A *Burkholderia pseudomallei* Mip-like Protein has Rapamycin Inhibitable Peptidyl-Prolyl Isomerase Activity and has Pleiotropic Effects on Virulence

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Abstract

Macrophage infectivity potentiators (Mips) are a group of virulence factors encoded by pathogenic bacteria such as *Legionella, Chlamydia* and *Neisseria*. Mips are part of the FK506-binding protein (FKBP) family that typically exhibit peptidyl-prolyl *cis-trans* isomerase (PPIase) activity, inhibitable by the immunosuppressants FK506 and rapamycin. Here we describe the identification and characterisation of BPSS1823, a Mip-like protein in the intracellular pathogen *Burkholderia pseudomallei*. Recombinant BPSS1823 protein has rapamycin-inhibitable PPIase activity, indicating that it is a functional FKBP. A mutant strain, generated by deletion of *BPSS1823* in *B. pseudomallei,* exhibited a reduced ability to survive within cells and significant attenuation *in vivo,* suggesting that BPSS1823 is important for *B. pseudomallei* virulence. In addition, pleiotropic effects were observed with a reduction in virulence mechanisms including resistance to host killing mechanisms, swarming motility and protease production.

Introduction

Burkholderia pseudomallei is a motile, Gram-negative bacillus and the causative agent of the disease melioidosis. Melioidosis is endemic in South East Asia and Northern Australia. Infection typically occurs by inoculation of the organism through skin legions, but infection by inhalation or ingestion of the organism has also been reported (5). Clinical presentation of melioidosis in humans varies from disseminated acute septicaemia to localised chronic infection (4). Pneumonic infection occurs in 60% of acute cases, resulting in significantly higher mortality rates (34). *B. pseudomallei* is listed as a category B agent by the US Center for Disease Control and Prevention (37). There is currently no vaccine available for prophylaxis and intrinsic antibiotic resistance makes treatment regimes complex.

Although the virulence mechanisms employed by *B. pseudomallei* have been extensively studied in recent years (1), many remain poorly defined. As an intracellular organism, *B. pseudomallei* is able to invade, replicate and spread directly from cell to cell (23, 24). In addition, bacteria can evade phagosome-lysosome fusion and destroy the phagosome membrane (18). However the mechanisms used by *B. pseudomallei* to avoid clearance are largely unknown.

FK506-binding proteins are ubiquitous in eukaryotes and prokaryotes which typically possess peptidyl-prolyl *cis/trans* isomerase (PPIase) activity and catalyse the folding of proline containing proteins. PPIase activity is inhibitable upon binding to the immunosuppressants FK506 or rapamycin (38). Although PPIases are widely distributed in bacteria, the functions of these proteins are poorly understood. In some bacteria, PPIases have been shown to play a role in virulence and have been termed macrophage infectivity potentiators (Mips) (7, 20; 27; 30). The best studied Mip is a 24 kDa FKBP from *Legionella pneumophila* (Lp-Mip) and has been shown to play a role in the invasion of human macrophages and virulence in guinea pigs (7, 8). Although Mips have been shown to be required for virulence in several pathogens, the cellular target(s) of Mip are yet to be elucidated. Furthermore, because of the potentially diverse functions of Mips it is unclear whether the *Legionella* Mip provides a paradigm for extrapolating the functions of Mips in other bacteria.

This study reports the identification of a Mip-like protein encoded by *B. pseudomallei* which possesses PPIase activity and is inhibitable by rapamycin. The Mip-like protein is required for intracellular survival and for virulence in a BALB/c mouse model of infection. In addition, inactivation of the Mip-like gene has pleiotropic effects on several known virulence mechanisms, providing new information on the role of bacterial Mips in disease.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1.

All strains were grown in LB broth at 37ºC overnight with agitation, unless otherwise stated. Antibiotics were used at the following final concentrations: kanamycin, 50 µg/ml; ampicillin, 50 µg/ml; chloramphenicol, 30 µg/ml; gentamycin 10 µg/ml – 30 µg/ml.

Construction of expression plasmid for production of recombinant BPSS1823

The open reading frame encoding *BPSS1823* was amplified by PCR using *B. pseudomallei strain* K96243 genomic DNA as template and the primers pET.F (CATATGACAGTCGTCACCACC) and pET.R (GGATCCTCAGACGTCGAGCAGTTC). The PCR product was inserted into the *NdeI/BamHI* site of pET15b expression plasmid (Novagen). The construct was transformed into *E. coli* strain BL21 (DE3) to allow expression of His_6 tagged BPSS1823 recombinant protein.

Purification of recombinant BPSS1823 protein

A single colony of *E. coli* BL12 (DE3) harbouring the expression construct was used to inoculate 2 L LB broth. This was incubated at 37ºC with agitation until the absorbance reached 0.4-0.6 at 600 nm. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and growth continued at 20ºC with agitation for 4 h. Cells were harvested by centrifugation at 8000 *x g* for 15 min at 4ºC then disrupted by sonication. Cell debris was pelleted by centrifugation at 8000 *x g* for 30 minutes at 4ºC. The supernatant was loaded onto a 1 ml Histrap FF column (GEhealthcare) and the recombinant protein eluted in 100 mM imidazole. Protein samples were separated by sodium dodecyl-sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) and purity examined by staining with Coomassie brilliant blue (Pierce Biotechnology). Protein concentration was determined using a bicinchoninic acid assay (Pierce Biotechnology). Imidazole was removed from the purified protein by dialysis against 10 mM PBS and samples frozen at -80ºC until use.

Peptidyl-prolyl isomerase assay

Peptidyl-prolyl *cis-trans* isomerase activity of recombinant BPSS1823 protein was determined by a protease coupled assay as described previously (14). Briefly, 10 nM BPSS1823 protein was incubated for 6 min at 10ºC in 1.2ml 35mM HEPEs buffer, pH 7.8 with succinyl-Ala-Phe-Pro-Phe-*p*-nitroanilide (10 mg/ml; Bachem).Chymotrypsin (Sigma) was added to the cuvette at a final concentration of 0.8 mg/ml and mixed. Hydrolysis of the substrate was measured at 390 nm using a Shimadzu 1800 UV/Vis spectrophotometer at 1 sec intervals until there was no further change in absorbance. For inhibition measurements, recombinant BPSS1823 protein was pre-incubated with varying concentrations of rapamycin from 30 nM – 1 nM for 6 min prior to the addition of substrate. At least three independent readings were taken at each data point. All data fitting and statistical analyses were performed using SPSS v16.0 (IBM)

The pseudo first order rate constant was calculated using equation 1; data from 10-50 sec (which were always after the lag phase, and before substrate became limiting) were taken, and k_{obs} calculated by linear regression.

(1) $$\ln[A_\infty - A_t] = -k_{obs}t + \ln[A_\infty - A_0]$$

The enzymatic rate was determined by comparing the observed rate to the uncatalysed rate (equation 2).

(2) $$k_{enz} = k_{obs} - k_{uncat}$$

The specificity constant k_{cat}/K_M for the enzyme was calculated using equation 3 (17): data were taken using 1 nM, 5 nM and 10 nM BPSS1823, and were fit using linear regression.

(3) $$\frac{k_{cat}}{K_M} = \frac{k_{enz}}{[PPIase]}$$

Data for inhibitor assays were fit to equation 4 (44) using least squares non-linear fitting. $v_0$and K_I^{app} were fit, using initial estimates based on the raw data and [E] was kept as constant.(4)

$$v = v_0 \frac{[E]-[I]-K+\sqrt{([E]-[I]-K)^2+4[E][K]}}{2[E]}$$

Mutant strain construction

B. pseudomallei deletion mutants were constructed as previously described (27). A 453 bp upstream flanking region including the start codon and a 311 bp downstream region including the stop codon were amplified from *B. pseudomallei* K96243 genomic DNA using primer pairs LFF/LFR (TCTAGAGCCGCCGACCTTTACATT/AGATCTGCTCGAATCGAACTTCTG) and RFF/RFR (AGATCTCTCGTGTTCGAAGTCGAA/TCTAGACCAGTTGGCTGTTGTCGG. Restriction sites were engineered into the primers to allow ligation of the flanks and insertion into the *XbaI* site of pDM4. The pDM4 construct was transformed into *E.coli* S17 λpir and conjugated into *B. pseudomallei* strain AI. Merodiploid integrants were identified using antibiotic selection and plated onto LB agar lacking sodium chloride but containing 10% sucrose. SacB counterselection was used to select for excision of vector DNA, resulting in an unmarked deletion. Colonies were screened for chloramphenical sensitivity and analyzed by PCR. Southern hybridization, using a Dig-labelled upstream flanking region to probe, was used to confirm a 171 bp deletion of *BPSS1823* and the strain termed *B. pseudomallei* AI Δ*BPSS1823*.

Complementation studies

The open reading frame encoding *BPSS1823* was amplified by PCR using *B. pseudomalleistrain* K96243 genomic DNA as template and the primers PBBR.F (GAATTCATGACAGTCGTCACCACC) and PBBR.R (TCTAGATCAGACGTCGAGCAGTTC). The PCR product was inserted into the *EcoRI/XbaI* restriction sites of pBBR1-MCS2. The complementation construct was transformed into *E.coli* S17 *λpir* and conjugated into *B. pseudomallei* AI Δ*BPSS1823* with the helper strain *E. coli* HB101 (pRK2013). Conjugates were selected for resistance on LB agar containing 700 µg/ml kanamycin and 50 µg/ml ampicillin and confirmed by colony PCR. For future experiments, the complemented mutant strain was grown in LB broth containing 200 µg/ml kanamycin and 1 mM IPTG to induce expression of BPSS1823.

Infection of cell lines

J774A.1 murine macrophages or A549 human epithelial cells were seeded onto a 24-well tissue culture plate at a concentration of 4 x 10^5 cells/ml in Dulbecco's Modified Eagels Medium (DMEM) supplemented with 1% L-glutamine and 10% fetal calf serum and incubated at 37 ºC and 5% CO_2 for approximately 16 h. *B. pseudomallei* strains were grown at 37ºC overnight then adjusted in Leibovitz L-15 medium with 10% fetal calf serum to an absorbance of 0.35-0.4 at 590 nm. Bacteria were serially diluted in L-15 medium, 1 ml was added to the cells at an MOI of 1 or 10 and incubated at 37ºC for 30 min or 1 h. Further dilutions were plated onto LB agar at the time of infection to allow for determination of the starting innoculum. Bacteria were removed and infected cells incubated with L15 containing 30 µg/ml gentamicin for 30 min at 37ºC. Antibiotic media was removed, serially diluted in PBS and plated onto LB agar to confirm extracellular killing. Cells were then incubated with

10 µg/ml gentamicin for 24 h. At 0, 2, 4 and 24 h post infection, cells were lysed with 1 ml dH_2O, serially diluted in PBS and plated onto LB agar to determine intracellular numbers.

Adhesion to A549 epithelial cells

A549 cells and bacteria were prepared as previously described. Cytochalasin-D (Sigma) was added to approximately 1 x 10^6 cells at a final concentration of 1 µg/ml and incubated at 37ºC with 5% CO_2 for 30 min. Cytochalasin-D was added to approximately 1 x 10^7 cfu/ml bacteria at a final concentration of 1 µg/ml. 1 ml treated bacteria was added to the pretreated cells at an MOI of 1:10 and incubated at 37ºC for 1h. Cells were then washed 3 times with warm PBS to remove non-adherent bacteria. Cells were lysed with 1 ml dH_2O, serially diluted in PBS and plated onto LB agar and incubated at 37ºC overnight. Cytochalasin-D was present throughout the assay.

Exposure to low pH

B. pseudomallei strains were grown at 37ºC overnight, adjusted to an absorbance of 0.01 at 590 nm and grown for 2 h at 37ºC with agitation. 100 µl of adjusted bacterial culture was inoculated into 10 ml LB broth at pH 4 or pH 7 (adjusted with HCl) and incubated at 37ºC overnight with agitation. At 0, 3 and 24 h post innoculation, 100 µl bacterial culture was removed and serially diluted, plated onto LB agar and incubated at 37ºC overnight.

Motility assay

B. pseudomallei strains were grown at 37ºC overnight. 1 µl of overnight culture was stabbed into 0.3% motility agar using a sterile inoculating loop and the plates incubated at 37ºC overnight. Bacterial spread was measured using a Scienceware® vernier calliper (Sigma).

Electron microscopy

B. pseudomallei strains were grown at 37ºC overnight. 2 ml of culture was pelleted at 15 000 *x g* for 5 min. Samples were fixed in 4% formalin for 24 h. Samples were stained with 2% w/v uranyl acetate and examined in a FEI CM12 transmission electron microscope operating at 80kV and images captured using a 1MP Keenview digital camera.

Protease assay

An overnight culture of *B. pseudomallei* was diluted 1:50 and grown at 37ºC. Following 24 h growth, 1 ml bacterial culture was removed and pelleted at 15 000 *x g* for 5 min. 100 µl supernatant was added to 100 µl azocasein (5 mg/ml; Sigma) and incubated at 37ºC for 1h. The reaction was stopped with 10% trichloroacetic acid (Sigma) and non-hydrolysed azocasesin pelleted at 10 000 *x g* for 15 min. The supernatant was added to 500 mM NaOH and read using a WPA Colourwave colourimeter (model C07500) at 440 nm.

Animals

Groups of six female BALB/c age-matched mice were housed together with free access to food and water and subjected to a 12h light/dark cycle. All studies involving animals were carried out according to the requirements of the Animal (Scientific Procedures) Act 1986 and the Codes of Practice for the Housing and Care of Animals used in Scientific Procedures 1989. For challenge with *B. pseudomallei,* animals were handled under biosafety level III containment.

***B. pseudomallei* challenge**

Groups of six mice were challenged with 6.3 x 10^6 cfu of *B. pseudomallei* AI or 2.5 x 10^6 cfu *B. pseudomallei* AI Δ*BPSS1823* intraperitoneally and infection was monitored for 5 weeks.

Humane endpoints were strictly observed so that animals presenting predetermined clinical signs indicative of a lethal infection were culled.

Isolation of bacteria from murine spleens

Following challenge with *B. pseudomallei*, remaining survivors were humanely culled. The spleens were aseptically removed and homogenized in 1 ml sterile PBS. Dilutions of the homogenates were plated onto LB agar to determine bacterial load.

Modelling of BPSS1823

The structure of BPSS1823 protein was modelled using MODELLER version 9.8 (12). Three structures (1FD9, 1FKB, 1ROT) were selected as templates. A structure based sequence alignment for these structures was produced using MAMMOTH-mult (31), and edited by hand. Structure based alignment of the sequence of BPSS1823 was performed using JOY (32) and FUGUE (40). 10 models were prepared using the high quality VTFM optimisation and MD/SA optimisation options. Models were scored according to MODELLER energy score, and Ramachandran plot quality judged by RAMPAGE (28).

Statistical analysis

For intracellular infection and pH exposure assays a Two-Way ANOVA and Bonferroni's post test were used to determine statistical significance between groups. For motility assays a One-Way ANOVA and Bonferroni's Multiple Comparison Test were used. For protease assays, an unpaired student's T-test was used. Survival curves were compared using a Log-rank (Mantel-Cox) Test. Significances were referred as follows: * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$. Statistical analyses were performed using either GraphPad Prism version 4.0 or Microsoft Office Excel 2003.

Results

Burkholderia pseudomallei encodes a Mip-like protein

The open reading frame *BPSS1823* from *B. pseudomallei* K96243 encodes a polypeptide of 113 amino acids annotated as a peptidyl-prolyl *cis-trans* isomerase (http://www.sanger.ac.uk/Projects/B_pseudomallei/). BLAST searches against a non-redundant NCBI protein database revealed sequence similarity between BPSS1823 and Mip proteins from a variety of bacterial intracellular pathogens.

BPSS1823 has 40%, 45% and 42% amino acid identity to *L. pneumophila* Mip (Lp-Mip), *Neisseria gonorrhoeae* Mip (Ng-Mip) and *Chlamydia trachomatis* Mip (Ct-Mip) respectively (Figure 1A). BPSS1823 does not contain a putative N-terminal dimerization domain but has high homology to the C-terminal PPIase domain possessed by other Mips, suggesting it could have PPIase activity. In addition, BPSS1823 possesses most residues required for PPIase activity in human FKBP12 (3, 21, 29)

To verify that BPSS1823 is likely to encode a Mip homologue, we modelled the structure of the protein (Figure 1B). This model predicts that, like Lp-Mip, BPSS1823 adopts a classical FKBP fold and has an active site that strikingly resembles the Lp-Mip active site. In addition, all of the residues that are highly conserved in the active site of FKBPs, appear conserved in sequence and space. This model gave us further confidence that BPSS1823 is a *bona fide* Mip homologue and that it might have a similar role to the Mips in other organisms.

B. pseudomallei Mip-like protein exhibits rapamycin-inhibitable PPIase activity

Purified recombinant His-tagged BPSS1823 protein had a molecular weight, determined by mass spectrometry, of 14436 Da. Size-exclusion chromatography (data not shown)

demonstrated that this protein is monomeric, consistent with the observation of the lack of an N-terminal dimerization domain in the sequence. Mip proteins from other bacteria have been shown to have PPIase activity which can be inhibited upon binding to FK506 and rapamycin (7, 20, 26, 30, 33). Recombinant BPSS1823 protein was tested for PPIase activity in an enzyme coupled assay by measuring *cis-trans* isomerisation of the tetrapeptide Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide (14). Using this substrate, the maximal activity of a highly purified enzyme fraction had a calculated specificity constant k_{cat}/K_m of 6.7 ± 0.4 x 10^6 M^{-1} s^{-1}. To examine the effect of rapamycin on the PPIase activity of BPSS1823, recombinant protein was incubated with increasing concentrations of rapamycin. The PPIase activity of BPSS1823 protein is inhibited by nanomolar concentrations of rapamycin, with a K_I of 3 ± 2 nM (Figure 2).

BPSS1823 is required for intracellular survival within, but not adhesion to, eukaryotic cells

To evaluate the role of BPSS1823 in *B. pseudomallei*, an in-frame deletion mutant was made in *B. pseudomallei* strain AI and the deletion confirmed by Southern hybridization (Figure 3, A). The parent strain or Δ*BPSS1823* mutant strain was used to infect phagocytic (J774A.1) or non-phagocytic (A549) cells. In J774A.1 macrophages, the numbers of parent bacteria or Δ*BPSS1823* mutant bacteria recovered 1 h after infection were similar. However, significantly fewer Δ*BPSS1823* mutant bacteria were recovered from cells 24 h post infection (Figure 3, C; $P<0.001$). In A549 epithelial cells, the number of Δ*BPSS1823* mutant bacteria recovered 1 h after infection was significantly lower than the number of parent bacteria (Figure 3, D; $P<0.01$). In addition, while the intracellular numbers of the parent strain increased 60-fold over 24 h, almost no replication of the mutant strain was observed (Figure 3, D; $P<0.001$). Re-introduction of the wildtype gene in *trans* fully restored the ability of the Δ*BPSS1823* mutant to survive and grow within both cells lines (Figure 3, C, D;

P<0.001), confirming that the defect was specific to BPSS1823 and not due to polar effects. Furthermore, the Δ*BPSS1823* mutant did not exhibit reduced growth in pH neutral LB broth (Figure 3, B) or increased sensitivity to gentamicin (data not shown).

We investigated whether BPSS1823 also played a role in adherence to A549 cells. Phagocytosis was inhibited pre-infection using cytochalasin-D and non-adherent bacteria were removed by washing with PBS. The number of adherent bacteria was determined 1 h after incubation of bacteria with cells and no significant difference between the parent and Δ*BPSS1823* mutant strain was observed (Figure 3, E).

BPSS1823 is involved in _B. pseudomallei_ resistance to low pH

To further characterise the role of BPSS1823 in intracellular survival, the Δ*BPSS1823* mutant strain was exposed to a range of environmental stresses, including osmotic stress (NaCl), peroxide stress (H_2O_2) and a range of pH conditions. There was no difference in the survival of parent or Δ*BPSS1823* mutant bacteria under osmotic or peroxide stress (data not shown). While the parent strain grew to a concentration of 10^7-10^9 cfu/ml in LB media adjusted to pH 4, 5, 6 or 7, the growth of the Δ*BPSS1823* mutant was significantly reduced by 24 h growth at pH 4 (Figure 4; P<0.001). Neither parent nor Δ*BPSS1823* mutant was able to grow in media at pH 3 or below (Figure 4, A).

Deletion of _BPSS1823_ renders _B. pseudomallei_ immotile and reduces protease production

PPIases have been shown to assist folding and chaperoning of outer membrane proteins (41). Therefore, membrane associated virulence mechanisms such as swarming motility and protease secretion were examined in.the Δ*BPSS1823* mutant. . While inoculation of *B. pseudomallei* AI into 0.3% agar resulted in a mean bacterial spread of 21.4 mm, inoculation

with the Δ*BPSS1823* mutant resulted in localised growth of 5.4 mm at the site of inoculation and significantly less bacterial spread (Figure 5, A, B; P<0.001).In addition, unlike *B. pseudomallei* AI, the Δ*BPSS1823* mutant did not produce flagella (Figure 5, B). Complementation of the Δ*BPSS1823* mutant strain fully restored bacterial motility and flagella formation, resulting in significantly increased bacterial spread compared to both *B. pseudomallei* AI Δ*BPSS1823* and *B. pseudomallei* AI (Figure 5, A, B P<0.001).

Secreted protease activity was determined by using azocasein as a substrate (2). While both strains exhibited protease activity, hydrolysis of azocasein was 4-fold less in the mutant strain (Figure 5, C, P<0.01). This indicates that BPSS1823 is required for production of putative virulence mechanisms in *B. pseudomallei*, such as swarming motility and protease production.

BPSS1823 is required for full virulence of _B. pseudomallei_ in a murine model of infection

The role of BPSS1823 in *B. pseudomallei* virulence *in vivo* was investigated by challenging BALB/c mice via the intraperitoneal route with 6.2 x 10^6 cfu *B. pseudomallei* AI or 2.5 x 10^6 cfu *B. pseudomallei* AI Δ*BPSS1823*. All mice challenged with *B. pseudomallei* AI had succumbed to infection by 1 day post challenge. In contrast, animals challenged with *B. pseudomallei AI* Δ*BPSS1823* had significantly increased survival with a MTTD of >35 days (Figure 6; P<0.001). The mice were monitored for 5 weeks post challenge, survivors culled and spleens aseptically removed. Colonies showing typical morphology to *B. pseudomallei* were recovered from a spleen from one out of three surviving mice, with a bacterial burden of <3 x 10^2 cfu/ml. Therefore, deletion of BPSS1823 significantly attenuated *B. pseudomallei* in mice, but low levels of viable bacteria were isolated from one mouse.

Discussion

Previous studies have shown that Mips are important virulence determinants in several intracellular pathogens (7, 20, 26, 30, 33). Despite the importance of Mip for bacterial pathogenesis, little is known about its specific role or intracellular target. In this study we describe the identification of a Mip-like protein from *B. pseudomallei,* which is important for virulence. In addition, we show for the first time that a functional Mip is important for enabling a more diverse range of virulence associated functions than previously reported for other Mips, including bacterial motility, protease production and acid tolerance.

Lp-Mip is a dimeric, outer membrane lipoprotein, containing an N-terminal dimerisation and chaperone domain, and a C-terminal PPIase domain (36). BPSS1823 shows significant sequence identity (> 40 %) to the Lp-Mip PPIase domain. Three-dimensional modelling of BPSS1823 indicated that the structure is highly conserved (Figure 1, B) and that all of the amino acids that are believed to contribute most significantly to enzyme activity are present (3, 7, 21, 29) These observations were confirmed by NMR and X-ray determination of the structure of BPSS1823 (Norville *et al.,* in preparation). These observations strongly suggest that BPSS1823 is a functional orthologue of Mip.

The k_{cat}/K_m of Lp-Mip is reported as 1.2 x 10^6, (25). We have shown that recombinant BPSS1823 exhibits PPIase activity which is >5 fold higher than that of Lp-Mip (k_{cat}/K_m = 6.7 ± 0.4 x 10^6 M^{-1} s^{-1}). As the same substrate was used to analyse PPIase activity in both cases (Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide), this observation is unlikely to be due to a difference in substrate specificity, instead this data may indicate at the importance of PPIase activity for the function of BPSS1823. Furthermore, this enzyme activity is inhibitable by rapamycin confirming that BPSS1823 belongs to the FKBP family of PPIases. Previous studies have questioned the importance of PPIase activity of Lp-Mip because its variants

showing a strongly reduced PPIase activity could complement *L. pneumophila* strains for intracellular survival in U937 cells and *A. castellani* (43). However, subsequent work on a parvulin-like PPIase indicated that vanishingly low levels of enzyme activity might suffice to ensure protection against loss of PPIase function (16). Consequently, targeting the PPIase domain of Lp-Mip with activity-neutralizing monoclonal antibodies inhibited *Legionella* infection of cells and FK506 or rapamycin inhibited transmigration of *L. pneumophila* across NCI-H292 lung epithelial cells (19, 42). In addition, removal of the PPIase domain of Lp-Mip attenuated virulence in guinea pig model of infection (25). The importance of PPIase activity for Mip-associated virulence and the availability of licensed PPIase inhibitors suggest that Mips represent novel antimicrobial targets for therapeutics (3). Further work to establish the role of PPIase activity in BPSS1823 function is required.

Inactivation of Lp-Mip resulted in reduced replication within macrophages and protozoa and attenuated virulence in a guinea pig model of infection (7, 8, 9). We report that the deletion of *BPSS1823* in *B. pseudomallei* results in reduced intracellular survival within eukaryotic cells and significant attenuation in a BALB/c mouse model of infection. The defects in intracellular survival may be partially explained by the observation that the Δ*BPSS1823* mutant was more sensitive to low pH conditions. Following bacterial infection of host cells, the phagosome acidifies to between pH 4 – 5 (11). Therefore, BPSS1823 may act on a protein that protects against acid stress, providing resistance to intracellular host killing mechanisms. In addition, deletion of *BPSS1823* resulted in reduced swarming motility and protease production. Previous studies report that flagella from *B. pseudomallei* is involved in invasion of cell lines and virulence in a BALB/c mouse model (6, 10, 22). Secreted proteases have also been shown to be important for *B. pseudomallei* pathogenesis in a rat model of lung infection, but not in a SWISS mouse model (15, 39). It may be hypothesised that BPSS1823 is acting to fold or export proteins required for

formation of the flagella complex or production of extracellular proteases. While deletion of BPSS1823 did not render *B. pseudomallei* avirulent, this can be explained by our *in vitro* data which indicates that the mutant exhibits defective rather than abolished infection of cells and virulence mechanisms. Therefore, it could be suggested that BPSS1823 is required for acute infection in BALB/c mice.

We have shown that *BPSS1823* encodes a Mip-like protein in *B. pseudomallei* which modulates a broader range of virulence-associated phenotypes than previously reported with other bacterial Mips. While the exact mechanism by which BPSS1823 functions remains unclear, the pleiotropic effects on virulence provide novel insights into the role of Mips in general. In addition, the identification of a Mip-like protein in *B. pseudomallei* has potential as a target for development of novel antimicrobial to treat melioidosis.

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Table 1 Bacterial strains used in this study

Strain	Description	Reference/source
E. coli BL21 (DE3)	BL21 with a λ DE3 lysogen	Invitrogen
E. coli S17-1 λpir	S17-1 with a λ prophage carrying the *pir* gene	35
E. coli HB101 (pRK2013)	HB101 containing pRK2013 Km^R	13

B. pseudomallei AI	K96243 derivative; unmarked deletion Δ*amrA;* Gm^S	Dr S. Harding, Dstl
B. pseudomallei AI Δ*BPSS1823*	K96243 derivative; unmarked deletion Δ*amrA ΔBPSS1823*; Gm^S	This study
B. pseudomallei Δ*BPSS1823 (PBBR-1823)*	K96243 derivative; unmarked deletion Δ*amrA BPSS1823*::pBBR1Mip; Gm^S Km^R	This study

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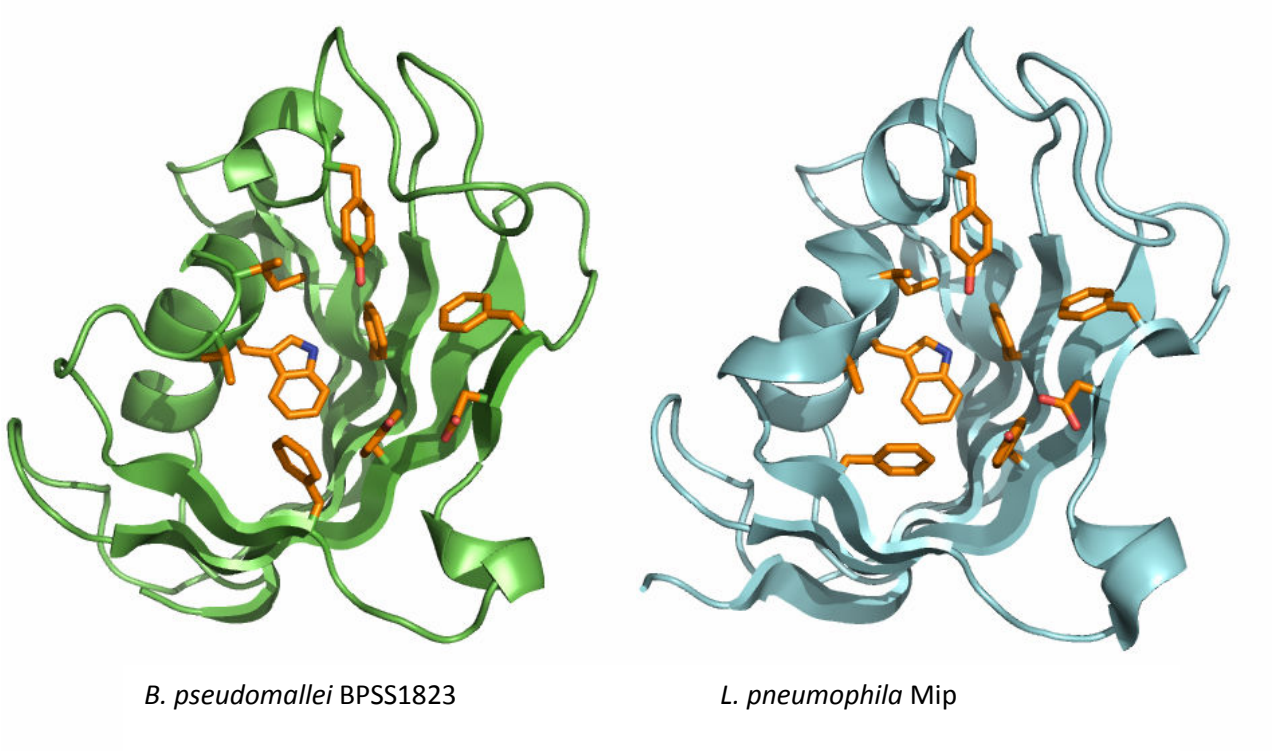
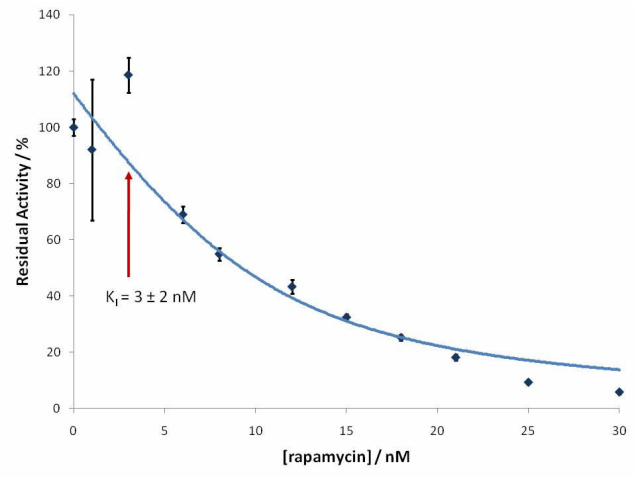
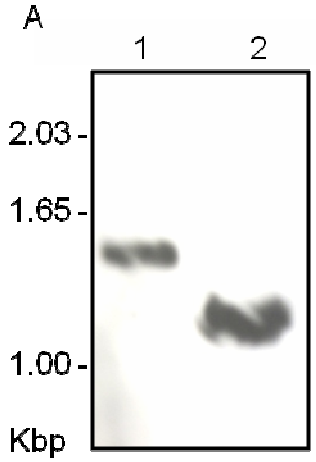
A

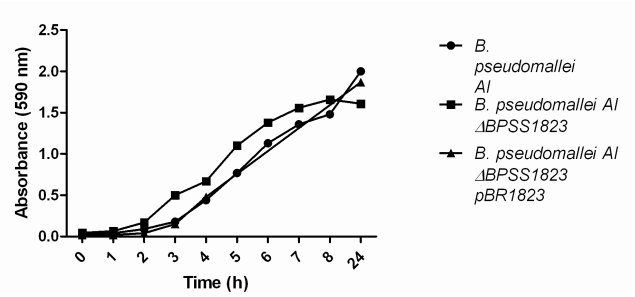


B. pseudomallei BPSS1823 *L. pneumophila* Mip

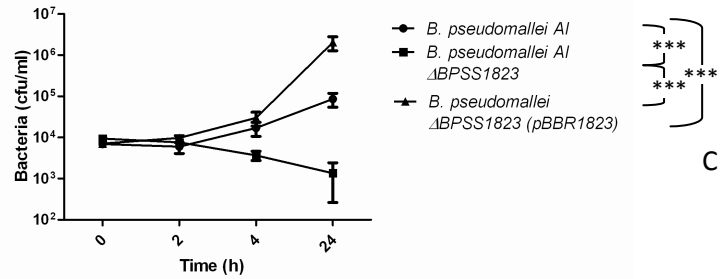
B


Residual Activity / %
[rapamycin] / nM
K_i = 3 ± 2 nM
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30

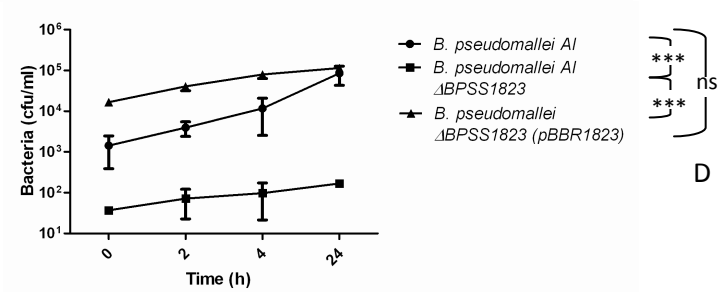

A
1
2
2.03
1.65
1.00
Kbp


Absorbance (590 nm)
Time (h)
B. pseudomallei AI
B. pseudomallei AI ΔBPSS1823
B. pseudomallei AI ΔBPSS1823 pBR1823

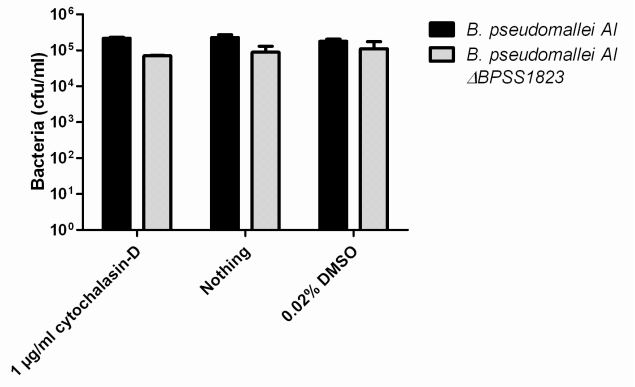
B


Bacteria (cfu/ml)
Time (h)
B. pseudomallei AI
B. pseudomallei AI ΔBPSS1823
B. pseudomallei ΔBPSS1823 (pBBR1823)
***
***
***

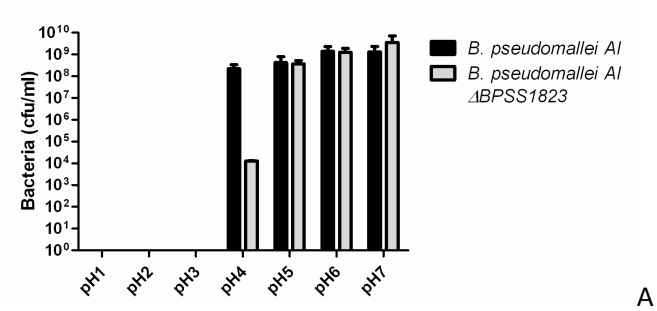
C


Bacteria (cfu/ml)
Time (h)
B. pseudomallei AI
B. pseudomallei AI ΔBPSS1823
B. pseudomallei ΔBPSS1823 (pBBR1823)
***
ns
***

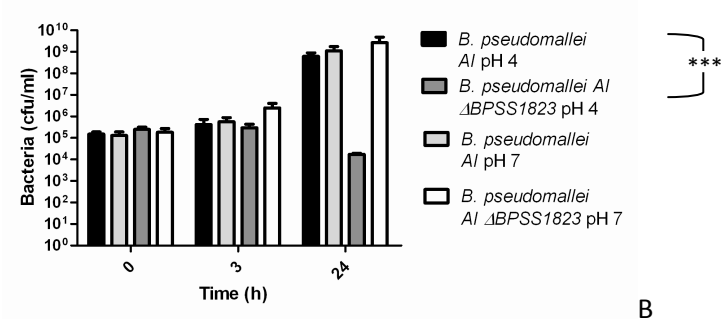
D


Bacteria (cfu/ml)
B. pseudomallei AI
B. pseudomallei AI ΔBPSS1823
1 µg/ml cytochalasin-D
Nothing
0.02% DMSO

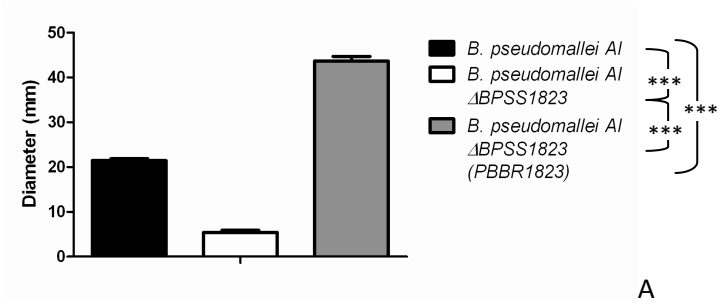
E


Bacteria (cfu/ml)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
pH1
pH2
pH3
pH4
pH5
pH6
pH7
B. pseudomallei AI
B. pseudomallei AI ΔBPSS1823

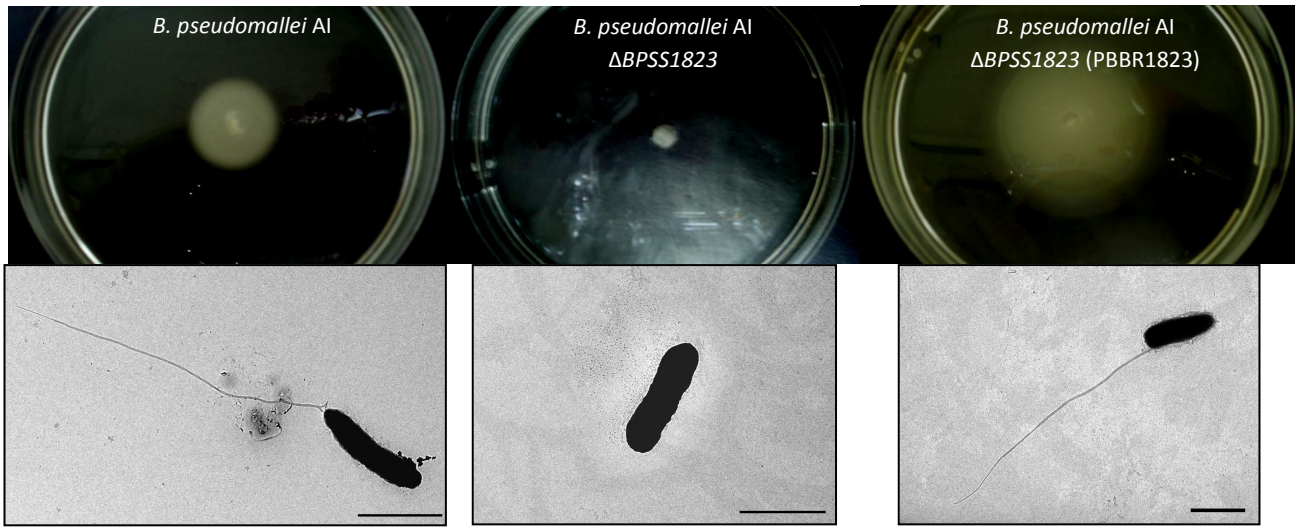
A


Bacteria (cfu/ml)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
10^{0}
0
3
24
Time (h)
B. pseudomallei AI pH 4
B. pseudomallei AI ΔBPSS1823 pH 4
B. pseudomallei AI pH 7
B. pseudomallei AI ΔBPSS1823 pH 7
***

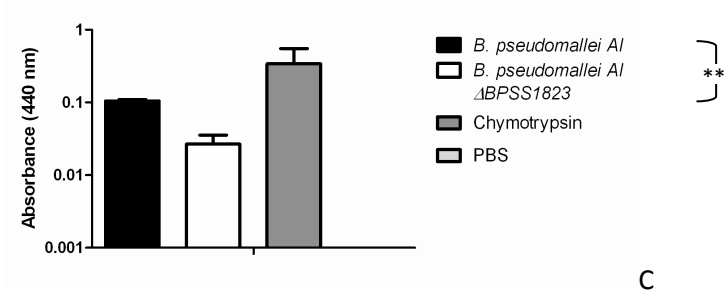
B


Diameter (mm)
50
40
30
20
10
0
B. pseudomallei AI
B. pseudomallei AI ΔBPSS1823
B. pseudomallei AI ΔBPSS1823 (PBBR1823)
***
***
***

A


B. pseudomallei AI
B. pseudomallei AI ΔBPSS1823
B. pseudomallei AI ΔBPSS1823 (PBBR1823)

B


Absorbance (440 nm)
1
0.1
0.01
0.001
B. pseudomallei AI
B. pseudomallei AI ΔBPSS1823
Chymotrypsin
PBS
**

C

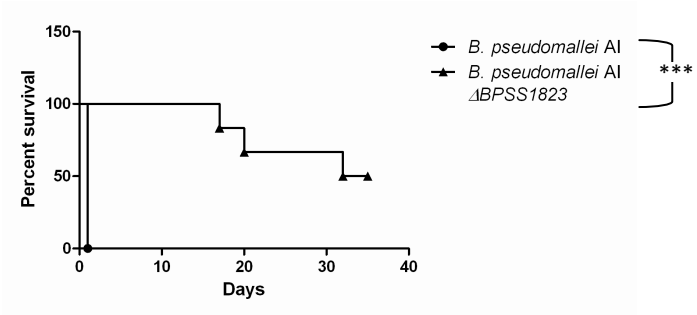

150
100
50
0
Percent survival
0
10
20
30
40
Days
B. pseudomallei AI
B. pseudomallei AI ΔBPSS1823
***

Figure Legends

Figure 1. BPSS1823 encodes a Mip-like protein

(A) Sequence alignment of BPSS1823, *L. pneumophila* (Lp), *T. cruzi* (Tc) and *N. gonorrhoeae* (Ng) Mips. Identical amino acids are shaded in grey. The PPIase domain of Lp-Mip is boxed in black. Residues shown to be required for PPIase activity in hFKBP12 are highlighted by asterisks. (B) Overview of the modelled structure of BPSS1823 (green), in comparison with *L. pneumophila* Mip (structure 1FD9, cyan). The nine most conserved amino acids in the active site are shown in orange.

Figure 2. Inhibition of PPIase activity of recombinant BPSS1823 by rapamycin

Increasing concentrations of rapamycin lead to dose-dependent inhibition of PPIase activity. The predicted inhibition curve for the fit data is shown. A K_I of 3 nM was calculated.

Figure 3. Intracellular survival kinetics of *B. pseudomallei* AI, *B. pseudomallei* AI Δ*BPSS1823* and *B. pseudomallei* AI Δ*BPSS1823 (PBBR-1823*)

(A) Southern hybridization of *B. pseudomallei* genomic DNA using a BPSS1823-specific DNA probe. Lane 1 – wildtype genomic DNA digested with *BamHI* and *ClaI* (1.55 kbp); 2 – mutant genomic DNA digested with *BamHI* and *ClaI* (1.38 kbp). (B) Growth of bacteria in neutral LB broth. Values are from a single experiment. (C) Intracellular survival in J774 macrophage-like cells (MOI 1). (D) Intracellular survival in A549 epithelial cells (MOI 10). (E) Adhesion to A549 epithelial cells. Values are the means from triplicate experiments $\pm$ standard errors. P values are shown for the comparison of intracellular bacteria at 24 h post infection.

Figure 4. Growth of *B. pseudomallei* AI and *B. pseudomallei* AI ∆*BPSS1823* at different pH

(A) Bacteria grown in media adjusted to pH 1-7 for 24 h. Values are the means from duplicate experiments ± standard errors. (B) Bacteria grown in pH 4 or 7 at 0, 3 and 24 h post inoculation. Values are the means from triplicate experiments ± standard errors. P values are shown for the comparison of intracellular bacteria at 24 h post infection.

Figure 5. Swarming motility and protease production of *B. pseudomallei* AI and *B. pseudomallei* AI ∆*BPSS1823*

(A) Diameter measurements of bacterial spread through 0.3% agar (B) Photographs of bacterial spread through 0.3% agar and representative electron micrographs showing flagella, scale bar = 2 µm. (C) Protease activity of bacteria using azocasein as a substrate. Values are the means from triplicate experiments ± standard errors. P values are shown for the comparison of strains.

Figure 6 *B. pseudomallei* AI ∆*BPSS1823* is significantly attenuated in a BALB/c mouse model of infection

Intraperitoneal infection of BALB/c mice (n=6) with 6.2 x 10^6 cfu *B. pseudomallei* AI or 2.5 x 10^6 cfu *B. pseudomallei* AI ∆*BPSS1823.*