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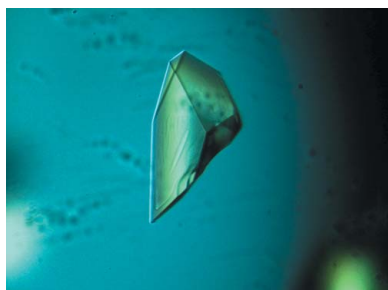
Crystallization and preliminary X-ray diffraction analysis of BipD, a virulence factor from *Burkholderia pseudomallei*

Burkholderia pseudomallei, the causative agent of melioidosis, possesses a protein-secretion apparatus that is similar to those found in *Salmonella* and *Shigella*. A major function of these secretion systems is to secrete virulence-associated proteins into target cells of the host organism. The BipD gene of *B. pseudomallei* encodes a secreted virulence factor that is similar in sequence and most likely functionally analogous to IpaD from *Shigella* and SipD from *Salmonella*. Thus, the BipD protein is likely to be a component of a type III protein-secretion system (TTSS) in *B. pseudomallei*. Proteins in the same class as BipD, such as IpaD and SipD, are thought to act as extracellular chaperones to help the hydrophobic translocator proteins enter the target cell membrane, where they form a pore and might even link the translocon pore with the secretion needle. There is evidence that the translocator proteins also bind an integrin which stimulates actin-mediated insertion of the bacterium into the host-cell membrane. Native BipD has been crystallized in a monoclinic crystal form that diffracts X-rays to 2.5 Å resolution. BipD protein which incorporates selenomethionine (SeMet-BipD) has also been expressed and forms crystals which diffract to a higher resolution of 2.1 Å.

1. Introduction

Burkholderia pseudomallei and *B. mallei* are intracellular bacteria that cause serious invasive infections in animals and are emerging as major causes of infectious disease in Southeast Asia (Dance, 2002). These bacteria cause the disease melioidosis, which is endemic to tropical and subtropical regions, particularly Southeast Asia and Northern Australia (Gan, 2005). Most commonly, this disease manifests itself clinically as abscesses, pneumonia and, at worst, as a fatal septicaemia, but has a complex spectrum of clinical manifestations, making it difficult to diagnose and treat effectively (Aldhous, 2005). *B. pseudomallei* is known to infect almost all types of cell and it appears able to evade and perhaps actively interfere with the host's immune system (Gan, 2005). Awareness of *B. pseudomallei* has increased in recent years since being identified and labelled as a potential biological terrorist weapon (Aldhous, 2005). This attention has now resulted in the completion of the genome sequence for *B. pseudomallei* (Holden *et al.*, 2004). Unlike most bacteria, the genome comprises two chromosomes of 4.07 and 3.17 Mbp, showing significant functional partitioning of genes between them. The large chromosome encodes many of the core functions associated with central metabolism and cell growth, whereas the small chromosome carries more accessory functions associated with adaptation and survival in different environments. At 7.24 Mbp, the *B. pseudomallei* genome is large in comparison with the typical prokaryotic genome, perhaps accounting for the bacterium's versatility and adaptability (Holden *et al.*, 2004).

Both *B. pseudomallei* and *B. mallei* possess a protein-secretion apparatus that is highly similar to those found in *Salmonella* and *Shigella* (Stevens *et al.*, 2002). A major function of these secretion systems is to secrete virulence-associated proteins into target cells of the host organism. Recently, it has been reported that disruption of the *bipD* gene, which is within a putative secretion-system locus, reduces the ability of *B. pseudomallei* to invade eukaryotic cells and reduces virulence in mice (Stevens *et al.*, 2002, 2004). The findings of



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this work indicate that the protein encoded by the BipD gene is an important secreted virulence factor of *B. pseudomallei*.

BipD is similar in sequence and most likely functionally analogous to IpaD from *Shigella* (26% identity) and SipD from *Salmonella* (27% identity). Thus, by analogy with these better studied organisms, the BipD protein is likely to be a component of a type III protein-secretion system (TTSS) in *B. pseudomallei*. TTSSs are large assemblies of proteins that span the inner bacterial membrane, the periplasmic space, the peptidoglycan layer, the outer bacterial membrane, the extracellular space and the target-cell membrane (Yip & Strynadka, 2006). The function of bacterial protein-secretion systems is to transport 'effector' and other proteins across the bacterial inner membrane and the outer envelope (Mecas & Strauss, 1996) in an ATP-dependent manner. There are five systems (types I–V) which operate in different ways and vary in complexity. Some involve a hollow tube or needle (the injectisome) through which the secreted proteins travel (Mota *et al.*, 2005). The TTSS injectisome varies between 45 and 80 nm in length depending on the bacterial species, is made by the polymerization of a major subunit and has a hollow interior of approximately 25 Å in diameter. The ring-like assembly that spans the membrane of the host cell is referred to as the translocon. This is formed by the initial secretion of a small number of proteins into the extracellular environment as a result of contact between the bacterium and the target cell (Pettersson *et al.*, 1996). The translocator proteins act to transport bacterial proteins across the plasma membrane into the host cell, which are then able to subvert or inhibit the cell's processes for the benefit of the bacterium. It appears that the translocator proteins form a pore in the lipid membrane of the target cell through which the effector proteins are able to pass (Blocker *et al.*, 2000).

It has been found that *B. pseudomallei* contains at least three loci encoding putative type III protein-secretion systems (Rainbow *et al.*, 2002), one of which shares homology with the TTSS of *Salmonella typhimurium* (Attree & Attree, 2001) and *Shigella flexneri* (Stevens *et al.*, 2002) and has been designated BSA (short for *Burkholderia* secretion apparatus; Hueck, 1998). The BSA effector proteins are termed Bop proteins and the translocators are termed Bip proteins, short for *Burkholderia* invasion proteins. In *Salmonella*, SipB, SipC and SipD have been shown to be required for the injection of effector proteins and the invasion of epithelial cells *in vitro* (Kaniga *et al.*, 1995) and likewise for IpaB, IpaC and IpaD from *Shigella* (Ménard *et al.*, 1994). Hence, it is thought that the *B. pseudomallei* homologues BipB, BipC and BipD perform a similar function.

Serum from melioidosis victims contains antibodies to BipD and the other putative translocator proteins. It has been shown that *B. pseudomallei* bacteria with mutated BipD show reduced virulence but are still able to cause fatal melioidosis (Stevens *et al.*, 2004). The BipD mutant exhibited impaired invasion of HeLa cells and reduced intracellular survival in murine macrophage-like cells and a marked reduction in actin-tail formation (Stevens *et al.*, 2002, 2004). Hence, there is evidence that BipD is involved in the actin polymerization that facilitates the escape of *B. pseudomallei* from endocytic vesicles during the initial infection and the subsequent escape of progeny bacteria into surrounding host cells. Observations made during these experiments support the idea that a functional TTSS is required for full virulence of *B. pseudomallei*. It has been suggested that proteins in the same class as BipD (*i.e.* IpaD and SipD) act as extracellular chaperones to help the hydrophobic translocators (equivalent to BipB and BipC) enter the target-cell membrane and might even link the translocon pore with the secretion needle (Mecas & Strauss, 1996). Since IpaD, as a complex with the hydrophobic translocators IpaB and IpaC, can bind the $\alpha_5\beta_1$ integrin, stimulating the phos-

phorylation of a focal adhesion kinase (Watarai *et al.*, 1996), it is possible that BipD acts in a similar way to aid actin-mediated insertion of the bacterium into the host-cell membrane.

The BipD protein from *B. pseudomallei* consists of 310 amino acids and has a molecular weight of 33 kDa. We have crystallized BipD in a monoclinic crystal form that diffracts X-rays to 2.5 Å resolution. We have also expressed BipD protein which incorporates selenomethionine (SeMet-BipD) for MAD data collection. The latter protein gave crystals of the same form which diffracted to an improved resolution of 2.1 Å.

2. Overexpression, purification and crystallization of native and selenomethionyl-BipD

We have generated a pGEX-4T (Pharmacia) plasmid construct encoding a fusion of BipD with glutathione-S-transferase (GST) to facilitate expression in *Escherichia coli* and purification by use of a GSTrap affinity column (GE Healthcare). Crystals of native BipD were obtained using the hanging-drop method with 5 µl of protein being mixed with 5 µl of each well solution on siliconized glass cover slips. The optimum well solution for crystallization consisted of 25% PEG 4000, 5 mM nickel chloride, 0.1 M glycine, 0.1 M sodium cacodylate pH 6.5. Crystals appeared after approximately nine weeks at either 277 or 295 K and were cryoprotected with 30% glycerol and mounted in mohair loops for flash-freezing in liquid ethane and storage under liquid nitrogen.

Initial attempts to express selenomethionyl-BipD (SeMet-BipD) involved growth of the cells on the 21 scale to mid-log phase in LB medium (including 50 µg ml⁻¹ ampicillin) followed by centrifugation to pellet them. The cells were resuspended in M9 minimal medium (containing ampicillin at the same concentration and 0.4% glucose) and grown for another 45 min to use up remaining amino acids. The cultures were then inoculated with the vitamins riboflavin, niacinamide, pyridoxine and thiamine each at final concentrations of 1 mg l⁻¹ and a mixture of all 20 amino acids each at final concentrations of 40 mg l⁻¹ except for methionine, which was replaced by selenomethionine. The cells were then shaken at 310 K for another 20 min prior to overnight induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). This procedure has the advantage of avoiding the use of a methionine-auxotrophic strain of *E. coli*. However, whilst with trial cultures SDS-PAGE had shown all the native protein expressed by the construct to be soluble, we found that virtually all of the SeMet-BipD was insoluble.

At this point, we used small trial cultures to experiment with a protocol to avoid the formation of inclusion bodies. This involved giving the cells a 25 min heat shock at 315 K followed by cooling on ice for 5 min and induction with a low concentration of IPTG (0.3 mM). To adapt this method for expression of SeMet protein, we followed the protocol for selenomethionine incorporation up to the point where the cells would have otherwise been induced and instead they were subjected to the above heat shock, cooling and induction. The cells were then grown at a low temperature (289 K) for 2 d and, as a precaution against oxidation of the SeMet-BipD, dithiothreitol (DTT) was included in the buffer used for sonication. SDS-PAGE gels showed that most of the protein was present in the soluble fraction, thus confirming the success of the method. The SeMet-protein was then expressed using 2 × 1 l cultures for purification of larger quantities for crystallization trials.

A crude initial purification of the SeMet-BipD by fractional ammonium sulfate precipitation was undertaken in which the ammonium sulfate concentration was raised to 30% saturation to

precipitate impurities and then raised to 60% to precipitate the fusion protein. The resuspended pellet was then dialysed against 50 mM Tris buffer pH 8.0 (1 mM DTT) prior to loading onto a GStrap FF (GE Healthcare) 5 ml affinity column *via* an AKTA prime low-pressure system. The column was washed with phosphate-buffered saline (PBS) pH 8.0 and 1 mM DTT to remove impurities and the SeMet-BipD was cleaved from its fusion partner (GST) by addition of 1.6 mg of thrombin to the column and incubation for 20 h. The column was then washed with PBS buffer to remove the SeMet-BipD and was regenerated by washing with reduced glutathione to remove the GST-fusion partner. The thrombin was removed from the SeMet-BipD by passage through a 1 ml benzamidine column (GE Healthcare). At all stages, the purity of the protein and success of the cleavage reaction was confirmed by SDS-PAGE. The final yield of SeMet protein was approximately 30 mg per litre of culture.

For crystallization experiments, SeMet-BipD was concentrated to 6 mg ml⁻¹ using a Vivaspin ultrafiltration spin column. Screening conditions were based on those used previously to obtain crystals of

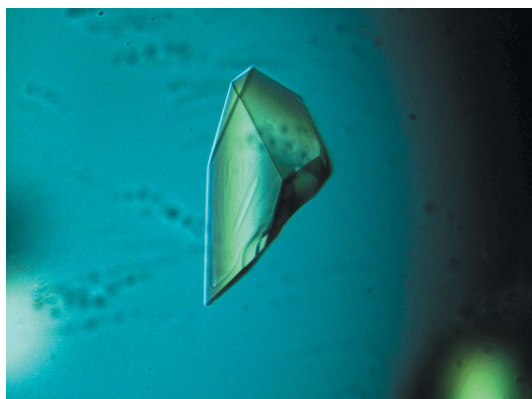


Figure 1
A crystal of selenomethionyl-BipD which diffracted to around 2.1 Å resolution. The crystal shown here is approximately 1.0 mm in its largest dimension.

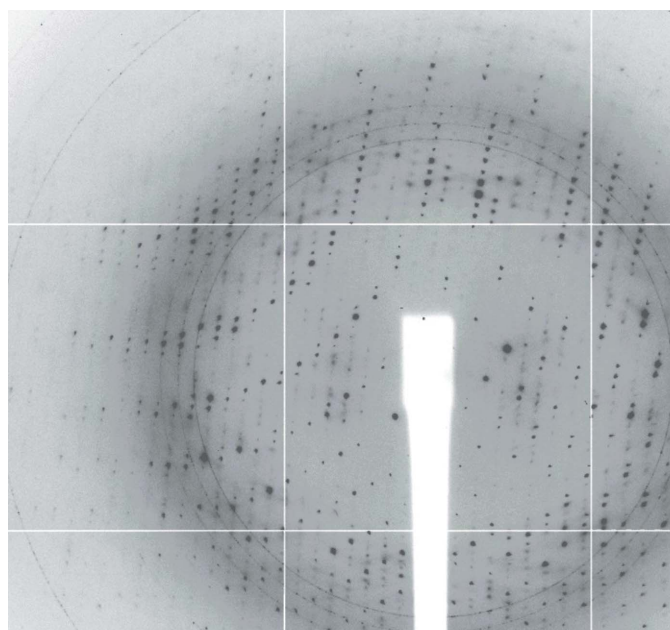


Figure 2
Part of a diffraction image obtained at ESRF (Grenoble) from a crystal of selenomethionyl-BipD showing diffraction spots to 2.1 Å resolution.

Table 1

Data-collection and processing statistics for native and selenomethionyl-BipD.

Values in parentheses are for the outer resolution shell.

Data set	Native	SeMet-BipD
Beamline	ID14-2 (ESRF)	ID23-1 (ESRF)
Wavelength (Å)	0.933	0.979
Space group	$P2_1$	$P2_1$
Unit-cell parameters		
a (Å)	53.8	53.5
b (Å)	56.4	56.2
c (Å)	85.0	84.2
β (°)	95.7	94.5
Resolution (Å)	36.3–2.5 (2.6–2.5)	38.7–2.1 (2.2–2.1)
R_{merge} (%)	5.6 (30.0)	8.6 (41.1)
Completeness (%)	99.0 (98.8)	97.5 (93.4)
Average $I/\sigma(I)$	18.5 (4.0)	10.4 (2.8)
Multiplicity	3.7 (3.8)	3.7 (3.5)

the native enzyme and were set up by the hanging-drop method. Within 2–3 weeks promising crystals of SeMet-BipD were obtained and following optimization of the crystallization conditions, significantly improved crystals were obtained under the following conditions: SeMet-BipD concentration 4 mg ml⁻¹, 20–25% PEG 4000, 5 mM nickel chloride, 0.1 M glycine, 0.1 M sodium cacodylate pH 5.0–5.5. Crystals of up to 1 mm in length grew within a period of four weeks (Fig. 1) and were flash-frozen as for the native enzyme.

3. X-ray diffraction analysis of BipD

The native and selenomethionyl-BipD crystals were taken to the European Synchrotron Radiation Facility (ESRF, Grenoble). A native crystal was found to diffract to a resolution of around 2.5 Å and larger SeMet-BipD crystals diffracted to a resolution of 2.1 Å (Fig. 2). Processing of the data sets using *MOSFLM* (Leslie, 2006), *SCALA* (Evans, 2006) and other programs from the *CCP4* program suite (Collaborative Computational Project, Number 4, 1994) indicated that the crystals belonged to the monoclinic space group $P2_1$. The selenomethionyl-BipD crystal had unit-cell parameters $a = 53.5$, $b = 56.2$, $c = 84.2$ Å, $\beta = 94.5^\circ$ and the lower resolution native data set yielded very similar values (Table 1). The processed selenomethionyl-BipD data set extends to 2.1 Å resolution with an overall R_{merge} of 8.6%, a completeness of 97.5% and a multiplicity of 3.7. The native data set to 2.5 Å resolution had an R_{merge} of 5.6%, a completeness of 99.0% and a multiplicity of 3.7. Use of the *MC* program (Collaborative Computational Project, Number 4, 1994) suggests that the crystals are likely to have either one BipD monomer (of 33 kDa) per asymmetric unit, corresponding to a solvent content of 68%, or two monomers per asymmetric unit, with a solvent content of 35%. Analysis of the structure is currently in progress.

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