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Expression, purification, crystallization and preliminary X-ray diffraction analysis of an essential lipoprotein implicated in cell-wall biosynthesis in *Mycobacteria*

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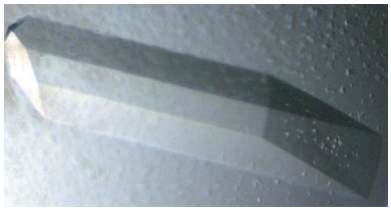

Mycobacterium tuberculosis is a renewed cause of devastation in the developing world. Critical to the success of this re-emerging pathogen is its unusual waxy cell wall, which is rich in rare components including lipoarabinomannan (LAM) and its precursors, the phosphatidylinositol mannosides (PIMs). Balanced synthesis of these related glycolipids is intrinsic to both cell-wall integrity and virulence in *M. tuberculosis* and presents a promising, albeit poorly defined, therapeutic target. Here, the expression, purification and crystallization of an essential 600-amino-acid lipoprotein, LpqW, implicated in this process are reported. Crystals of LpqW were grown using 20–24%(*w*/*v*) PEG 4000, 8–16%(*v*/*v*) 2-propanol, 100 m*M* sodium citrate pH 5.5 and 10 m*M* DTT. A complete data set was collected at 2.4 Å using synchrotron radiation on a crystal belonging to space group *C*222, with unit-cell parameters $a = 188.57$, $b = 312.04$, $c = 104.15$ Å. Structure determination is under way.

1. Introduction

Tuberculosis (TB) claims the lives of over two million people each year, with more than a quarter of deaths in Africa alone. The epidemic has rapidly worsened in this region over the past 15 y owing to poor compliance with TB treatment programs and widespread coincidence of HIV/AIDS. This has also fostered rampant multi-drug resistance in the causative bacterium, *Mycobacterium tuberculosis*, and intensified the need to develop new drugs against this agent. The unusual waxy cell wall of *M. tuberculosis* is essential to its survival and pathogenicity and thus holds significant potential as a target for such drugs.

The cell wall of *Mycobacteria* spp. consists of a peptidoglycan core with covalently linked layers of arabinogalactan and mycolic acid (Brennan & Nikaido, 1995). This complex structure forms a hydrophobic barrier, complemented by glycolipids including lipoarabinomannan (LAM) and its abundant precursors, the phosphatidylinositol mannosides (PIMs; Brennan & Nikaido, 1995). By actively blocking maturation of phagosomes containing internalized bacilli while permitting fusion events with early endosomes, these lipoglycans are responsible for the survival and persistence of *M. tuberculosis* in the host macrophage (Fratti *et al.*, 2003; Vergne *et al.*, 2004).

PIMs consist of a phosphatidylinositol anchor glycosylated with one to six mannose residues (PIM1–PIM6) and modified by extra fatty-acyl chains. Addition of further mannose residues gives lipomannans (LMs) and these intermediates are in turn glycosylated with branching arabinose chains to form LAMs (Besra *et al.*, 1997). Although the bifurcation of the synthesis of PIMs and LAM is not defined, it is thought to arise from the addition of either α(1–2)- or α(1–6)-linked mannose to the last common intermediate, PIM4, in the periplasmic space (Morita *et al.*, 2004).

Recently, a putative lipoprotein comprised of 600 amino-acid residues (62.9 kDa), termed LpqW, has been implicated in controlling the divergence point of the PIMs/LAM biosynthetic pathways (Kovacevic *et al.*, 2005). Moreover, saturation mutagenesis studies revealed that the corresponding gene (Rv1166c) was essential to *M. tuberculosis* (Sassetti *et al.*, 2003), suggesting that different mycobacterial species exhibit subtleties in their dependencies on the PIMs/LAM metabolic machinery.

Table 1
Data-collection statistics.

The values in parentheses are for the highest resolution bin (approximate interval 0.1 Å).

Temperature (K)	100
X-ray source	APS, BioCars 14-BM-C
Detector	Quantum 4 CCD
Space group	*C*222
Unit-cell parameters (Å)	a = 188.6, b = 312.0, c = 104.1
Resolution (Å)	2.4
Total No. of observations	439493
No. of unique observations	115857
Multiplicity	3.8
Data completeness (%)	97.1 (93.0)
No. of data > $2\sigma(I)$	91071 (4632)
$\langle I/\sigma(I)\rangle$	17.9 (2.1)
$R_{\rm merge}$ (%)	8.1 (57.7)

† $R_{\rm merge} = \sum |I_{hkl} - \langle I_{hkl}\rangle| / \sum_{hkl}$.

To delineate the structure and function of LpqW, the soluble ectodomain of the *M. smegmatis* homologue was expressed in *Escherichia coli*. Crystals of LpqW were obtained that diffract to 2.4 Å resolution. The details of the expression, purification, crystallization and preliminary diffraction analysis are reported here.

2. Materials and methods

2.1. Cloning of LpqW ectodomain

The *lpqW* gene was PCR amplified from *M. smegmatis* mc^{2}155 genomic DNA using primers *A* (5′-GGAAT**TCCAT**ATGTGCAC-GGTGAGCCCGCCTCCC-3′) and *B* (5′-GCC**GAATTC**TACTTGG-TCTTCGTCCAGTC-3′) and Proofstart DNA Polymerase (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The primers were designed to allow expression of *lpqW* without the putative signal peptide such that the N-terminal amino acid of the recombinant protein, after the vector-derived leader sequence (MGSSH$_6$SSGLVPRGSHM), is the cysteine to which the lipid is likely to be attached in mycobacteria. Restriction-enzyme sites for *Nde*I and *Eco*RI (bold) were incorporated into primers *A* and *B*, respectively, for cloning purposes. The PCR product was purified using the UltraClean 15 DNA Purification Kit (Mo Bio Laboratories, CA, USA). *Nde*I/*Eco*RI-digested PCR product was then cloned into *Nde*I/*Eco*RI-digested pET28b (Novagen, WI, USA). A positive clone

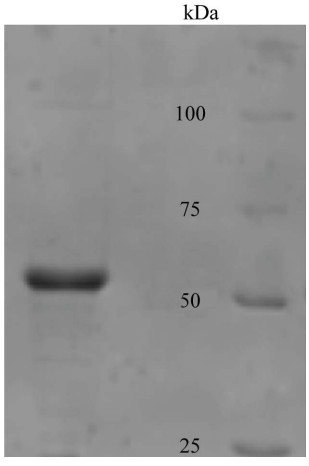

Figure 1
Reducing SDS–PAGE of LpqW purified using Ni–NTA and size-exclusion chromatography (lane 1). The positions of the molecular-weight markers are indicated in lane 2. The gel was stained using Coomassie Brilliant Blue.

was sequenced using T7 promoter and terminator primers. The resulting construct, encoding a 620-amino-acid protein with a N-terminal hexahistidine tag, was used to transform the *E. coli* BL21 strain.

2.2. Protein expression and purification

BL21 cells were grown at 310 K to an OD_{600} of 0.5 in Luria–Bertani medium containing kanamycin (34 µg ml^{-1}). Protein expression was induced by the addition of 0.5 m*M* isopropyl-β-D-thiogalactopyranoside. Cells were harvested after 4 h incubation by centrifugation at 6000*g* for 20 min at 277 K, resuspended in lysis buffer (20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 0.2 m*M* phenylmethylsulfonyl fluoride, 10 m*M* β-mercaptoethanol) and homogenized by passage through a French press (Avestin, Ottawa, Canada). Lysates were centrifuged at 10 000*g* for 15 min at 277 K and applied onto a 2 ml column of Ni–NTA agarose (Qiagen) in equilibration buffer (20 m*M* imidazole, 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl). The column was washed with five column volumes of equilibration buffer and LpqW was eluted in 200 m*M* imidazole, 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl. The protein was further purified by chromatography on a calibrated 16/60 Superdex 200 prep-grade column (Amersham-Pharmacia, Uppsala, Sweden) in 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2 m*M* dithiothreitol (DTT), concentrated and quantified by spectrophotometric analysis.

2.3. Crystallization and data collection

Crystallization experiments were performed at room temperature by the hanging-drop vapour-diffusion technique. Solutions of purified LpqW were concentrated to 10–15 mg ml^{-1} (in 20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 2 m*M* DTT) and screened in the commercially available PEG/Ion Screen and Crystal Screens 1 and 2 (Hampton Research, CA, USA).

Diffraction-quality crystals of LpqW were grown by mixing equal volumes of 20 mg ml^{-1} protein solution and reservoir buffer consisting of 20–24%(*w*/*v*) polyethylene glycol (PEG) 4000, 8–16%(*v*/*v*) 2-propanol, 100 m*M* sodium citrate pH 5.5, 10 m*M* DTT. Crystals were equilibrated in cryoprotectant solution comprising 25%(*w*/*v*) PEG 4000, 12%(*v*/*v*) 2-propanol, 100 m*M* Tris pH 7.0 for 12 h and flash-cooled directly in liquid nitrogen prior to diffraction analysis.

A complete native data set was collected at the BioCars beamline (Advanced Photon Source, Chicago) using a Quantum 4 charge-coupled device detector. The native data set comprised 260 images (0.5° oscillation, 5 s exposure per oscillation). Diffraction data were processed and analysed using the *HKL* package (Table 1) (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Protein expression and purification

Following cell lysis, the majority of the LpqW protein was in soluble form. Protein eluted from the Ni–NTA chromatography medium using 200 m*M* imidazole was approximately 80% pure, as estimated by SDS–PAGE. A polypeptide of approximate molecular weight 65 kDa was observed that matched the predicted size (65.1 kDa) of LpqW including an N-terminal hexahistidine tag and the additional residues encoded by pET28b. Yields of pure protein were typically 6 mg per litre of culture. The protein was applied directly onto a calibrated S200 size-exclusion column. The majority of

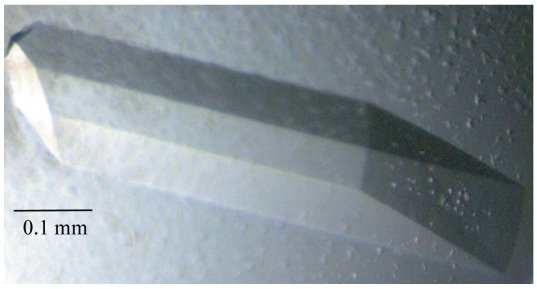

Figure 2
Diffraction-quality crystal of LpqW.

LpqW eluted from the column in monomeric form (Fig. 1) and was concentrated to 20 mg ml^{-1} for crystallization trials.

3.2. Crystallization and data collection

Crystals were obtained in two of the conditions screened and improved orthorhombic crystals grew in conditions based on Crystal Screen formulation No. 40. Freezing conditions that included a range of cryoprotectant agents at various concentrations were screened and resulted in crystals of very poor diffraction quality [high mosaicity (greater than 1.5°) and low resolution (less than 4 Å)]. However, single-step dehydration in reservoir buffer containing an increased concentration (total 25%) of the precipitant PEG 4000 for 12 h, followed by flash-cooling directly in liquid nitrogen, extended the diffraction limit of the crystals with a much reduced mosaicity (less than 0.6°).

Crystals of LpqW that grew in the optimized condition (Fig. 2) reproducibly diffracted to beyond 3 Å in-house. A complete native data set to 2.4 Å resolution was collected on a crystal belonging to the orthorhombic space group *C*222, with unit-cell parameters a = 188.57, b = 312.04, c = 104.15 Å. Assuming the presence of three monomers per asymmetric unit, the calculated V_M (Matthews, 1968) and solvent content for the native crystal were 3.9 Å^3 Da^{-1} and 68% respectively. Nonetheless, there is no biochemical evidence to suggest that LpqW functions as an oligomer.

The three-dimensional structure of LpqW will provide insight into the regulatory mechanisms that ensure balanced synthesis of PIMs and LAM in the cell wall of *M. tuberculosis*. LpqW has been proposed to function at the point where the biosynthetic pathways for these molecules bifurcate. As the maintenance of this equilibrium is important for both cell-wall integrity and pathogenesis, this will not only serve intrinsic interest in mycobacterial cell-wall biosynthesis, but also potentially elucidate targets for and thus inform the design of novel anti-tuberculosis drugs. Structure determination is under way.

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