallization conditions, shiny single crystals were obtained using sitting-drop vapour diffusion by mixing equal volumes (2 µl) of protein solution (25 mg ml^{-1}) and reservoir solution composed of 23%(*w*/*v*) PEG 8000, 0.04 *M* monobasic potassium phosphate pH 4.5 and 3%(*v*/*v*) 1,4-dioxane. The best crystals grew within 7 d to final dimensions of approximately 0.2 × 0.2 × 0.4 mm (Fig. 1). The Lmo0540 crystal diffracted to 1.5 Å resolution and belonged to space group *C*2, with unit-cell parameters $a = 82.5$, $b = 75.7$, $c = 75.9$ Å, $\alpha = \gamma = 90$, $\beta = 121.8^{\circ}$. The diffraction data set was 98.5% complete, with an $R_{\rm merge}$ of 5.0% (Table 1). Assuming one Lmo0540 molecule per asymmetric unit, the Matthews parameter $V_{\rm M}$ (Matthews, 1968) was 2.78 Å^3 Da^{-1}, corresponding to a solvent content of 55.8%. In order to determine the structure, molecular replacement was attempted using the structure of the DD-peptidase from *Streptomyces* sp. R61 (27% sequence identity for 325 aligned amino acids) as a search model in the program *MOLREP* (Vagin & Teplyakov, 2010), but this method was not successful. In order to solve the structure, selenomethione-substituted Lmo0540 is being prepared for phasing by multiple anomalous dispersion (MAD).

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