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Overexpression, purification and crystallization of bacteriocin LlpA from *Pseudomonas* sp. BW11M1

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LlpA is a bacteriocin produced by *Pseudomonas* sp. BW11M1 that shows remarkable similarity to a family of mannose-binding plant lectins. A His-tagged version of LlpA was recombinantly produced in *Escherichia coli* and purified to homogeneity. Single crystals were grown by vapour diffusion and belong to space group $P2_12_12$, with unit-cell parameters a = 150.5, b = 154.5, c = 34.2 Å. The crystals diffract to at least 2.2 Å using synchrotron radiation.

1. Introduction

Bacteriocins are peptides or proteins that can kill or inhibit the growth of related bacterial species, but not the producer strain (James *et al.*, 1996). They are found in almost every bacterial species examined to date and within a given species tens or even hundreds of different kinds of bacteriocins can be produced (Riley & Wertz, 2002). It is thought that bacteriocin production serves to increase the competitiveness and diversity of bacteria in their ecological niche (Kirkup & Riley, 2004). Bacteriocins range from small peptides produced mainly by Gram-positive bacteria (Hechard & Sahl, 2002) to high-molecular-weight complexes that resemble defective bacteriophages, such as the R- and F-type pyocins of *Pseudomonas aeruginosa* (Michel-Briand & Baysse, 2002). Owing to the great variety in their chemical structure, bacteriocins can affect different essential functions of the bacterial cell (transcription, translation, replication and cell-wall biosynthesis), but most of them act by forming membrane channels or pores that destroy the energy potential of sensitive cells (Moll *et al.*, 1999; Lakey & Slatin, 2001).

Very little is known about bacteriocin production by bacteria occupying the rhizosphere, a zone of intense microbial activity surrounding plant roots. Fluorescent *Pseudomonas* are common rhizosphere-inhabiting bacteria that can promote plant health (Lucy *et al.*, 2004). From the rhizosphere isolate *Pseudomonas* sp. BW11M1, we have identified a novel type of antibacterial protein (bacteriocin LlpA) that is not related in sequence to any other previously characterized *Pseudomonas* bacteriocin. LlpA is a potent growth inhibitor of the rhizosphere strain *P. putida* GR12-2R3, as well as some phytopathogenic pseudomonads (Parret *et al.*, 2003). Sequence alignments indicate that this protein consists of two domains related to the monocot mannose-binding lectins (MMBLs; Parret *et al.*, 2003). MMBL domains (called B_lectin domains in the Protein Families database; http://www.sanger.ac.uk/Software/Pfam) are widespread among monocotyledonous plants (Van Damme *et al.*, 1998) but absent in dicotyledonous plants. Recently, two mannose-binding lectins, each with one MMBL or B_lectin domain, were isolated from the pufferfish *Fugu rubripes*. Both these pufflectins can bind parasitic nematodes, suggesting that these proteins contribute to the parasite-defence system in *Fugu* (Tsutsui *et al.*, 2003).

Genome analysis has indicated that LlpA is the first functionally characterized member of a family of bacterial proteins with diverse composite structures but that have one or two MMBL domains in common (Parret *et al.*, 2003). A second bacteriocin member of this family was identified as albusin B from the ruminal bacterium *Ruminococcus albus* 7 that inhibits the growth of *R. flavus* (Chen *et al.*, 2004). Despite their sequence similarities to mannose-binding lectins, neither LlpA nor albusin B possess mannose-binding activity. However, there are many cases in which proteins closely related to either plant or animal lectins lack carbohydrate-binding activity but have acquired a different function. Examples include the MMBL-related curculin (Barre *et al.*, 1997), the arcelins (Hamelryck *et al.*, 1996) and α-amylase inhibitors (Bompard-Gilles *et al.*, 1996) from the legume lectin family, antifreeze proteins from the C-type lectin family (Loewen *et al.*, 1998) and Charcot–Leyden crystal protein from the galectin family (Leonidas *et al.*, 1995).

It is expected that the crystal structure of LlpA together with further biochemical characterization will provide insight into the antibacterial action of this protein and other members of the lectin-like bacteriocin protein family. Moreover, structure determination of LlpA could reveal whether or not bacteriocin activity involves carbohydrate recognition. We

report here the large-scale expression of recombinant LlpA and the first diffraction data of its crystals as a first step towards the elucidation of the structure and function of this unusual bacteriocin.

2. Material and methods

2.1. Cloning, expression and purification

The *llpA* gene was amplified from *Pseudomonas* sp. BW11M1 by high-fidelity PCR using 5′-modified primers to generate terminal restriction sites for subsequent cloning into the N-terminal His-tag fusion vector pET28a (Novagen). The sequences of the oligonucleotides were as follows (restriction sites are in bold): pseu-730-5′*Nde*I, 5′-TA**C ATA TG**G CAG GTC GTA CCC GCA TT-3′; pseu-731-5′*Xho*I, 5′-TA**C TCG AG**T CAG AAG TGC CAG GTC CAG AT-3′. The amplified fragments were cloned into pCRII-TOPO (Invitrogen) after addition of 3′-A overhangs to create the plasmid pCMPG6029. The 835 bp *Nde*I/*Xho*I fragment from pCMPG6029 was cloned behind a T7 promoter in pET28a and the integrity of the resulting fusion pCMPG6056 was confirmed by cycle sequencing using two internal *llpA* primers. *Escherichia coli* BL21(DE3) cells (Novagen) containing pCMPG6056 were grown in Luria–Bertani medium supplemented with 20 µg ml^{-1} kanamycin at 310 K until the cell density reached an OD_{600} of 0.6–0.8. The culture was then induced with a final concentration of 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 290 K. The cells were harvested by centrifugation (4000*g*, 20 min, 277 K) and frozen at 253 K. The following day the cell pellets were thawed on ice for 15 min and resuspended in lysis buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, 10 m*M* imidazole pH 8). Cells were disrupted by adding lysozyme (Sigma) to a final concentration of 1 mg ml^{-1}, followed by incubation at 310 K for 30 min. Complete lysis was achieved by sonicating the lysate on ice 10 times for 10 s with 10 s pauses at 200–300 W. The soluble lysate fraction was cleared by centrifugation (30 min, 10 000*g*, 277 K) and mixed with Ni–NTA agarose (Qiagen) equilibrated with lysis buffer. His-tagged proteins were allowed to bind to the matrix overnight at 277 K with gentle shaking. After five washing steps with buffer containing 50 m*M* NaH_2PO_4, 300 m*M* NaCl, 20 m*M* imidazole pH 8, bound proteins were eluted with 250 m*M* imidazole buffer. Buffer exchange and removal of contaminating proteins from the elution fractions was achieved on a Superdex 200 gel-filtration column (Amersham Biosciences) run with 20 m*M* bis-tris propane pH 7.0 supplemented with 200 m*M* NaCl. Fractions containing pure recombinant LlpA were identified by analysis on 12.5% SDS–PAGE and protein concentration was determined by A_{280} using a molar extinction coefficient of 62 910 M^{-1} cm^{-1} calculated according to Pace *et al.* (1995). This two-step purification procedure yielded about 75 mg LlpA per litre of culture.

2.2. Crystallization and X-ray analysis

Prior to crystallization trials, the same protein solution as above was concentrated to 10 mg ml^{-1} by ultrafiltration using a Vivaspin concentrator (Vivascience) with a 10 kDa cutoff. This protein solution is stable for several weeks at room temperature, but not at 277 K, where precipitation was observed. Also, freezing and subsequent thawing of this solution leads to irreversible aggregation. Crystallization conditions were screened by the hanging-drop vapour-diffusion method. 2 µl protein solution was mixed with 2 µl precipitant solution and equilibrated against 500 µl precipitant solution using the Hampton Research Crystal Screen I kit and the Hampton Research Malonate Grid Screen kit.

Crystals were either frozen directly in the X-ray beam [using 20–40%(*v*/*v*) glycerol added to the mother liquor as a cryoprotectant] or mounted in glass capillaries for data collection at room temperature. X-ray data were collected at EMBL station BW7A of the DESY synchrotron (Hamburg, Germany). The wavelength was 0.9184 Å and a MAR CCD detector was used. The data were indexed and processed with the *HKL* suite of programs (Otwinowski & Minor, 1997).

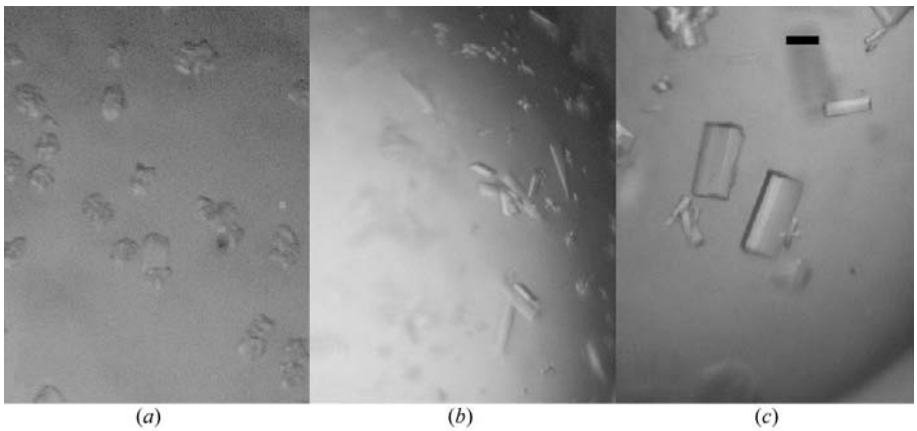

Figure 1
Crystals of LlpA. (*a*) Crystals grown in Hampton Crystal Screen condition No. 2. (*b*) Crystals grown in Hampton Crystal Screen condition No. 7. (*c*) Crystals grown in Hampton Crystal Screen condition No. 25 and used for data collection. The small black bar in (*c*) indicates as size 0.2 mm. All three panels are shown on the same scale.

3. Results and discussion

LlpA was produced recombinantly as a 295-amino-acid protein consisting of the 276 amino acids of the naturally occurring protein from *Pseudomonas* preceded by 19 additional amino acids that contain a His tag and a thrombin-cleavage site. This tag was not removed, as this results in a lower yield of protein, but did not seem to interfere with crystallization. The total molecular weight of this recombinant protein is 31 999 Da. A preliminary screen using Hampton Research Crystal Screen I resulted in small intergrown crystals in conditions Nos. 2 (0.4 *M* potassium/sodium tartrate) and 7 (0.1 *M* sodium cacodylate pH 6.5, 1.4 *M* sodium acetate) after two weeks (Figs. 1*a* and 1*b*). Preliminary attempts to vary the precipitant and protein concentrations did not lead to any immediate improvement. After one month, large (0.15 × 0.2 × 0.3 mm) single crystals appeared in condition No. 25 (0.1 *M* imidazole pH 6.5, 1 *M* sodium acetate). These could easily be reproduced, with the largest crystals formed at a sodium acetate concentration of 1.3 *M* (Fig. 1*c*).

Because all crystals grew in high-salt conditions, a malonate grid screen was also tried. This again lead to small intertwinned crystals similar to those obtained under condition No. 2 of Hampton Research Crystal Screen I.

The crystals grown from condition No. 25 were chosen for X-ray analysis. Initially, data collection at cryogenic temperatures was attempted. Of the different cryoprotectants tried (glycerol, malonate, 2-propanol and PEG 4000), only glycerol did not lead to visual damage to the crystals upon examination under a light microscope. Malonate turned out to be highly destructive even

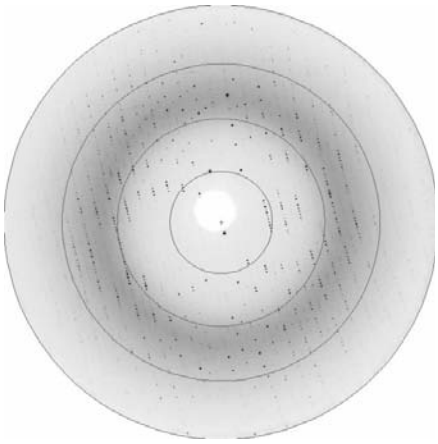

Figure 2
Diffraction pattern of a LlpA crystal of dimensions 0.25 × 0.25 × 0.4 mm mounted in a glass capillary and exposed for 10 s on beamline BW7A of the DESY synchrotron, Hamburg using a 1° rotation. The circles correspond to 7.9, 3.9, 2.6 and 2.0 Å resolution, respectively.

Table 1
Data-collection statistics for LlpA.

Values in parentheses are for the highest resolution shell.

Resolution range (Å)	20.0–2.2 (2.28–2.2)
Total No. reflections collected	210976 (19009)
No. unique reflections	41385 (4097)
Data completeness (%)	99.9 (99.7)
Total φ range covered (°)	130
Mosaicity (°)	0.08
R_{sym} (%)	6.4 (58.2)
$\langle I/\sigma(I)\rangle$	16.2 (3.3)

after 1–2 s despite the fact that the crystals were grown using a salt as precipitant. Even with the 'best' cryoprotectant, glycerol, the diffraction pattern was poor and indicative of very high mosaicity. Attempts to improve the mosaicity by annealing completely destroyed all diffraction. Therefore, a crystal was mounted in a glass capillary and briefly exposed to X-rays at room temperature. This led to a high-quality diffraction pattern extending to 2.0 Å with an apparent mosaicity of 0.1° (Fig. 2). Using short exposure times of 5 s per degree, a full data set was collected. Although some radiation damage did occur, the final data were usable to a resolution of 2.2 Å. From analysis of the systematic absences, the space group was determined to be $P2_12_12$. The refined unit-cell parameters are a = 150.5, b = 154.5, c = 34.2 Å. Full data-collection statistics are given in Table 1. The size of the unit cell suggests either two (V_M = 2.96 Å^3 Da^{-1}) or possibly three (V_M = 1.97 Å^3 Da^{-1}) monomers in the asymmetric unit.

LlpA has been proposed to be related to the mannose-binding lectins from monocot plants, the crystal structures of several of which have been determined (Hester & Wright, 1996; Wood *et al.*, 1999; Sauerborn *et al.*, 1999; Chandra *et al.*, 1999). The percentage sequence identity is low (*e.g.* 22% identity with *Allium sativum* agglutinin; see Fig. 2 in Parret *et al.*, 2003) and structure determination by molecular replacement is unlikely. Therefore, we intend to screen for suitable heavy-atom derivatives and solve the structure by MIR or to produce an SeMet-labelled variant.

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References

Barre, A., Van Damme, E. J. M., Peumans, W. J. & Rougé, P. (1997). *Plant Mol. Biol.* **33**, 691–698.
Bompard-Gilles, C., Rousseau, P., Rougé, P. & Payan, F. (1996). *Structure*, **4**, 1441–1452.
Chandra, N. R., Ramachandraiah, G., Bachhawat, K., Dam, T. K., Surolia, A. & Vijayan, M. (1999). *J. Mol. Biol.* **285**, 1157–1168.
Chen, J., Stevenson, D. M. & Weimer, P. (2004). *Appl. Environ. Microbiol.* **70**, 3167–3170.
Hamelryck, T. W., Poortmans, F., Goossens, A., Angenon, G., Van Montagu, M., Wyns, L. & Loris, R. (1996). *J. Biol. Chem.* **271**, 32796–32802.
Hechard, Y. & Sahl, H. G. (2002). *Biochimie*, **84**, 545–557.
Hester, G. & Wright, C. S. (1996). *J. Mol. Biol.* **262**, 516–531.
James, R., Kleanthous, C. & Moore, G. R. (1996). *Microbiology*, **142**, 1569–1580.
Kirkup, B. C. & Riley, M. A. (2004). *Nature (London)*, **428**, 412–414.
Lakey, J. H. & Slatin, S. L. (2001). *Curr. Top. Microbiol. Immunol.* **257**, 131–161.
Leonidas, D. D., Elbert, B. L., Zhou, Z., Leffler, H., Ackerman, S. J. & Acharya, K. R. (1995). *Structure*, **3**, 1379–1393.
Loewen, M. C., Gronwald, W., Sönnichsen, F. D., Sykes, B. D. & Davies, P. L. (1998). *Biochemistry*, **37**, 17745–17753.
Lucy, M., Reed, E. & Glick, B. R. (2004). *Antonie van Leeuwenhoek*, **86**, 1–25.
Michel-Briand, Y. & Baysse, C. (2002). *Biochimie*, **84**, 499–510.
Moll, G. N., Konings, W. N. & Driessen, A. J. (1999). *Antonie Van Leeuwenhoek*, **76**, 185–198.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pace, C. N., Vajdos, F., Fee, L. & Grimsley, G. (1995). *Protein Sci.* **4**, 2411–2423.
Parret, A. H. A., Schoofs, G., Proost, P. & De Mot, R. (2003). *J. Bacteriol.* **185**, 897–908.
Riley, M. A. & Wertz, J. (2002). *Annu. Rev. Microbiol.* **56**, 117–137.
Sauerborn, M. K., Wright, L. M., Reynolds, C. D., Grossmann, J. G. & Rizkallah, P. J. (1999). *J. Mol. Biol.* **290**, 185–199.
Tsutsui, S., Tasumi, S., Suetake, H. & Suzuki, Y. (2003). *J. Biol. Chem.* **278**, 20882–20889.
Van Damme, E. J. M., Peumans, W. J., Barre, A. & Rougé, P. (1998). *Crit. Rev. Plant Sci.* **17**, 575–692.
Wood, S. D., Wright, L. M., Reynolds, C. D., Rizkallah, P. J., Allen, A. K., Peumans, W. J. & Van Damme, E. J. (1999). *Acta Cryst.* D**55**, 1264–1272.