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Article *in* Acta Crystallographica Section F Structural Biology and Crystallization Communications · December 2014

DOI: 10.1107/S2053230X14025278 · Source: PubMed

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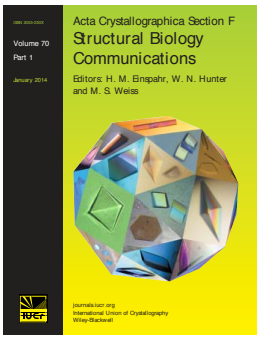
Acta Cryst. (2014). F70, 1697–1700



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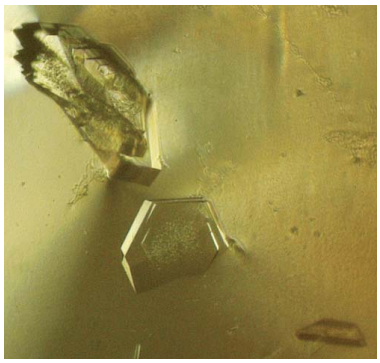
Received 22 September 2014
Accepted 18 November 2014



Melioidosis is an infectious disease caused by the pathogenic bacterium Burkholderia pseudomallei. Whole-genome sequencing revealed that the B. pseudomallei genome includes 5855 coding DNA sequences (CDSs), of which ~25% encode hypothetical proteins. A pathogen-associated hypothetical protein, BPSL1038, was overexpressed in Escherichia coli, purified and crystallized using vapour-diffusion methods. A BPSL1038 protein crystal that grew using sodium formate as precipitant diffracted to 1.55 Å resolution. It belonged to space group $C222_1$, with unit-cell parameters a = 85.36, b = 115.63, c = 46.73 Å. The calculated Matthews coefficient (V_M) suggests that there are two molecules per asymmetric unit, with a solvent content of 48.8%.

1. Introduction

Burkholderia pseudomallei is a pathogenic Gram-negative bacterium that causes melioidosis, also known as 'Whitmore's disease', a condition with high fatality rates in humans and animals. Melioidosis is endemic in Southeast Asia (Laos, Malaysia, Myanmar and Thailand) and Northern Australia (Cheng & Currie, 2005; James et al., 2013). The detailed molecular mechanism for the pathogenicity of this disease is still largely unknown, although several putative virulence factors associated with quorum-sensing systems and type III and type VI secretion systems, as well as bacterial toxins, have been hypothesized to be involved (Gamage et al., 2011; Stevens et al., 2002; Burtnick et al., 2011; Cruz-Migoni et al., 2011). In 2002, B. pseudomallei was classified as a category B bioterrorism agent by the Centers for Disease Control and Prevention (CDC), USA (Rotz et al., 2002). B. pseudomallei is highly resistant to clinically used antibiotics such as ceftazidime, clavulanic acid, trimethoprim/sulfamethoxazole and doxycycline (Schweizer, 2012). Moreover, there is no effective vaccine against this pathogen currently available for humans.

The genome of B. pseudomallei strain K96243 consists of two circular replicons of 4.07 and 3.17 Mb that include 5855 coding DNA sequences (CDSs), with approximately 25% of the CDSs still considered to be hypothetical genes (Holden et al., 2004; Winsor et al., 2008). To further understand the biology of B. pseudomallei through its encoded hypothetical proteins, we report here the crystallization and preliminary X-ray analysis of the conserved hypothetical protein BPSL1038. The BPSL1038 protein (NCBI GeneID 3093799, UniProt Q63W52) consists of 88 amino-acid residues and was predicted to contain a Bin/Amphiphysin/Rvs (BAR) domain (Marchler-Bauer et al., 2013). Members of the BAR domain protein superfamily are known to play an important role in regulating dynamin-mediated membrane fission (Daumke et al., 2014) and have recently been shown to be able to induce membrane curvature of organelles in cells (Suarez et al., 2014). BPSL1038 has been classified as a pathogen-associated protein (Winsor et al., 2008). Homologues have also been found in the B. pseudomallei strains BPC006 and MSHR305 isolated from melioidosis patients (Fang et al., 2012; Stone et al., 2013).

The BPSL1038 protein was overexpressed using a pET-based Escherichia coli expression system, purified using Ni^{2+}-affinity and size-exclusion chromatography (SEC) and crystallized using vapour-diffusion methods. The protein crystal diffracted to 1.55 Å resolution

Table 1
Macromolecule-production information.

Source organism	B. pseudomallei
DNA source	Chemically synthesized (GenScript, USA)
Cloning vector	pUC57
Expression vector	pET-28b
Expression host	E. coli strain BL21-Rosetta gami (DE3)
Complete amino-acid sequence of the construct produced†	MGSSHHHHHHSSGLVPRGSHMAGNLVIVCRDQDADAFDQLMQ–EYGSFQTRLSSTAWYLNMNIVPETLQEDILERVGKYTTLY–IFEATSVTYNTIDSNAAETLSTLFGE

† The His-tag fusion sequence at the N-terminus of recombinant BPSL1038 is underlined.

and belonged to space group $C222_1$. Work is in progress on the preparation of selenomethionine-derivatized protein crystals in order to obtain experimental phases for structure determination.

2. Materials and methods

2.1. Macromolecule production

The bpsl1038 gene of B. pseudomallei strain K96243 was synthesized and cloned into the pUC57 vector (GenScript, USA) between the NdeI and HindIII sites within the vector multiple cloning site (MCS). The bpsl1038 gene was subsequently subcloned into the pET-28b vector using these restriction enzymes to produce a construct that contains a 6×His tag at the N-terminus (Table 1). The pET-28b-BPSL1038 construct was transformed into E. coli strain BL21-Rosetta gami (DE3). A single colony of the transformant was inoculated into Luria–Bertani (LB) medium containing 50 mg ml^{-1} kanamycin. The culture was grown at 310 K in an incubator shaker at 200 rev min^{-1} to an OD_{600} of 0.5–0.8 prior to induction with isopropyl β-d-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The culture was allowed to grow at 310 K for an additional 3–4 h before the cells were harvested.

The bacterial pellet was resuspended in lysis solution (10 ml per gram of cell paste) consisting of 25 mM Tris–HCl pH 7.5, 100 mM sodium chloride, 20 mM imidazole, 20 mM β-mercaptoethanol. The cells were disrupted using a sonicator (Qsonica; 20 cycles of 35% amplitude for 15 s each). Soluble protein was separated from cell debris by centrifugation at 17 968g for 30 min. The supernatant was filtered through a 0.2 mm PVDF membrane filter and then applied onto an Ni^{2+}–NTA-coupled HisTrap HP 5 ml column (GE Healthcare, USA). The binding buffer used was the same as the lysis solution. The BPSL1038 protein was eluted using a linear gradient to 25 mM Tris–HCl pH 7.5, 100 mM sodium chloride, 500 mM imidazole, 20 mM β-mercaptoethanol. The protein eluted at an imidazole concentration of ~110 mM. Fractions containing BPSL1038 protein were pooled and concentrated using a Vivaspin concentrator fitted with a 3 kDa molecular-weight cutoff filter (Sartorius, Germany). The concentrated BPSL1038 protein was subjected to size-exclusion chromatography using a HiLoad 16/600 Superdex 75 pg column (GE Healthcare, USA) pre-equilibrated in 25 mM Tris–HCl pH 7.5, 100 mM sodium chloride, 20 mM β-mercaptoethanol. SDS–PAGE was used to verify that the BPSL1038 protein had been purified to homogeneity. The protein was stained with Coomassie Brilliant Blue R-250 staining solution. Protein concentration was assessed by means of the Bradford assay (Bio-Rad, USA).

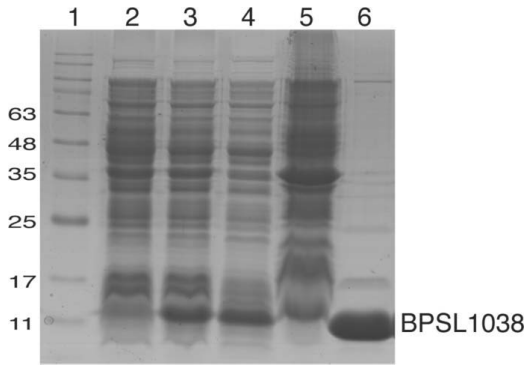


Figure 1
SDS–PAGE (12%) analysis of overexpressed and Ni^{2+}–NTA affinity-purified BPSL1038. Lane 1, BlueRAY prestained protein ladder (GeneDirex, Taiwan; labelled in kDa); lane 2, total protein of crude extract without IPTG induction (negative control); lane 3, total protein of crude extract after IPTG induction; lane 4, supernatant of crude extract after IPTG induction; lane 5, pellet of crude extract after IPTG induction; lane 6, eluate from Ni^{2+}–NTA affinity-column purification.

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion for initial crystal screening and hanging-drop vapour diffusion for crystal optimization
Plate type	96-well MRC plates for initial crystal screening and 24-well plates for crystal optimization
Temperature (K)	293
Protein concentration (mg ml^{-1})	10
Buffer composition of protein solution	25 mM Tris–HCl pH 7.5, 100 mM sodium chloride, 20 mM β-mercaptoethanol
Composition of reservoir solution	0.1 M sodium acetate pH 4.6, 2 M sodium formate
Volume and ratio of drop	1.0 µl for initial crystal screening and 2.0 µl for optimization; 1:1 protein:reservoir solution
Volume of reservoir	80 µl for initial crystal screening and 1.0 ml for optimization

2.2. Crystallization

The size-exclusion chromatography-purified BPSL1038 protein was concentrated to 10 mg ml^{-1} using a Vivaspin concentrator fitted with a 3 kDa molecular-weight cutoff filter (Sartorius, Germany). Crystallization screening was initiated using the sitting-drop vapour-diffusion method in 96-well MRC crystallization plates (Molecular Dimensions) with the Crystal Screen and Crystal Screen 2 kits (Hampton Research). The drops, which consisted of 0.5 µl BPSL1038 protein (10 mg ml^{-1}) and 0.5 µl reservoir solution, were equilibrated against 80 µl reservoir solution at 293 K (Table 2). Crystals were observed using reservoir solution consisting of 0.1 M sodium acetate pH 4.6, 2 M sodium formate. The condition was further optimized using the hanging-drop vapour-diffusion method in 24-well trays with drops made up of equal volumes of protein solution and precipitant solution (1:1 µl) and equilibrated against 1 ml reservoir solution at 293 K. Crystals grew to dimensions of ~30 × 100 × 200 µm after 3–4 d incubation.

2.3. Data collection and processing

The BPSL1038 crystals were flash-cooled with liquid nitrogen after soaking in cryoprotectant solution consisting of 25 mM Tris–HCl pH 7.5, 100 mM sodium chloride, 20 mM imidazole, 20 mM β-mercaptoethanol, 0.1 M sodium acetate pH 4.6, 2.2 M sodium formate, 20% glycerol for approximately 5 min at room temperature. X-ray diffraction data were collected under a nitrogen-gas stream at 100 K using a PILATUS detector on beamline I02 at Diamond Light Source, Didcot, England at a wavelength of 0.9795 Å. A total of 960 images were collected with an oscillation of 0.25° per image (Table 3). The data were processed and indexed using iMosflm (v.7.1.1; Battye

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Station I02, Diamond Light Source
Wavelength (Å)	0.9795
Temperature (K)	100
Detector	PILATUS
Crystal-to-detector distance (mm)	308.83
Rotation range per image (□)	0.25
Total rotation range (□)	240
Exposure time per image (s)	0.25
Space group	$C222_1$
a, b, c (Å)	85.36, 115.63, 46.73
□, □, □ (□)	90, 90, 90
Mosaicity (□)	0.3
Resolution range (Å)	28.08–1.55 (1.58–1.55)
Total No. of reflections	258112 (7866)
No. of unique reflections	33704 (1480)
Completeness (%)	99.1 (89.8)
Multiplicity	7.7 (5.3)
h□/□(□)i	12.3 (2.9)
$R_{r.i.m.}$	0.090 (0.616)
Overall B factor from Wilson plot (Å^2)	15.7

et al., 2011) and were scaled and merged with AIMLESS (Evans & Murshudov, 2013).

3. Results and discussion

Recombinant BPSL1038 protein with a 6□His tag at the N-terminus was overexpressed and purified to homogeneity. The protein appeared as a single band corresponding to a molecular weight of □12 kDa on an SDS–PAGE gel (Fig. 1), which is in good agreement with its calculated molecular weight using ProtParam (Gasteiger et al., 2005). The results of size-exclusion chromatography (SEC) showed that the BPSL1038 protein may exist as dimers or trimers in solution, with the molecular weight falling between those of myoglobin (17 kDa) and ovalbumin (44 kDa) (Supplementary Fig. S1[1]). Nonetheless, other oligomeric forms of BPSL1038 protein cannot be excluded since the shape of the protein may also influence the SEC results. Secondary-structure prediction suggests that BPSL1038 is likely to be a member of the □+□ class of proteins, with four □-helices and four □-strands (Supplementary Fig. S2).

The purified recombinant BPSL1038 was concentrated to □10 mg ml□1 prior to crystallization. Initial crystallization hits were identified in a crystallization screen condition consisting of 2.0 M sodium formate with 0.1 M sodium acetate buffer pH 4.6. Larger crystals (□30 □ 100 □ 200 mm) were grown using sodium formate in the range 1.8–2.0 M by the hanging-drop method in 24-well plates. BPSL1038 crystals tend to grow in the form of plate clusters (Fig. 2). Nevertheless, it was possible to obtain single-crystal plates useful for data collection by breaking the clusters using acupuncture needles.

One BPSL1038 protein crystal diffracted to □1.55 Å resolution at Diamond Light Source (Fig. 3). The data were processed and indexed using iMosflm (v.7.1.1) and scaled and merged with AIMLESS (Evans & Murshudov, 2013). A native data set with 99.1% completeness was collected. The POINTLESS (Evans, 2006) indexing suggested that the crystal belonged to space group $C222_1$, with unit-cell parameters a = 85.36, b = 115.63, c = 46.73 Å, □ = □ = □ = 90□. Calculation of possible Matthews coefficients (V_M) gave a likely value of 2.40 Å^3 Da□1, suggesting that the crystal contains 48.8% solvent with two molecules in the asymmetric unit. The crystallographic parameters and data-collection statistics are shown in Table 3. The calculated V_M value and the retention time obtained from size-exclusion chromatography consistently indicated that BPSL1038 might be present as two monomers in the asymmetric unit and as dimers in aqueous solution, respectively. To find noncrystallographic symmetry (NCS) within the asymmetric unit, MOLREP (Vagin & Teplyakov, 2010) was used to calculate a general self-rotation function (Crowther, 1972) plotted with □ angles of 60, 90, 120 and 180□ using a high-resolution data cutoff at 3 Å (Supplementary Fig. S3). The rotation function only showed significant peaks at □ = 180□ corresponding to the three perpendicular twofold crystallographic axes of the assigned space group $C222_1$ (Supplementary Fig. S3). Furthermore, the absence of a significant off-origin peak in the native Patterson suggests that noncrystallographic twofold axes were not present. Therefore, if BPSL1038 is present as dimers in solution as indicated by SEC, one possibility is that the dimers are built up by the twofold crystallographic axis within the crystal. It is also possible that

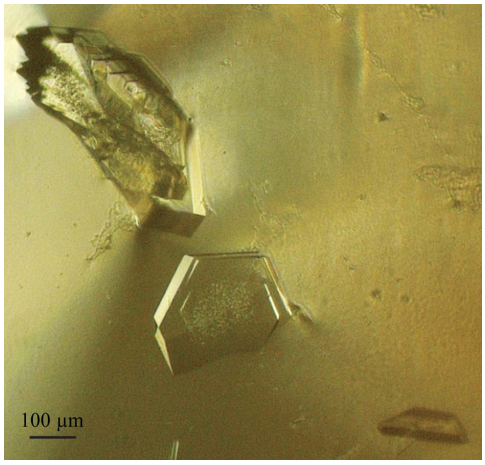


Figure 2
Crystals of BPSL1038 grown by the vapour-diffusion method against a reservoir consisting of 2.0 M sodium formate, 0.1 M sodium acetate buffer pH 4.6.

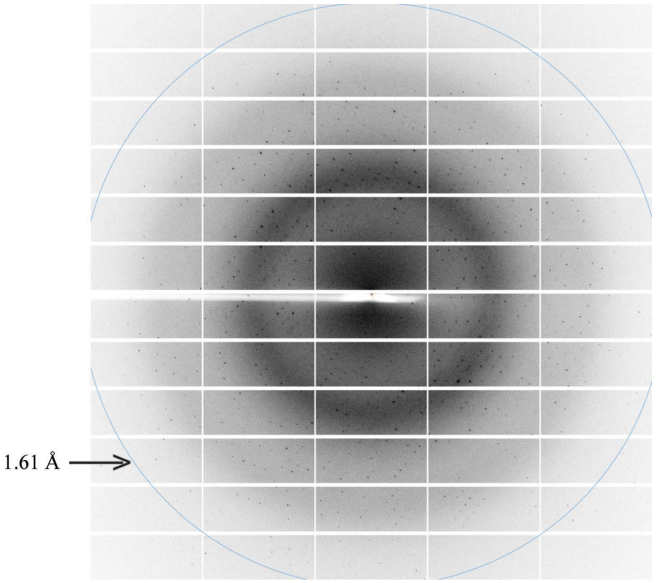


Figure 3
A diffraction image of a 0.25□ oscillation from the data collected for the BPSL1038 crystal. The diffraction extends to a resolution of 1.61 Å, as indicated by the blue line.

[1] Supporting information has been deposited in the IUCr electronic archive (Reference: US5069).

the two monomers in the asymmetric unit constitute dimers with no or imperfect twofold symmetry.

A homology search using BLAST (Altschul et al., 1997) revealed that no homologous structures (>30%) are available for BPSL1038 (data not shown). Therefore, we are currently producing selenomethionine-labelled BPSL1038 protein crystals in an attempt to solve the structure using experimental phases. Such approaches, involving the structure solution of a coding region originally annotated as a hypothetical protein that is uniquely conserved in Burkholderia, previously resulted in the successful functional characterization of the Burkholderia lethal factor 1 (BLF1) toxin (Cruz-Migoni et al., 2011) and the B. pseudomallei virulence factor $PilO2_{Bp}$ (Lassaux et al., 2014). It is hoped that the BPSL1038 structure will shed light on the biological function of this protein and its role in B. pseudomallei pathogenesis.

4. Related literature

The following reference is cited in the Supporting Information for this article: Kelley & Sternberg (2009).

The authors would like to acknowledge Universiti Kebangsaan Malaysia for financial support through the DIP-2012-13 and GGPM-2012-069 grants. This research also supported by the Ministry of Education, Malaysia through ERGS/1/2011/STG/UKM/01/15 grant. We thank Diamond Light Source, Didcot, England for synchrotron beam time and we acknowledge Juan Sanchez-Weatherby and Thomas Sorensen for assistance at station I02 during data collection. We thank Miguel Ortiz Lombardía for reading the manuscript and providing cogent comments.

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