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

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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 *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 *BPSL0918* gene was disrupted. *BPSL0918* encodes a putative FK-506-binding protein (FKBP) representing a family of proteins that typically possess peptidyl-prolyl isomerase (PPIase) activity. A *B. pseudomallei* Δ *BPSL0918* 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, *B. pseudomallei* Δ *BPSL0918* was significantly attenuated in a murine model of infection. Structural modelling confirmed a modified FKBP fold of the *BPSL0918*-encoded protein but unlike virulence-associated FKBP from other pathogenic bacteria, recombinant *BPSL0918* protein did not possess PPIase activity *in vitro*. In accordance with this observation *BPSL0918* exhibits several mutations in residues that have been proposed to mediate PPIase activity in other FKBP. To our knowledge this *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.

Received 22 February 2011

Revised 3 June 2011

Accepted 14 June 2011

INTRODUCTION

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 septicaemia to chronic localized infection. The infection can become latent, and reactivation has been recorded 62 years after primary exposure (Ngaay *et al.*, 2005). *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 *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 & Aumüller, 2003). FKBP typically possess peptidyl-prolyl isomerase (PPIase) activity, which is thought to have evolved to catalyse the slow, rate-limiting *cis-trans* isomerization of Xaa-Pro peptide bonds during protein folding (Brandts *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 *et al.*, 1989; Schreiber, 1991). Several studies have shown that FKBP play a role in mediating bacterial

Abbreviations: BMM, bone-marrow-derived macrophage; FKBP, FK-506-binding protein; Mip, macrophage infectivity potentiator; PPIase, peptidyl-prolyl isomerase.

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 Ratchatani, 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 obtained from Invitrogen. *B. pseudomallei* was grown on Columbia agar or Luria–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* Tn5(pOT182) 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(pRK2013). 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 Ptk2 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 (CCCGAATTCGTATTGCATGCTGCTGACCTC) and reverse primer 0918compR (CCCAAGCTTCTCGACGACGTACTTG-TTGTG) (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-Tn7T-Zeo-BPSL0918) and two *E. coli* helper strains [*E. coli* Hb101(pRK2013) 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 OD₆₅₀ of 0.01. For growth curve analysis, OD₆₅₀ was measured at the indicated time points. For *BPSL0918* expression analysis, bacteria were collected by centrifugation when the OD₆₅₀ 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 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 microlitres 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 (CATATGAGCCTCATCGACCTT) and 0918R (GGATCCTT-ACAGAATCCCCGATGAT). 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 His₆ 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 bicinchoninic 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 and an HP1100 Series LC system (Agilent Technologies). All data acquisition, mass spectrometer control and post-run processing were carried out by using MassLynx and MaxEnt software (Micromass).

Enzyme-coupled PPIase assay. To determine if BPSL0918 protein had PPIase 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 PPIase 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/anhedrous 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.

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, 2KO7, 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 VTFM 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 microlitres 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.

Etest. Imipenem Etest strips were obtained from bioMérieux and used according to the manufacturer's instructions.

Statistical analysis. To determine significant differences between groups, either a Student's *t*-test or one-way ANOVA test was used as indicated for each experiment. Survival curves were compared by using the log rank Kaplan–Meier test. Statistical analyses were performed by using either GraphPad Prism version 4.0 or Microsoft Office Excel 2003.

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* Δ *BPSL0918* 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* Δ *BPSL0918* 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

Fig. 1. (a, b) Growth kinetics of *B. pseudomallei* wild-type (WT) E8 and *B. pseudomallei* Δ BPSL0918 in LB medium (a) and minimal Vogel-Bonner medium (b). (c) *In vitro* expression of BPSL0918 in LB and Vogel-Bonner medium. Lanes: 1, *B. pseudomallei* wild-type DNA; 2, 3, RNA (2) and cDNA (3) from bacteria grown in LB medium; 4, 5, RNA (4) and cDNA (5) from bacteria grown in Vogel-Bonner medium.

B. pseudomallei Δ BPSL0918 (Fig. 2a). Thus, the ability of *B. pseudomallei* Δ BPSL0918 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* Δ BPSL0918 was also significantly reduced 24 h after initial infection (Fig. 2b). Complementation of the mutant fully restored the ability of *B. pseudomallei* Δ BPSL0918 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* Δ BPSL0918 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

Fig. 2. Comparison of the intracellular survival kinetics of *B. pseudomallei* wild-type (WT) E8, mutant Δ BPSL0918 and complemented mutant Δ BPSL0918 : Tn7-BPSL0918. (a) Intracellular survival in BMMs (m.o.i. ~15:1). (b) Intracellular survival in HeLa cells (m.o.i. ~10:1). *P*-values were calculated using either one-way ANOVA (a) or Student's *t*-test (b) and are shown for the comparison of intracellular bacteria at 24 h after infection. ***P* < 0.01, ****P* < 0.001. Values are means $\pm$ SD from triplicate determinations. Data were obtained at least three times and representative experiments are shown.

B. pseudomallei Δ BPSL0918 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* Δ BPSL0918 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

Fig. 3. Mortality curves of BALB/c mice after intranasal infection. (a) Intranasal infection of BALB/c mice ($n=10$) with *B. pseudomallei* wild-type (WT) E8, mutant $\Delta BPSL0918$ and complemented mutant $\Delta BPSL0918$:Tn7-BPSL0918 (infection dose ~30–90 c.f.u. per animal). Pooled data from two independent experiments are shown. (b) Intranasal infection of BALB/c mice ($n=5$) with *B. pseudomallei* WT and mutant $\Delta BPSL0918$ (infection dose ~800 c.f.u. per animal). Data from a single experiment are shown.

FkpB-type peptidyl-prolyl *cis-trans* isomerase (Holden *et al.*, 2004). A search against the non-redundant NCBI database revealed sequence homology between *BPSL0918* and FKBP 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, 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 FKBP, including *E. coli* SlyD (Hottenrott *et al.*, 1997), *Thermus thermophilus* SlyD (Löw

Fig. 4. Modelling of the structure of *BPSL0918*. (a) Overview of the modelled structure of *BPSL0918* (green), in comparison with representative parent structures from *Homo sapiens* (structure 1FKB; cyan) and *Thermus thermophilus* (structure 3CGM; magenta). Note that secondary structure is well conserved in the model, indicating that the model is likely to be a good quality representation of the protein structure. (b) Highlight of the FKBP active site. The model of *BPSL0918* bound to rapamycin is shown, with rapamycin shown as white sticks. The surface of *BPSL0918* is shown coloured according to conservation: cyan, identical side chain to active homologues; magenta, non-identical side chain but may be functionally equivalent; orange, non-identical side chain and unlikely to be functionally equivalent. The nine most important side chains for catalysis are shown in stronger colours. (c) The putative peptide-binding groove of SlyD is conserved in *BPSL0918*. The surface of the insert-in-fold (IF) domain of *BPSL0918* (green) and *Thermus thermophilus* SlyD (magenta) are shown. The proposed peptide binding groove in the SlyD structure and the equivalent location in *BPSL0918* are indicated by arrows.

et al., 2010) and *Methanococcus thermolithotrophicus* FKBP (Suzuki *et al.*, 2003). The additional subdomain adds a general chaperone activity to FKBP, 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 FKBP 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 FKBP12, 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 substrate to the aqueous buffer, the

equilibrium shifts back to 10:90. However, while human FKBP12 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 FKBP12 (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* FKBP-like protein is enzymically inactive.

β -Lactam sensitivity is increased in *B. pseudomallei* Δ BPSL0918

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, Δ BPSL0918 and complemented mutant strains against the

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 ($\pm$ SD) of three independent experiments. A paired Student's *t*-test was used to determine statistical significances. ND, Not done.

Test group	Activity (mAU min ⁻¹)	
	Standard PPIase assay	Protease-free PPIase assay
Control (without PPIase)	14.9 ($\pm$ 0.1)	2.6 ($\pm$ 0.9)
Human FKBP12 (Suc-Ala-Phe-Pro-Phe-4-nitroanilide)	24.7 ($\pm$ 1.7)*	15.9 ($\pm$ 4.4)*
BPSL0918 (Suc-Ala-Phe-Pro-Phe-4-nitroanilide)	14.6 ($\pm$ 1.7)	1.4 ($\pm$ 1.2)
BPSL0918 (Suc-Ala-Ala-Pro-Phe-4 nitroanilide)	13.1 ($\pm$ 2.1)	ND
BPSL0918 (Suc-Ala-Leu-Pro-Phe-4 nitroanilide)	13.2 ($\pm$ 1.3)	ND

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

BPSL0918	MSLI DLAEVKPGS-----HVTLLHYRLALADGACTVNTFS-DKFAITL	40
hFKBP12	-MGVQVETI SPQDGRTFPKRGQT CVVHYTGMLLEDGKKFDSSRDNRKFEKF	49
BPSL0918	LLGAGQLAPSLLEELI GLKAGDHSTFRLAPEHAFGPRNPDMI QRVSLATL	90
hFKBP12	MLGKQEVLRQWEEGVAQMSVQRAKLTI SPDYAYG-----	84
BPSL0918	RENGMI GDDFAPGDLI EFNAPDGGRYAGVLKEI GETSALFDNHLPLAGQA	140
hFKBP12	-----ATG---HPGI PPH-----A---T-	97
BPSL0918	LTTEVNI IGI L	151
hFKBP12	LVFDVLELLKLE	108

Fig. 5. Alignment of human FKBP12 and BPSL0918 protein sequences. Identical amino acids are highlighted by a black background. Asterisks indicate residues important for PPIase activity in hFKBP12. Boxed residues (F36, D37, F46, I56, W59, Y82) are mutated in BPSL0918 to residues that are unlikely to be functionally equivalent to those in hFKBP12 and are therefore likely to affect PPIase activity (for further details see main text).

β -lactam antibiotic imipenem by two different methods. The MIC of imipenem was significantly reduced in *B. pseudomallei* Δ BPSL0918 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* Δ BPSL0918 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 FKBP-like protein, a class of proteins which typically possess PPIase activity. FKBP has been shown to be important virulence factors in several intracellular pathogens (Cianciotto *et al.*, 1989; Moro *et al.*, 1995; Lundemose *et al.*, 1993; Leuzzi *et al.*, 2005). Due to the role of these FKBP 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 NCL-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 FKBP-like protein of *B. pseudomallei*. As

there are limitations to the standard enzyme-coupled assay because only one direction of the reversible isomerization is measured (*cis*→*trans*), and some PPIases are highly susceptible to proteolytic degradation (Wülfing *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 the human FKBP12 that were shown to be important

Fig. 6. Imipenem sensitivity testing of *B. pseudomallei* wild-type (WT) strain E8, mutant Δ BPSL0918 and complemented mutant Δ BPSL0918:Tn7-BPSL0918. (a) Microtitre broth dilution assay at 37 and 30 °C. Values are the means $\pm$ SD of triplicate determinations. One out of three independent experiments with similar results is shown. One-way ANOVA was used to determine statistical significances. (b) Imipenem Etest. Pooled data from three independent experiments are shown. Student's *t*-test was used to determine statistical significance. ****P* < 0.001.

for PPIase activity (Ikura & Ito, 2007). FKBP 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 enzymically 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 domains, 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; Burtneck and Woods, 1999). Interestingly, we found that *B. pseudomallei* Δ BPSL0918 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.

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Edited by: H. Hilbi