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Purification, crystallization and preliminary crystallographic analysis of *Streptococcus pyogenes* laminin-binding protein Lbp

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The laminin-binding protein Lbp (Spy2007) from *Streptococcus pyogenes* (a group A streptococcus) mediates adhesion to the human basal lamina glycoprotein laminin. Accordingly, Lbp is essential in *in vitro* models of cell adhesion and invasion. However, the molecular and structural basis of laminin binding by bacteria remains unknown. Therefore, the *lbp* gene has been cloned for recombinant expression in *Escherichia coli*. Lbp has been purified and crystallized from 30%(*w*/*v*) PEG 1500 by the sitting-drop vapour-diffusion method. The crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 42.62$, $b = 92.16$, $c = 70.61$ Å, $\beta = 106.27^\circ$, and diffracted to 2.5 Å resolution.

1. Introduction

The Gram-positive bacterium *Streptococcus pyogenes* is one of the most common and versatile human pathogens. It causes a broad range of minor diseases such as pharyngitis, erysipelas and pyodermas, which are readily controlled by antibiotics. Since the 1980s, however, *S. pyogenes* has gained new attention as severe and often lethal diseases such as acute rheumatic fever, necrotizing fasciitis and streptococcal toxic shock syndrome have re-emerged in the western world (reviewed in Cunningham, 2000). Moreover, streptococcal rheumatic heart disease is a major issue in less developed countries and also in certain populations within developed countries such as the Aboriginal community in Australia and the Maori community in New Zealand (reviewed in Carapetis *et al.*, 2005).

A crucial step in the infection of human host tissue by *S. pyogenes* is the recognition and binding of host structures by bacterial adhesion molecules. *S. pyogenes* has been shown to bind to a variety of human cell-surface and extracellular matrix (ECM) proteins, for instance fibronectin (Courtney *et al.*, 1992), collagen (Visai *et al.*, 1995), vitronectin (Valentin-Weigand *et al.*, 1988), CD46 receptor (Okada *et al.*, 1995) and laminin (Switalski *et al.*, 1984).

A mediator of adherence to laminin has only recently been discovered in all serotypes of *S. pyogenes* (Terao *et al.*, 2002; Elsner *et al.*, 2002) and in the closely related species *S. agalactiae* (a group B streptococcus; Spellerberg *et al.*, 1999). In *S. pyogenes* serotype M1, this mediator is called laminin-binding protein Lbp and corresponds to gene number *spy2007* in the serotype M1 strain SF370. In both *S. pyogenes* and *S. agalactiae*, Lbp is tethered to the bacterial surface by an N-terminal lipid anchor (Spellerberg *et al.*, 1999; Elsner *et al.*, 2002). Based on homology searches, Lbp is a member of a family of extracellular metal-binding receptors (MBR) that are part of ABC-type permease complexes. However, in contrast to the other family members, Lbp is not encoded in one operon together with a corresponding permease and an ATPase (Elsner *et al.*, 2002).

Evidence points to Lbp having a role as a laminin-binding molecule: the binding of *S. pyogenes* and *S. agalactiae* to laminin is significantly decreased by deletion/mutation of the *lbp* gene (Spellerberg *et al.*, 1999; Elsner *et al.*, 2002) or by preincubation of either laminin or bacteria with recombinant Lbp (Spellerberg *et al.*, 1999; Wahid *et al.*, 2005). Furthermore, Lbp seems to be essential in

adhesion to and/or invasion of eukaryotic cells *in vitro*, depending on the cell type used. Again, adhesion and/or invasion can be reduced either by mutation of *lbp* or by preincubation with Lbp or anti-Lbp antibody (Elsner *et al.*, 2002; Terao *et al.*, 2002; Tenenbaum *et al.*, 2007).

Since laminin is a major part of the basal lamina, a type of ECM supporting the epithelia and endothelia, the binding of laminin by bacteria may be a key step in bacterial migration through these tissues. However, the structural basis of laminin binding by bacteria is still unknown. Therefore, we expressed, purified and crystallized Lbp. A structure of Lbp might provide important new information on its binding mechanisms, particularly in comparison with known MBR structures such as TroA (PDB code 1toa; Lee *et al.*, 1999), ZnuA (PDB code 1pq4; Banerjee *et al.*, 2003), MntC (PDB code 1xvl; Rukhman *et al.*, 2005) and PsaA (PDB code 1psz; Lawrence *et al.*, 1998). As Lbp has been shown to be immunogenic (Wahid *et al.*, 2005) and to occur in all *S. pyogenes* serotypes, its structure also may guide the development of an efficient vaccine to prevent severe streptococcal infections.

2. Methods and results

2.1. Cloning, expression and purification

The gene *lbp*, without the region coding for the N-terminal signal peptide sequence, was amplified from genomic *S. pyogenes* serotype M1 (strain SF370) DNA by PCR using the primers spy2007_forw (5′-**GGC AGC GGC GCG** AAC CCC AAA CAG CCT ACG C-3′) and spy2007_rev (5′-**GAA AGC TGG GTG** TTA CTT CAA CTG TTG ATA GAG-3′). The product of this PCR was used in a second round of PCR ('nested PCR') with the primers gateway_forw (5′-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC *GAA AAC CTG TAT TTT CAG* ***GGC*** **AGC GGC GCG**-3′) and gateway_rev (5′-GGG GAC CAC TTT GTA CAA **GAA AGC TGG GTG**-3′). Bold nucleotides indicate the adapter sequence for the nested PCR; italicized residues indicate a sequence encoding the recognition site for the recombinant tobacco etch virus (TEV) protease. The second-round PCR product was cloned into a pDONR221 vector through a recombinase reaction (Gateway Technology, Invitrogen). The resulting construct, termed pDONR221_spy2007, was sequenced by the University of Auckland Centre for Genomics and Proteomics. Its insert was transferred into a pDEST17 vector through another recombinase reaction (Gateway Technology, Invitrogen) which confers an N-terminal His_6-tag (MSYYH$_6$LESTSLYKKAG). This final construct was termed pDEST17_spy2007.

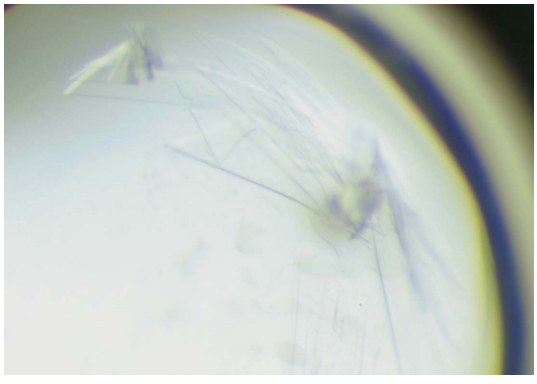

Figure 1
Crystals of Lbp [drop: 700 nl 37 mg ml^{-1} Lbp in PBS + 0.002%(*w*/*v*) NaN_3 and 700 nl 30%(*w*/*v*) PEG 1500] grown at 291 K overnight. The dimensions of the largest crystal plate are ∼200 × 40 µm.

pDEST17_spy2007 was transformed into *Escherichia coli* BL21 (DE3) cells for heterologous overexpression. Cultures of *E. coli* BL21 (DE3) pDEST17_spy2007 were grown in auto-inducing ZYM-5052 medium (Studier, 2005) containing 100 µg ml^{-1} ampicillin at 310 K for 2 h and then at 301 K for 20 h with shaking. Cells were harvested by centrifugation (20 min, 3800*g*, 277 K), resuspended in 50 m*M* bis-Tris propane–HCl pH 9.0, 500 m*M* NaCl, 0.002%(*w*/*v*) NaN_3 (buffer *A*) with Complete Protease Inhibitor Cocktail Mini Tablets (EDTA-free, Roche) at 277 K and lysed by ultrasonication on ice. The lysate was cleared by sedimentation of insoluble matter (centrifugation at 20 000*g* for 30 min at 277 K).

Lbp was enriched by immobilized metal-affinity chromatography (IMAC) employing TALON resin (Clontech) with cobalt: the cleared lysate was added to TALON resin in a gravity-flow column. Bound protein was washed with 20 column volumes (CV) of buffer *A* and eluted with three CV of buffer *A* + 10 m*M* imidazole and 6–9 CV of 50 m*M* imidazole. Eluted protein was incubated with rTEV protease (in a ratio of 1 mg rTEV protease per 20 mg eluted protein) and 5 m*M* dithiothreitol (DTT) under concomitant dialysis against 10 m*M* phosphate pH 7.4, 137 m*M* NaCl, 2.7 m*M* KCl (phosphate-buffered saline; PBS) + 5 m*M* DTT and 0.002%(*w*/*v*) NaN_3 at 277 K overnight. The digest was applied onto TALON resin and the flow-through containing digested protein was recovered and concentrated by ultrafiltration in Vivaspin-20 concentrators (molecular-weight cutoff 5 kDa). Finally, Lbp was applied onto a HiLoad 16/60 Superdex 200 prep-grade (Amersham Biosciences) column pre-equilibrated in PBS + 0.002%(*w*/*v*) NaN_3 at 277 K. A small proportion of Lbp was eluted at a retention volume of 78 ml and the majority at 88 ml. Fractions of the peaks at 88 ml were assessed by SDS–PAGE and dynamic light scattering (DLS) using Dynapro-MS (Wyatt): typically, Lbp was more than 95% pure and monodisperse (C_p/R_h < 10%). The best fractions were concentrated to 37 mg ml^{-1} in PBS + 0.002%(*w*/*v*) NaN_3 as measured by absorption at 280 nm and assuming a calculated extinction coefficient of 27 390 M^{-1} cm^{-1} (*PROTPARAM*; Gasteiger *et al.*, 2005). The yield of Lbp was estimated to be 10–15 mg per litre of culture.

The final purified protein comprised 286 amino-acid residues, representing residues 6–287 of the mature protein together with a four-residue N-terminal extension (GSGA) that remained after cleavage of the His_6-tag. The identity of the purified protein as Lbp was confirmed by mass spectrometry (LC-MS/MS) by the University of Auckland Centre for Genomics and Proteomics. DLS data pointed to a monomeric state of Lbp in solution (PBS). The protein could be stored briefly at 277 K or long-term at 193 K without precipitation.

2.2. Crystallization

The crystallization properties of Lbp were screened using a Cartesian Honeybee nanolitre dispensing robot (Genomic Solutions) and a local 480-condition crystallization screen (Moreland *et al.*, 2005). Experiments were carried out by sitting-drop vapour diffusion at 291 K using 96-well Intelli-Plates (Hampton Research) with 100 µl reservoir solution. 100 nl reservoir solution was added to 100 nl protein solution [37 mg ml^{-1} Lbp, PBS + 0.002%(*w*/*v*) NaN_3]. Plate-shaped crystals grew overnight or within 2 d at 291 K using a reservoir solution consisting of 30%(*w*/*v*) PEG 1500.

Fine screens around this condition were performed using Intelli-Plates (Hampton Research) and varying the PEG 1500 concentration and drop size. Single plate-shaped crystals were obtained using 30%(*w*/*v*) PEG 1500 with drops containing 700 nl protein solution

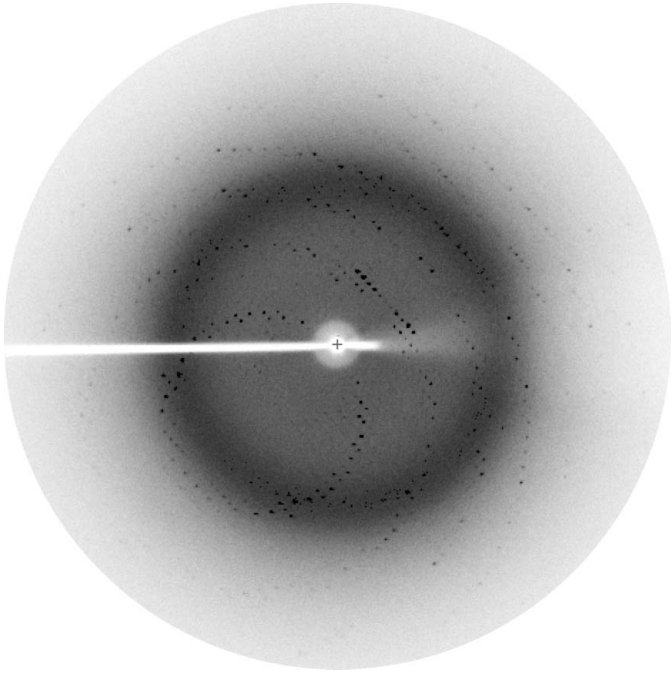

Figure 2
Diffraction pattern of Lpb at 0.5° to a resolution of 2.45 Å.

[37 mg ml^{-1} Lbp, PBS + 0.002%(*w*/*v*) NaN_3] and 700 nl reservoir solution [30%(*w*/*v*) PEG 1500] at 291 K overnight (Fig. 1). Further screening with different crystallization methods, alternative crystallization solutions and additives yielded no change in crystal morphology.

2.3. Data collection and processing

Crystals of Lbp were transferred into Paratone-N prior to flash-cooling in liquid nitrogen. X-ray diffraction data were collected in-house (Micromax-007HF, Rigaku; MAR345DTB, MAR Research) under cooling by a nitrogen-gas stream at 100 K (Cobra Oxford Cryosystems). The data were processed with *MOSFLM* (Leslie, 1992) and scaled using *SCALA* (Evans, 2006). The statistics are summarized in Table 1.

3. Results and discussion

A complete 360° data set of good quality could be collected to 2.45 Å resolution from a crystal of dimensions 160 × 40 µm (Fig. 2), despite a relatively high mosaicity which is probably a consequence of the tendency of the crystals to grow as overlapping stacked plates. The diffraction symmetry established the crystals as monoclinic, space group $P2_1$ (from systematic absences), with unit-cell parameters a = 42.62, b = 92.16, c = 70.61 Å, β = 106.27° and two Lbp molecules per asymmetric unit, as judged from a Matthews coefficient of 2.14 Å^3 Da^{-1} (solvent content 42.6%). The availability of the structures of other proteins from the MBR family, such as TroA, ZnuA, MntC and PsaA, suggests that the structural analysis could be undertaken by molecular replacement, despite the relatively low sequence identity (20–30% identity on a pairwise basis).

Table 1
Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Wavelength (Å)	1.5418
No. of images	720
Oscillation angle (°)	0.5
Exposure time (min)	10
Resolution range (Å)	29.09–2.45 (2.58–2.45)
Total No. of observations	141445 (20392)
Unique reflections	19354 (2823)
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 42.62, b = 92.16, c = 70.61, α = 90, β = 106.27, γ = 90
Matthews coefficient (Å^3 Da^{-1})	2.14
Mosaicity (°)	1.32
Molecules per ASU	2
Solvent content (%)	42.6
Completeness (%)	99.9 (100.0)
Mean $I/\sigma(I)$	18.8 (3.0)
R_{merge} (%)	9.4 (52.8)

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