A novel FK-506-binding-like protein that lacks peptidyl-prolyl isomerase activity is involved in intracellular infection and \textit{in vivo} virulence of \textit{Burkholderia pseudomallei}

Isobel H. Norville, 1 Katrin Breitbach, 2 Kristin Eske-Pogodda, 2 Nicholas J. Harmer, 3 Mitali Sarkar-Tyson, 1 Richard W. Titball 3 and Ivo Steinmetz 2

1 Defence Science and Technology Laboratory, Porton Down, Salisbury SP4 0JQ, UK
2 Friedrich Loeffler Institute of Medical Microbiology, Ernst-Moritz-Arndt University Greifswald, Greifswald, Germany
3 School of Biosciences, University of Exeter, Exeter EX4 4QJ, UK

\textit{Burkholderia pseudomallei} is a facultative intracellular bacterial pathogen causing melioidosis, an often fatal infectious disease that is endemic in several tropical and subtropical areas around the world. We previously described a Ptk2 cell-based plaque assay screening system of \textit{B. pseudomallei} transposon mutants that led to the identification of several novel virulence determinants. Using this approach we identified a mutant with reduced plaque formation in which the \textit{BPSL0918} gene was disrupted. \textit{BPSL0918} encodes a putative FK-506-binding protein (FKBP) representing a family of proteins that typically possess peptidyl-prolyl isomerase (PPIase) activity. A \textit{B. pseudomallei ΔBPSL0918} mutant showed a severely impaired ability to resist intracellular killing and to replicate within primary macrophages. Complementation of the mutant fully restored its ability to grow intracellularly. Moreover, \textit{B. pseudomallei ΔBPSL0918} was significantly attenuated in a murine model of infection. Structural modelling confirmed a modified FKBP fold of the \textit{BPSL0918}-encoded protein but unlike virulence-associated FKBP from other pathogenic bacteria, recombinant \textit{BPSL0918} protein did not possess PPIase activity \textit{in vitro}. In accordance with this observation \textit{BPSL0918} exhibits several mutations in residues that have been proposed to mediate PPIase activity in other FKBP. To our knowledge this \textit{B. pseudomallei} FKBP represents the first example of this protein family which lacks PPIase activity but is important in intracellular infection of a bacterial pathogen.

\textbf{INTRODUCTION}

\textit{Burkholderia pseudomallei} is the causative agent of melioidosis and is endemic in tropical and subtropical regions such as Northern Australia and South East Asia, where it can be isolated from soil and water (Inglis & Sagripanti, 2006). Bacteria enter the body primarily by inoculation through skin lesions and occasionally by inhalation (Currie & Jacups, 2003). Clinical presentation of the disease in humans varies from acute septicemia to chronic localized infection. The infection can become latent, and reactivation has been recorded 62 years after primary exposure (Ngauy \textit{et al.}, 2005). \textit{B. pseudomallei} is considered to be a biological warfare threat and is listed as a category B agent by the US Centers for Disease Control and Prevention (Rotz \textit{et al.}, 2002). There is no vaccine available for prophylaxis and despite current treatment using an antibiotic regimen, mortality is reported as 50\% (White, 2003).

FK-506-binding proteins (FKBPs) are present in both prokaryotes and eukaryotes and are known to be involved in various cellular functions such as protein folding, protein chaperoning and cell division (Fischer & Aumu¨ller, 2003). FKBPs typically possess peptidyl-prolyl isomerase (PPIase) activity, which is thought to have evolved to catalyse the slow, rate-limiting \textit{cis-trans} isomerization of Xaa-Pro peptide bonds during protein folding (Brandts \textit{et al.}, 1975). PPIase activity was first observed in human FKBP12 protein, and inhibition of PPIase activity occurs upon binding to the macrolide immunosuppressants FK506 or rapamycin (Harding \textit{et al.}, 1989; Schreiber, 1991). Several studies have shown that FKBPs play a role in mediating bacterial
virulence in various intracellular pathogens (Cianciotto et al., 1989; Lundemose et al., 1993; Moro et al. 1995; Horne et al., 1997; Leuzzi et al., 2005). In this context it was shown that a *Legionella pneumophila* strain lacking a 24 kDa FKBP, which exhibited characteristic PPIase activity (Fischer et al., 1992), was reduced in its ability to infect macrophages and was therefore named macrophage infectivity potentiator (Mip) (Cianciotto et al., 1989). The PPIase activity of Mip was also shown to be required for full virulence in a *Legionella* infection model (Wintermeyer et al., 1995; Köhler et al., 2003; Helbig et al., 2003).

The present study reports the identification of a novel FKBP-like protein in *B. pseudomallei* which, in contrast to the previously described virulence-associated FKBP of pathogenic bacteria, lacks characteristic PPIase activity in vitro, but is essential for intracellular replication and in vivo virulence of the pathogen.

**METHODS**

**Bacterial strains, media and reagents.** *B. pseudomallei* E8 is a soil isolate from the area surrounding Ubon Ratchathani, north-east Thailand (Wuthiekanun et al., 1996), and was obtained from N. J. White (Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand). *B. pseudomallei* K96243 is a clinical isolate obtained from S. Songsivilai (Siriraj Hospital, Bangkok, Thailand). *Escherichia coli* BL21(DE3) was selected for further analysis and termed mutant strain (see below) were carried out at the Friedrich Loeffler Institute, Wiga. *B. pseudomallei* was grown on Columbia agar or Lon–Bertani (LB) agar plates, and LB broth or modified Vogel–Bonner broth to be inserted behind base 169 of the selected for further analysis and termed mutant strain (see below) were carried out at the Friedrich Loeffler Institute, Wiga. *B. pseudomallei* was grown on Columbia agar or Lon–Bertani (LB) agar plates, and LB broth or modified Vogel–Bonner broth (Pilatz et al., 2006) was used as liquid medium. When appropriate, antibiotics were added at the following concentrations: 25 μg chloramphenicol ml⁻¹, 25 μg kanamycin ml⁻¹ and 12.5 μg tetracycline ml⁻¹ for *E. coli* SM10(pOT182), 100 μg streptomycin ml⁻¹ and 50 μg tetracycline ml⁻¹ for *B. pseudomallei* TnsOT182 mutants, 100 μg ampicillin ml⁻¹ for *E. coli* DH5α(pTNS3), 50 μg ampicillin ml⁻¹ for *E. coli* BL21(DE3)(pET15b), and 35 μg kanamycin ml⁻¹ for *E. coli* HB101(pRK233). All chemicals were obtained from Sigma-Aldrich unless stated otherwise. All kits were obtained from commercial sources and were used in accordance with the manufacturer’s instructions.

**B. pseudomallei** Tn5-OT182 mutagenesis and plaque assay screening. *B. pseudomallei* E8 was mutagenized with Tn5-OT182 and examined for mutants exhibiting defects in plaque formation in a plaque-assay screen using Plk2 cells as previously described (Pilatz et al., 2006). In one of the mutants that exhibited reduced plaque formation compared with the wild-type strain, the transposon insertion site was identified as described (Pilatz et al., 2006) and found to be inserted behind base 169 of the *BPSL0918* locus. This mutant was selected for further analysis and termed *B. pseudomallei* Δ*BPSL0918*. All experiments with *B. pseudomallei* Δ*BPSL0918* and the complemented mutant strain (see below) were carried out at the Friedrich Loeffler Institute of Medical Microbiology under biosafety level 3 conditions.

**Complementation of B. pseudomallei Δ*BPSL0918**. To complement *B. pseudomallei* Δ*BPSL0918* we used the mini-Tn7 system as described by Choi et al. (2008). The forward primer 0918compF (CCCCGAAGTCGATCAGGATGGTGTT) and reverse primer 0918compR (TCCAGGTTTCTGACGCTTGGTT) (restriction sites underlined) were used to amplify a 791 bp fragment of *B. pseudomallei* E8 genomic DNA containing the *BPSL0918* coding region plus 147 bp upstream of the translational start and 188 bp downstream of the stop codon. Following digestion this fragment was cloned into the EcoRI/HindIII sites of the pUC18T mini-Tn7T-Zeo vector (Choi et al., 2008), transformed into *E. coli* DH5α, and delivered into *B. pseudomallei* Δ*BPSL0918* by four parental matings using the donor strain (*E. coli* DH5α pUC18T mini-Tn7-T-Zeo-BPSL0918) and two *E. coli* helper strains (*E. coli* Hb101[pRK2103] and *E. coli* DH5α[pTNS3]) as described by Choi et al. (2008). In brief, bacteria were cultured with the respective appropriate antibiotics overnight in LB medium. One hundred microlitres each of the cultures was mixed with 600 μl 10 mM MgSO₄ and centrifuged at 7000 g for 2 min. Supernatant was removed and cells were washed in 1 ml 10 mM MgSO₄ and finally resuspended in 30 μl 10 mM MgSO₄. Cells were added on a pre-warmed 0.45 μm filter on LB agar containing 4 % (v/v) glycerol and incubated for 8 h at 37 °C. Cells were then harvested, resuspended in 2 ml PBS and spread onto LB agar containing 4 % (v/v) glycerol, 2 mg zeocin ml⁻¹ and 15 μg polymyxin B ml⁻¹ for selection of complemented mutants. Successful insertion of *BPSL0918* in the recipient strain *B. pseudomallei* Δ*BPSL0918* was verified by PCR.

**In vitro growth and expression analysis of BPSL0918**. Bacterial colonies were harvested from Colombia blood agar plates and precultured in 15 ml LB or Vogel–Bonner medium for 16 h at 37 °C in a shaking incubator. Cultures were then transferred in 100 ml of the respective medium and adjusted to an OD₅₆₀ of 0.01. For growth curve analysis, OD₅₆₀ was measured at the indicated time points. For BPSL0918 expression analysis, bacteria were collected by centrifugation when the OD₅₆₀ reached 0.3–0.7. Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. Reverse transcription was performed using the Stratascript cDNA Synthesis kit (Stratagene) and samples were subjected to PCR by standard molecular biology techniques.

**Generation and infection of bone-marrow-derived macrophages.** Murine bone-marrow-derived macrophages (BMMs) were generated under serum-free conditions from BALB/c mice as previously described (Eske et al., 2009). Briefly, tibias and femurs were aseptically removed, and bone marrow cells were flushed out with sterile PBS and centrifuged at 150 g for 10 min. Cells were resuspended in RPMI medium containing 5 % Panexin BMM (PAN Biotech), recombinant murine granulocyte-macrophage colony-stimulating factor (2 ng ml⁻¹; PAN Biotech) and 50 μM mercaptoethanol and cultivated for at least 10 days at 37 °C and 5 % CO₂. Twenty-four hours prior to infection experiments, cells were seeded in 48-well plates (1.5 × 10⁵ cells per well) and infected with either *B. pseudomallei* wild-type strain E8, *B. pseudomallei* Δ*BPSL0918* or the complemented mutant at an m.o.i. of 15:1 for 30 min. Cells were then washed twice with PBS, and medium containing 100 μg kanamycin ml⁻¹ was added to each well. At the indicated time points (time zero was taken as 20 min after incubation in antibiotic-containing medium) the number of intracellular c.f.u. was determined as previously described (Eske et al., 2009).

**Infection of HeLa cells.** HeLa cells were cultivated and infected as previously described (Pilatz et al., 2006), with minor modifications. Cells were seeded in 48-well plates approximately 24 h prior to infection. Bacteria were centrifuged onto cells at 300 g for 10 min and incubated for 30 min at 37 °C. Cells were then washed with PBS and cell culture medium containing 250 μg kanamycin ml⁻¹ was added to each well. At the indicated time points the number of intracellular c.f.u. was determined.

**Murine infection model.** Female 8- to 12-week-old BALB/c mice were obtained from Charles River Wiga. All in vivo studies were approved by the local authority. Animals were maintained under specific pathogen-free conditions and were provided with food and water ad libitum. Bacteria were grown for 18–20 h in LB broth and diluted in PBS to the required concentration. Prior to intranasal application mice were anaesthetized with a mixture of ketamin hydrochloride and xylazine hydrochloride. Thirty microlitres of the...
bacterial suspension was inoculated per animal into both nostrils. The mortality of animals was monitored daily. To confirm that spleens, livers and lungs from infected mice were sterile, the organs were aseptically removed, homogenized in 0.5–1 ml sterile PBS containing 0.5 % Tergitol/1 % BSA, transferred to 5 ml LB broth and incubated for 48 h. One hundred microorganisms of this organ suspension was then plated onto Ashdown agar and incubated for 48 h.

**Construction of BPSL0918 expression plasmid.** The BPSL0918 gene (513 bp) was amplified from *B. pseudomallei* K96243 genomic DNA (100 % identical to BPSL0918 from strain E8) using primers 0918F (CATATGAGCCCTACGCAGCTT) and 0918R (GGATCC-TGACGAAATCCTCCTGGTATG). The PCR product was cloned into the *NdeI/BamHI* sites of the pET15b expression plasmid (Novagen) for expression of recombinant protein with an N-terminal His tag. This construct was confirmed by nucleotide sequencing prior to transformation into *E. coli* BL21(DE3).

**Expression and purification of recombinant BPSL0918.** *E. coli* BL21(DE3) harbouring the BPSL0918 expression construct was grown at 37 °C to an OD600 of 0.4–0.6. Expression was induced by IPTG at a final concentration of 1 mM and the bacteria were subsequently cultured at 20 °C for 20 h. Cells were harvested by centrifugation at 8000 g for 15 min at 4 °C, resuspended in PBS and disrupted by sonication four times for 30 s. Cell debris was pelleted at 8000 g for 30 min and the cell supernatant was loaded onto a nickel affinity chromatography column (GE Healthcare). Purified BPSL0918 protein was eluted in 100 mM imidazole and purity was examined by SDS-PAGE followed by Coomassie brilliant blue staining. Protein concentrations were determined using a biocinchoninic acid assay (Pierce Biotechnology). Imidazole was removed from purified protein by buffer exchange leaving the protein in 10 mM PBS (Dulbecco A) prior to being stored at −80 °C. UV and circular dichroism spectra were determined at room temperature using a Chirascan spectrophotometer (Applied Photophysics) in the regions 400–230 and 260–180 nm. Protein secondary structure content was assessed by using the principal components regression method (Malik, 1997). LC-MS analyses were carried out with a Quattro II tandem quadrupole mass spectrometer by using the principal components regression method (Malik, 1997). LC-Chirascan spectrophotometer (Applied Photophysics) in the regions 400–circular dichroism spectra were determined at room temperature using a Shimadzu 1800 UV/Vis spectrophotometer for 300 s at 5 s intervals. The synthetic substrate succinyl-Ala-Pro-Phe-leucinamide (10 mg ml−1) was added to the cuvette and mixed. The solvent jump was measured at 330 nm in a Shimadzu 1800 UV/Vis spectrophotometer for 300 s at 5 s intervals. Human FKBP12 (Sigma) was used as a positive control.

**Enzyme-coupled PPlase assay.** To determine if BPSL0918 protein had PPlase activity, an enzyme-coupled assay was carried out as described by Fischer et al. (1984). Briefly, 100 nM recombinant protein was incubated for 6 min at 10 °C in 1.2 ml 35 mM HEPES buffer, pH 7.8, with 25 mg chymotrypsin ml−1. The synthetic substrate succinyl-Ala-Xaa-Pro-Phe-p-nitroanilide (Xaa=Pro, Leuor Ala) (10 mg ml−1; Bachem) was added to the cuvette and mixed. Hydrolysis of the substrate was measured at 390 nm in a Shimadzu 1800 UV/Vis spectrophotometer for 300 s at 5 s intervals. Human FKBP12 (Sigma) was used as a positive control.

**Protease-free PPlase assay.** The protease-free assay was performed as described by Janowski et al. (1997). Briefly, 400 nM recombinant protein was incubated for 6 min at 10 °C in 1.5 ml 35 mM HEPES buffer, pH 7.8. The synthetic substrate succinyl-Ala-Phe-Pro-Phe-p-nitroanilide (10 mg ml−1) was dissolved in 0.48 M LiCl/anhydrous trifluoroethanol. This substrate was added to the cuvette and mixed. The solvent jump was measured at 330 nm in a Shimadzu 1800 UV/Vis spectrophotometer for 300 s at 5 s intervals. Human FKBP12 (Sigma) was used as a positive control.

**Modelling of the structure of BPSL0918.** The structure of BPSL0918 was modelled by using MODELLER version 9.7 (Eswar et al., 2006). Four structures (1IX5, 3CGM, 2K07, 1FKB) were selected as templates. A structure-based sequence alignment for these structures was produced by using MAMMOTH-mult (Lupyan et al., 2005) and carefully edited by hand. Structure-based alignment of the sequence of BPSL0918 was performed by using JOY (Mizuguchi et al., 1998) and FUGUE (Shi et al., 2001). Models were prepared for the apo-protein, and for a complex with rapamycin. In each case, 10 models were prepared using the high-quality VTREF optimization and MD/SA optimization options. Models were scored according to MODELLER energy score and Ramachandran plot quality as judged by RAMPAGE (Lovell et al., 2003).

**Imipenem sensitivity assays.** Microtitre broth dilution assay. Bacteria grown on Colombia blood agar were inoculated in PBS, adjusted to a McFarland standard of 0.5 and diluted 1:100 in LB broth; 50 µl of this suspension was added to each well of a 96-well microtitre plate. Fifty microorganisms of an imipenem solution in LB broth was added to obtain final concentrations of 0.375–2 µg ml−1. The plates were incubated at 30 or 37 °C. Inhibition of bacterial growth was considered to have occurred when the wells did not show any turbidity, and minimal inhibition concentration (MIC) was determined 48 h after incubation.

**Results**

**Identification of BPSL0918, a gene important for the intracellular lifestyle of *B. pseudomallei***

A previous screening of *B. pseudomallei* transposon mutants for defects in the intracellular life cycle by using the plaque- assay screening approach led to the identification of a broad range of genes that were associated with virulence in mice (Pilatz et al., 2006). By extending this screening we identified a mutant showing reduced plaque formation that proved to have a defect in the BPSL0918 gene. As *B. pseudomallei* ΔBPSL0918 did not show restricted growth in either LB or in minimal Vogel–Bonner medium compared with the wild- type strain (Fig. 1a, b), we conjectured that its defect in the intracellular life cycle is not due to a growth defect per se. *B. pseudomallei* expressed BPSL0918 during *in vitro* growth in LB as well as in minimal Vogel–Bonner medium, as verified by RT-PCR (Fig. 1c).

**BPSL0918 is important for intracellular replication of *B. pseudomallei***

To further analyse the role of BPSL0918 in *B. pseudomallei* infection, we investigated the invasion and intracellular survival kinetics of *B. pseudomallei* ΔBPSL0918 in phagocytic and non-phagocytic cells. We could not detect a defect of the mutant in its ability to invade macrophages (Fig. 2a) or HeLa cells (Fig. 2b), and formation of actin tails in HeLa cells was also not impaired (data not shown). However, the number of intracellular bacteria of the wild-type strain had increased about 100-fold 24 h after initial infection of macrophages, whereas almost no replication was found in...
B. pseudomallei ΔBPSL0918 (Fig. 2a). Thus, the ability of B. pseudomallei ΔBPSL0918 to replicate intracellularly was significantly impaired compared with the wild-type strain. Similar results were also obtained in non-phagocytic HeLa cells, in that the intracellular replication of B. pseudomallei ΔBPSL0918 was also significantly reduced 24 h after initial infection (Fig. 2b). Complementation of the mutant fully restored the ability of B. pseudomallei ΔBPSL0918 to replicate intracellularly in macrophages (Fig. 2a), suggesting that direct rather than unspecific polar side effects of the BPSL0918 protein are responsible for the observed phenotype. Thus, we suggest that BPSL0918 is an essential component required to replicate intracellularly in phagocytic and non-phagocytic cells.

**BPSL0918 is important for full virulence of B. pseudomallei in mice**

To assess whether BPSL0918 is important for full virulence, we examined B. pseudomallei ΔBPSL0918 in a murine model of melioidosis. BALB/c mice that were challenged by the intranasal route with 30–90 c.f.u. per animal of B. pseudomallei ΔBPSL0918 completely survived the observed period of 4 months. In contrast, mice infected with the wild-type strain all died within 10 days (Fig. 3a; P<0.001). Additionally, in the complemented mutant strain the virulence was at least partially restored, indicating that BPSL0918 is essential for full virulence of B. pseudomallei (Fig. 3a). Tissues from the spleens, livers and lungs of five surviving mice infected with B. pseudomallei ΔBPSL0918 were examined for remaining bacteria and no viable B. pseudomallei could be detected. However, mice that were infected with a higher dose of the mutant (800 c.f.u. intranasally) died within 1 week after challenge (Fig. 3b). Thus, disruption of BPSL0918 led to significant attenuation in mice but did not render B. pseudomallei completely avirulent.

**BPSL0918 encodes an FKBP-like protein in B. pseudomallei**

BPSL0918 encodes a 151 amino acid polypeptide, annotated in the B. pseudomallei K96243 genome as a putative FKBP-like protein.
FKBP involved in B. pseudomallei virulence

FKBP-type peptidyl-prolyl cis–trans isomerase (Holden et al., 2004). A search against the non-redundant NCBI database revealed sequence homology between BPSL0918 and FKBPs from the closely related species Burkholderia mallei (peptidyl-prolyl cis–trans isomerase, FKBP-type, 100% identity) and Burkholderia thailandensis (peptidyl-prolyl cis–trans isomerase, FKBP-type, 97% identity). Other homologues have been found in E. coli (putative FKBP-type peptidyl-prolyl cis–trans isomerase, FKBP-type, 40% identity), Salmonella typhimurium (FKBP-type peptidyl-prolyl cis–trans isomerase, 37% identity), Schizosaccharomyces pombe (FKBP-type peptidyl-prolyl cis–trans isomerase Fkh1, 27% identity) and also in humans (FKBP12, 28% identity). Further bioinformatic analysis suggests that amino acids 8–150 of BPSL0918 encode an FKBP-like domain (Gough et al., 2001).

To verify that BPSL0918 is likely to form an FKBP fold, we modelled the structure of the protein (Fig. 4). The model predicts that BPSL0918 adopts a modified FKBP fold, with an additional subdomain (the insert-in-fold or IF domain) added to the FKBP core. This fold is common to a number of active FKBPs, including E. coli SlyD (Hottenrott et al., 1997), Thermus thermophilus SlyD (Löw et al., 2010) and Methanococcus thermolithotrophicus FKB (Suzuki et al., 2003). The additional subdomain adds a general chaperone activity to FKBPs, providing an enhanced role in protein folding (Knappe et al., 2007; Weininger et al., 2009). The overall fold appears well conserved with parent structures, reflecting the strong homology between BPSL0918 and other FKBPs and indicating that it is highly likely that this model reflects
the true fold. In particular, the proposed peptide binding cavity is well conserved (Löw et al., 2010).

Recombinant BPSL0918 protein lacks characteristic PPIase activity

FKBPs characteristically exhibit peptidyl-prolyl cis–trans isomerase activity, which is frequently linked to the protein function (Fischer & Aumüller, 2003). To determine whether BPSL0918 has PPIase activity, His-tagged recombinant protein was purified and the molecular mass was confirmed as 18.5 kDa by mass spectrometry. Analysis of the spectrum by circular dichroism for the secondary structure content indicated that this protein contained approximately 11.5 % α-helices and 32.5 % β-sheet. Recombinant BPSL0918 was then tested in a standard enzyme-coupled assay by using isomer-specific protolysis of a test peptide (Fischer et al., 1984). Chymotrypsin rapidly cleaves the nitroanilide from 90 % of the peptide in the trans conformation whilst the remaining 10 % is a cis conformer that undergoes slow spontaneous isomerization. Human FKBPI2, which was used as a positive control in these assays, had a significant effect on the rate of reaction [a mean change of 9.85 milli-absorbance units (mAU); lower limit of 7.67 mAU, upper limit of 12.04 mAU, 95 % CI; P<0.05]. In contrast, the presence of BPSL0918 protein did not catalyse the cis–trans isomerization of any test substrate with the structure Suc-Ala-Xaa-Pro-Phe-4-nitroanilide (a mean change of –0.25 mAU; lower limit of –2.54 mAU, upper limit of 2.03 mAU, 95 % CI; no significant differences; Table 1).

As some PPIases cannot be measured by this standard assay due to susceptibility to proteolytic degradation by α-chymotrypsin (Janowski et al., 1997), a protease-free assay was used to confirm that BPSL0918 protein lacks PPIase activity. Suc-Ala-Phe-Pro-Phe-4-nitroanilide was dissolved in 0.48 M LiCl/anhydrous trifluoroethanol, which shifts the cis:trans ratio up to 40:60 (Kofron et al., 1991). Upon addition of the aqueous buffer, the equilibrium shifts back to 10:90. However, while human FKBPI2 had a significant effect on the rate of reaction by using Suc-Ala-Phe-Pro-Phe-4 nitroanilide as a substrate (mean change of 13.28 mAU; lower limit of 8.38 mAU, upper limit of 18.18 mAbs, 95 % CI; P<0.05), BPSL0918 had no measurable effect (mean change of –1.25 mAU; lower limit of –3.54 mAU, upper limit of 1.04 mAU, 95 % CI; no significant difference). These data provide further evidence that recombinant BPSL0918 does not possess PPIase activity.

BPSL0918 harbours several mutations likely to be responsible for the lack of PPIase activity

Examination of the protein sequence of BPSL0918 revealed that the majority of the active site residues that have been proposed to mediate PPIase activity for FKBPs are not conserved in BPSL0918. Extensive experiments on active FKBPs suggest that the most important include Y26, F36, D37, F46, V55, I56, W59, Y82 and F99 [numbering according to that for FKBPI2 (Ikura & Ito, 2007; Ceymann et al., 2008; Löw et al., 2010)]. Of these, six are mutated in BPSL0918 and in five cases to another residue that is unlikely to be functionally equivalent (Fig. 5). This might explain why the B. pseudomallei FKB-like protein is enzymically inactive.

β-Lactam sensitivity is increased in B. pseudomallei ΔBPSL0918

Due to the lack of PPIase activity of BPSL0918, we speculate that this protein might have a function as a chaperone, as shown for other FKBPs. In this context it was noted that the gene downstream of BPSL0918 encodes a LspH/LytB homologue, a key enzyme involved in the non-mevalonate pathway used for isoprenoid biosynthesis (Cunningham et al., 2000). As LytB was shown to be associated with penicillin tolerance (Gustafson et al., 1993), we tested the sensitivity of B. pseudomallei wild-type, ΔBPSL0918 and complemented mutant strains against the

<table>
<thead>
<tr>
<th>Test group</th>
<th>Activity (mAU min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard PPIase assay</td>
</tr>
<tr>
<td>Control (without PPIase)</td>
<td>14.9 (±0.1)</td>
</tr>
<tr>
<td>Human FKBPI2 (Suc-Ala-Phe-Pro-Phe-4-nitroanilide)</td>
<td>24.7 (±1.7)*</td>
</tr>
<tr>
<td>BPSL0918 (Suc-Ala-Phe-Pro-Phe-4-nitroanilide)</td>
<td>14.6 (±1.7)</td>
</tr>
<tr>
<td>BPSL0918 (Suc-Ala-Ala-Pro-Phe-4 nitroanilide)</td>
<td>13.1 (±2.1)</td>
</tr>
<tr>
<td>BPSL0918 (Suc-Ala-Leu-Pro-Phe-4 nitroanilide)</td>
<td>13.2 (±1.3)</td>
</tr>
</tbody>
</table>

*Statistically significant difference compared with the control (P<0.05).

Table 1. Rate of cis–trans isomerization of substrates with the structure Suc-Ala-Xaa-Pro-Phe-4-nitroanilide

Activity was assayed with the substrates indicated, using both a standard enzyme-coupled assay to monitor the rate of release of p-nitroanilide upon addition of chymotrypsin, and a protease-free assay to monitor a solvent jump of the substrate. Values are means (±SD) of three independent experiments. A paired Student’s t-test was used to determine statistical significances. ND, Not done.
β-lactam antibiotic imipenem by two different methods. The MIC of imipenem was significantly reduced in B. pseudomallei ΔBPSL0918 in the microtitre broth dilution assay at 30 and 37 °C. In the complemented mutant strain the MIC of imipenem was at least partially restored (Fig. 6a). Increased sensitivity of B. pseudomallei ΔBPSL0918 was also clearly seen by using Etest strips (Fig. 6b). Similar results were obtained with ceftazidime (data not shown).

**DISCUSSION**

*B. pseudomallei* produces a large range of cell surface-associated and secreted virulence factors, many of which are still poorly characterized. We recently identified novel genes that are involved in the intracellular lifestyle and *in vivo* virulence of *B. pseudomallei* by screening transposon mutants in a Ptk2 cell-based plaque assay screening system (Pilatz et al., 2006). In the present study, this approach allowed us to identify a mutant strain in which the disrupted gene is BPSL0918; this mutant exhibited reduced replication within mammalian cells and was attenuated *in vivo*. BPSL0918 therefore encodes a crucial determinant in *B. pseudomallei* pathogenesis.

To assess a putative function for BPSL0918 in *B. pseudomallei* virulence, bioinformatic analysis and modelling was carried out. Sequence and structural analysis indicated that BPSL0918 encodes an FKB-like protein, a class of proteins which typically possess PPIase activity. FKBPs have been shown to be important virulence factors in several intracellular pathogens (Ciaccio et al., 1989; Moro et al., 1993; Lundemose et al., 1993; Leuzzi et al., 2005). Due to the role of these FKBPs in invasion into macrophages, they have been named macrophage infectivity potentiators (Mips). The importance of PPIase activity of Mips was demonstrated by specifically targeting the PPIase domain with monoclonal antibodies that inhibited *Legionella* infection of protozoa and human macrophages (Helbig et al., 2003). In addition, the PPIase inhibitors FK506 and rapamycin were reported to inhibit transmigration of *L. pneumophila* across NCI-H292 lung epithelial cells (Wagner et al., 2007). Active PPIase activity was also shown to play a role in Mip-mediated infection of macrophages by *Chlamydia trachomatis* (Lundemose et al., 1993).

Although it resembled a Mip, we could not detect PPIase activity in the FKB-like protein of *B. pseudomallei*. As there are limitations to the standard enzyme-coupled assay because only one direction of the reversible isomerisation is measured (cis→trans), and some PPIases are highly susceptible to proteolytic degradation (Wülfling et al., 1994), we additionally tested PPIase activity of BPSL0918 in a protease-free assay. However, no activity of recombinant BPSL0918 was observed in either of the assay systems, with various substrates. A possible explanation for the lack of PPIase activity is that the amino sequence of BPSL0918 contains mutations in several residues equivalent to those in hFKBP12 and are therefore likely to affect PPIase activity (for further details see main text).
for PPIase activity (Ikura & Ito, 2007). FKBP s from a wide range of species have been extensively studied and at least nine amino acids have been shown to be important for PPIase activity (Ikura & Ito, 2007; Ceymann et al., 2008; Löw et al., 2010). In the amino acid sequence of BPSL0918, six of these (F36, D37, F46, I56, W59 and Y82) are mutated to residues that are unlikely to be functionally equivalent: residue W59 forms the base of the substrate-binding cavity and the W → L mutation alone has been shown to reduce the PPIase activity of FKBP12 by 50% (Ikura & Ito, 2007). Residues D37 and Y82 provide the main source of hydrogen-bonding interactions within the active site of FKBP12 (Löw et al., 2010) and the substitution of these residues in the predicted active site of BPSL0918 is likely to result in minimal PPIase activity. Furthermore, residues F36, F46 and I56 provide part of the hydrophobic bowl of the active site (Ceymann et al., 2008) and the mutation of these to smaller side chains is likely to reduce the effectiveness of the hydrophobic pocket in binding to the transition state. The lack of conservation of these important functional residues supports the experimental data demonstrating that recombinant BPSL0918 is enzymatically inactive.

Several reports describe that the PPIase activity itself was not necessarily required for full physiological function of proteins (Behrens et al., 2001; Zhang et al., 2007; Weininger et al., 2009). SurA, a parvulin homologue exhibiting PPIase activity (Rouvière and Gross, 1996), was first described in E. coli (Tormo et al., 1990) and is required for full virulence in some bacteria (Sydenham et al., 2000; Justice et al., 2005, 2006). Beside its PPIase activity, SurA was also shown to function as a chaperone that is involved in the maturation of outer-membrane proteins (Rouvière & Gross, 1996; Lazar & Kolter, 1996). In a surA mutant strain complemented with a SurA variant lacking PPIase functions, features associated with chaperone-mediated functions were restored, suggesting that the chaperone role of SurA is independent of its PPIase activity (Behrens et al., 2001; Watts & Hunstad, 2008). We therefore speculate that the primary function of BPSL0918 might be as a chaperone that, like SurA, works independently of PPIase activity. In this context it is noteworthy that the gene downstream of BPSL0918 encodes a homologue of LspH/LytB, a key enzyme involved in the non-mevalonate pathway used for isoprenoid biosynthesis (Cunningham et al., 2000). LytB has been shown to be involved with penicillin tolerance and outer-membrane protein formation (Gustafson et al., 1993; Burtnick and Woods, 1999). Interestingly, we found that B. pseudomallei ΔBPSL0918 was significantly more susceptible than the wild-type strain to β-lactam antibiotics. These data support the notion that BPSL0918 may act on LspH as a chaperone. Unfortunately, when we expressed B. pseudomallei LspH in E. coli, it gave only insoluble protein in all the conditions that we tested. It has therefore not been possible to test this hypothesis. However, the structural model of BPSL0918 indicates that the protein has a subdomain which has been previously shown to be important for FKBP chaperone function (Suzuki et al., 2003; Knappe et al., 2007; Weininger et al., 2009). The presence of point mutations in the BPSL0918 active site raises the possibility that a selective pressure induced loss of enzyme activity whilst maintaining its important function. Further studies are needed to determine specific functions of BPSL0918 that might explain its involvement in B. pseudomallei pathogenesis.

ACKNOWLEDGEMENTS

I. N. and M. S.-T. were supported by funding from the UK Ministry of Defence. K. E.-P. was supported by a grant of the Graduate College 840 (Deutsche Forschungsgemeinschaft) to I. S. We are grateful to Dr Tom Laws for assistance with statistical analysis and Helga Schalimow for technical assistance.

REFERENCES


FKBP involved in B. pseudomallei virulence


Edited by: H. Hilbi