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A *Burkholderia pseudomallei* Macrophage Infectivity Potentiator-Like Protein Has Rapamycin-Inhibitable Peptidylprolyl Isomerase Activity and Pleiotropic Effects on Virulence▽

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Macrophage infectivity potentiators (Mips) are a group of virulence factors encoded by pathogenic bacteria such as *Legionella*, *Chlamydia*, and *Neisseria* species. Mips are part of the FK506-binding protein (FKBP) family, whose members typically exhibit peptidylprolyl *cis-trans* isomerase (PPIase) activity which is inhibitable by the immunosuppressants FK506 and rapamycin. Here we describe the identification and characterization 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.

Burkholderia pseudomallei is a motile, Gram-negative bacillus and the causative agent of the disease melioidosis. Melioidosis is endemic in Southeast Asia and Northern Australia. Infection typically occurs by inoculation of the organism through skin lesions, but infection by inhalation or ingestion of the organism has also been reported (10). Clinical presentation of melioidosis in humans varies from disseminated acute septicemia to localized 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 U.S. Centers for Disease Control and Prevention (37). There is currently no vaccine available for prophylaxis, and intrinsic antibiotic resistance makes treatment regimens 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 (17). However, the mechanisms used by *B. pseudomallei* to avoid clearance are largely unknown.

FK506-binding proteins (FkBPs) are ubiquitous in eukaryotes and prokaryotes; they typically possess peptidylprolyl *cis-trans* isomerase (PPIase) activity and catalyze the folding of proline-containing proteins. PPIase activity is inhibitable upon binding to the immunosuppressants FK506 and 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*, which 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 is 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 37°C 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; and gentamicin, 10 μg/ml to 30 μg/ml.

Construction of an expression plasmid for production of recombinant BPSS1823. The open reading frame *BPSS1823* was amplified by PCR using *B. pseudomallei* strain K96243 genomic DNA as a template and the primers pET.F (CATATGACAGTCGTCACCACC) and pET.R (GGATCCTCAGACGTCGAGCAGTTC). The PCR product was inserted into the NdeI/BamHI site of the pET15b expression plasmid (Novagen). The construct was transformed into

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TABLE 1. Bacterial strains used in this study

Species and strain	Description	Reference or source
E. coli		
BL21(DE3)	BL21 with a λ DE3 lysogen	Invitrogen
S17-1 λpir	S17-1 with a λ prophage carrying the *pir* gene	35
HB101(pRK2013)	HB101 containing pRK2013 Km^r	13
B. pseudomallei		
AI	K96243 derivative; unmarked deletion Δ*amrA*; Gm^s	S. Harding, Dstl
AI Δ*BPSS1823*	K96243 derivative; unmarked deletion Δ*amrA* Δ*BPSS1823*; Gm^s	This study
Δ*BPSS1823* (*PBBR-1823*)	K96243 derivative; unmarked deletion Δ*amrA* Δ*BPSS1823*::pBBR1Mip; Gm^s Km^r	This study

Escherichia 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) harboring the expression construct was used to inoculate 2 liters of LB broth. This was incubated at 37°C with agitation until the absorbance reached 0.4 to 0.6 at 600 nm. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and growth continued at 20°C with agitation for 4 h. Cells were harvested by centrifugation at 8,000 × *g* for 15 min at 4°C and then disrupted by sonication. Cell debris was pelleted by centrifugation at 8,000 × *g* for 30 min at 4°C. The supernatant was loaded onto a 1-ml Histrap FF column (GE Healthcare) 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). The protein concentration was determined using a bicinchoninic acid assay (Pierce Biotechnology). Imidazole was removed from the purified protein by dialysis against 10 mM phosphate-buffered saline (PBS) and samples frozen at −80°C until use.

Peptidylprolyl isomerase assay. The peptidylprolyl *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 10°C in 1.2 ml 35 mM 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/visible spectrophotometer at 1-s intervals until there was no further change in absorbance. For inhibition measurements, recombinant BPSS1823 protein was preincubated with various concentrations of rapamycin from 30 nM to 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 the equation $\ln (A_\infty - A_t) = -k_{obs}t + \ln (A_\infty - A_0)$; data from 10 to 50 s (which were always after the lag phase and before substrate became limiting) were taken, and k_{obs} was calculated by linear regression. The enzymatic rate was determined by comparing the observed rate to the uncatalyzed rate using the equation $k_{enz} = k_{obs} - k_{uncat}$. The specificity constant k_{cat}/K_m for the enzyme was calculated using the equation $(k_{cat}/K_m) = (k_{enz}/[\text{PPIase}])$ (18); data were taken using 1 nM, 5 nM, and 10 nM BPSS1823 and were fit using linear regression. Data for inhibitor assays were fit to the equation $v = v_0 \frac{[E] - [I] - K + \sqrt{([E] - [I] - K)^2 + 4[E][K]}}{2[E]}$ (43) using least-squares nonlinear fitting. v_0 and K_I^{app} were fit using initial estimates based on the raw data, and [E] was kept constant.

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/AGATCTGCTCGAATCG AACTTCTG) and RFF/RFR (AGATCTCTCGTGTTCGAAGTCGAA/TCTA GACCAGTTGGCTGTTGTCGG). 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 chloramphenicol sensitivity and analyzed by PCR. Southern hybridization, using a digoxigenin (DIG)-labeled upstream flanking region to probe, was used to confirm a 171-bp deletion of *BPSS1823*, and the strain was termed *B. pseudomallei* AI Δ*BPSS1823*.

Complementation studies. The open reading frame *BPSS1823* was amplified by PCR using *B. pseudomallei* strain K96243 genomic DNA as a template and the primers PBBR.F (GAATTCATGACAGTCGTCACCACC) and PBBR.R (TC TAGATCAGACGTCGAGCAGTTC). The PCR product was inserted into the EcoRI/XbaI restriction sites of pBBR1-MCS2. The complementation construct was transformed into *E. coli* S17 λ*pir* 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 4×10^5 cells/ml in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% L-glutamine and 10% fetal calf serum and incubated at 37°C with 5% CO_2 for approximately 16 h. *B. pseudomallei* strains were grown at 37°C overnight then adjusted in Leibovitz L-15 medium with 10% fetal calf serum to an absorbance of 0.35 to 0.4 at 590 nm. Bacteria were serially diluted in L-15 medium, and 1 ml was added to the cells at a multiplicity of infection (MOI) of 1 or 10 and incubated at 37°C 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 inoculum. Bacteria were removed, and infected cells incubated with L-15 containing 30 μg/ml gentamicin for 30 min at 37°C. Antibiotic medium 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 postinfection, cells were lysed with 1 ml distilled water (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 described above. Cytochalasin D (Sigma) was added to approximately 1×10^6 cells at a final concentration of 1 μg/ml and incubated at 37°C with 5% CO_2 for 30 min. Cytochalasin D was added to approximately 1×10^7 CFU/ml bacteria at a final concentration of 1 μg/ml. One milliliter of treated bacteria was added to the pretreated cells at an MOI of 1:10 and incubated at 37°C for 1 h. The cells were then washed 3 times with warm PBS to remove nonadherent bacteria. Cells were lysed with 1 ml dH_2O, serially diluted in PBS, plated onto LB agar, and incubated at 37°C overnight. Cytochalasin D was present throughout the assay.

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

Motility assay. *B. pseudomallei* strains were grown at 37°C overnight. One microliter of overnight culture was stabbed into 0.3% motility agar using a sterile inoculating loop and the plates incubated at 37°C overnight. Bacterial spread was measured using a Scienceware vernier caliper (Sigma).

Electron microscopy. *B. pseudomallei* strains were grown at 37°C overnight. Two milliliters of culture was pelleted at 15,000 × *g* for 5 min. Samples were fixed in 4% formalin for 24 h. Samples were stained with 2% (wt/vol) uranyl acetate and examined in an FEI CM12 transmission electron microscope operating at 80 kV, and images were captured using a 1MP Keenview digital camera.

Protease assay. An overnight culture of *B. pseudomallei* was diluted 1:50 and grown at 37°C. Following 24 h of growth, 1 ml bacterial culture was removed and

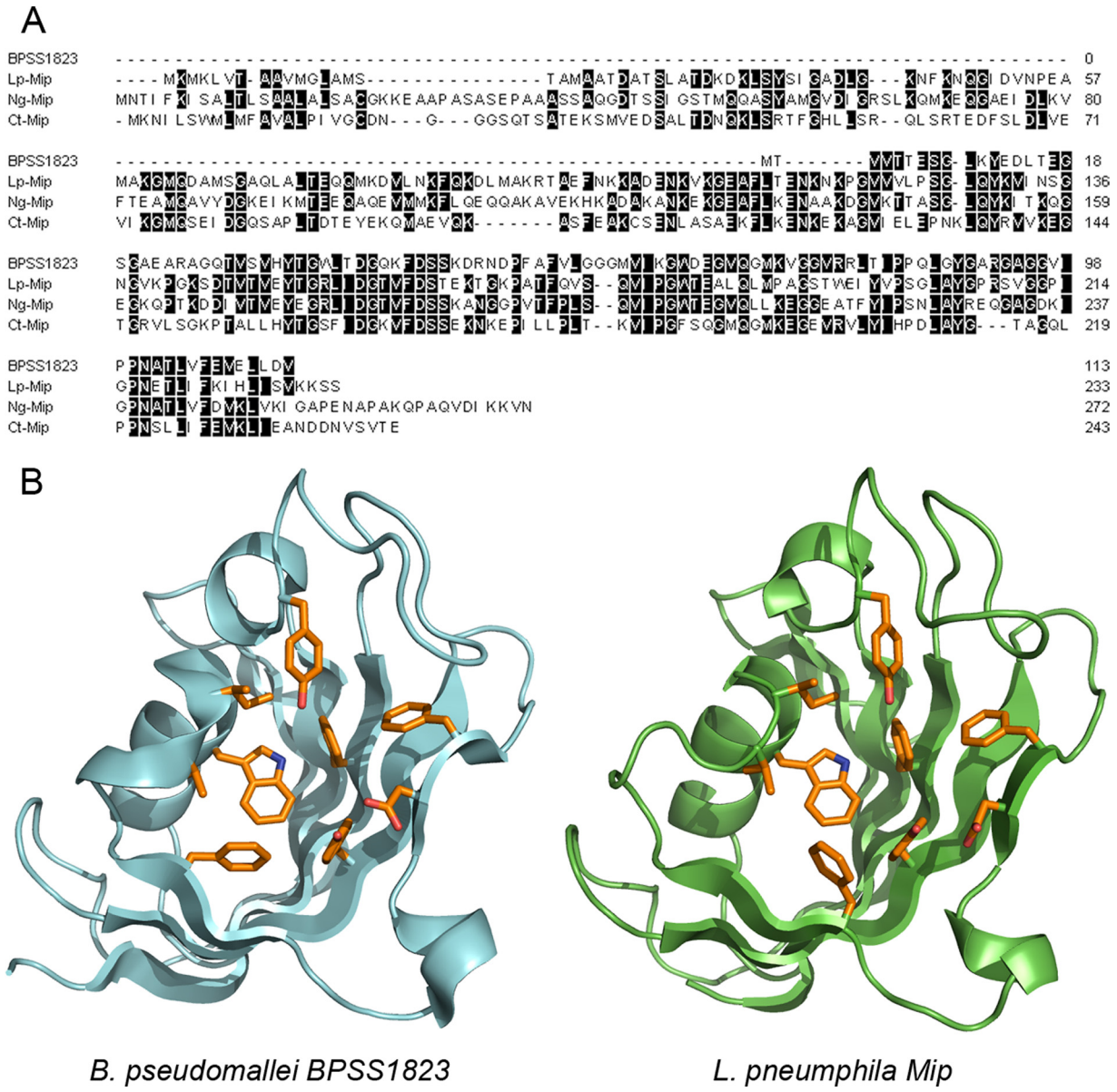

FIG. 1. *BPSS1823* encodes a Mip-like protein. (A) Sequence alignment of BPSS1823 and *L. pneumophila* (Lp), *T. cruzi* (Tc), and *N. gonorrhoeae* (Ng) Mips. Identical amino acids are shaded in gray. (B) Overview of the modeled structure of BPSS1823 (green) in comparison with *L. pneumophila* Mip (structure 1FD9, cyan). The nine most conserved amino acids in the active site are shown in orange.

pelleted at 15,000 × *g* for 5 min. One hundred microliters of supernatant was added to 100 μl azocasein (5 mg/ml; Sigma) and incubated at 37°C for 1 h. The reaction was stopped with 10% trichloroacetic acid (Sigma) and nonhydrolyzed azocasesin pelleted at 10,000 × *g* for 15 min. The supernatant was added to 500 mM NaOH and read using a WPA Colorwave colorimeter (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 12-h 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 6.3×10^6 CFU of *B. pseudomallei* AI or 2.5×10^6 CFU of *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.

Modeling of BPSS1823. The structure of BPSS1823 protein was modeled using MODELLER version 9.8 (12). Three structures (1FD9, 1FKB, and 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). Ten 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 was judged by RAMPAGE (28).

Statistical analysis. For intracellular infection and pH exposure assays, a two-way analysis of variance (ANOVA) and Bonferroni's posttest were used to determine statistical significance between groups. For motility assays, a one-way

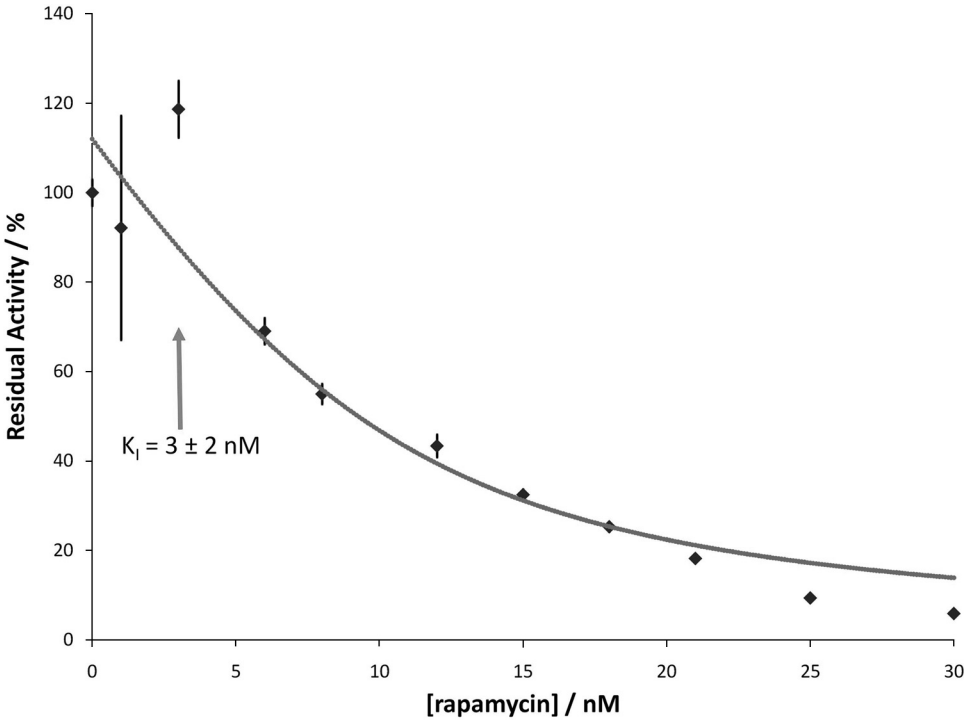

FIG. 2. Inhibition of PPIase activity of recombinant BPSS1823 by rapamycin. Increasing concentrations of rapamycin lead to dose-dependent inhibition of PPIase activity. The predicted inhibition curve for the fit data is shown. A K_I of 3 nM was calculated.

ANOVA and Bonferroni's multiple-comparison test were used. For protease assays, an unpaired Student *t* test was used. Survival curves were compared using a log rank (Mantel-Cox) test. Significances are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. 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 peptidylprolyl *cis-trans* isomerase (http://www.sanger.ac.uk/Projects/B_pseudomallei/). BLAST searches against a nonredundant 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, *Neisseria gonorrhoeae* Mip, and *Chlamydia trachomatis* Mip, respectively (Fig. 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 that 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 modeled the structure of the protein (Fig. 1B). This model predicts that, like *L. pneumophila* Mip, BPSS1823 adopts a classical FKBP fold and has an active site that strikingly resembles the *L. pneumophila* Mip active site. In addition, all of the residues that are highly conserved in the active site of FKBPs appear to be 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 role similar to that of the Mips in other organisms.

The *B. pseudomallei* Mip-like protein exhibits rapamycin-inhibitable PPIase activity. Purified recombinant His-tagged BPSS1823 protein had a molecular mass, determined by mass spectrometry, of 14,436 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* isomerization 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 $6.7 \times 10^6 \pm 0.4 \times 10^6\ M^{-1}\ s^{-1}$. 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 (Fig. 2).

BPSS1823 is required for intracellular survival or replication 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 (Fig. 3A). The parent strain or the Δ*BPSS1823* mutant strain was used to infect phagocytic (J774A.1) or nonphagocytic (A549) cells. In J774A.1 macrophages, the numbers of parent bacteria or Δ*BPSS1823* mutant bacteria recovered 1 h after infection were

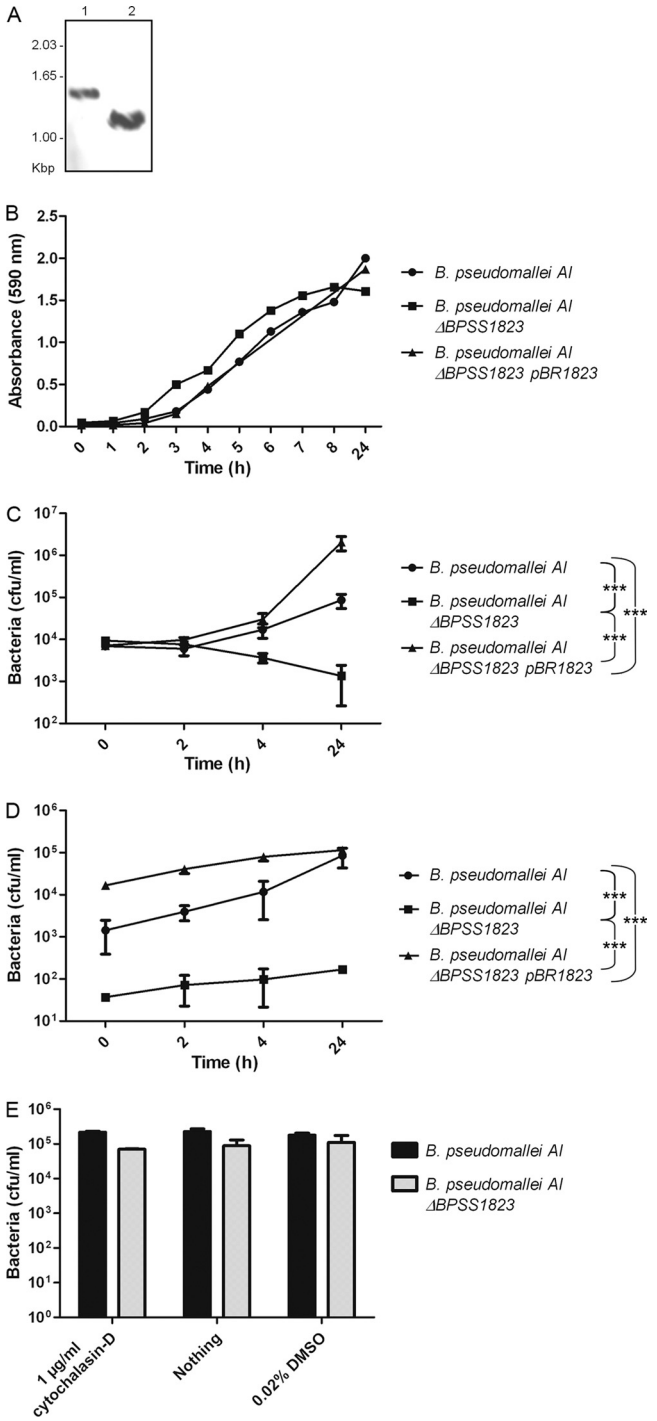

FIG. 3. Intracellular survival kinetics of *B. pseudomallei* AI, *B. pseudomallei* AI Δ*BPSS1823*, and *B. pseudomallei* AI Δ*BPSS1823* (*PBBR-1823*). (A) Southern hybridization of *B. pseudomallei* genomic DNA using a BPSS1823-specific DNA probe. Lane 1, wild-type genomic DNA digested with BamHI and ClaI (1.55 kbp); lane 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 of 1). (D) Intracellular survival in A549 epithelial cells (MOI of 10). (E) Adhesion to A549 epithelial cells. Values are the means from triplicate experiments ± standard errors. *P* values are shown for the comparison of intracellular bacteria at 24 h postinfection.

similar. However, significantly fewer Δ*BPSS1823* mutant bacteria were recovered from cells at 24 h postinfection (Fig. 3C) ($P < 0.001$). 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 (Fig. 3D) ($P < 0.01$). 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 (Fig. 3D) ($P < 0.001$). Reintroduction of the wild-type gene in *trans* fully restored the ability of the Δ*BPSS1823* mutant to survive and grow within both cell lines (Fig. 3C and D) ($P < 0.001$), confirming that the defect was specific to BPSS1823 *in vitro* and not due to polar effects. Furthermore, the Δ*BPSS1823* mutant did not exhibit reduced growth in LB broth at neutral pH (Fig. 3B) or increased sensitivity to gentamicin (data not shown).

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

BPSS1823 is involved in resistance of *B. pseudomallei* to low pH. To further characterize 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 to 10^9 CFU/ml in LB medium adjusted to pH 4, 5, 6, or 7, the growth of the Δ*BPSS1823* mutant was significantly reduced by 24 h of growth at pH 4 (Fig. 4) ($P < 0.001$). Neither the parent nor the Δ*BPSS1823* mutant was able to grow in medium at pH 3 or below (Fig. 4A).

Deletion of *BPSS1823* renders *B. pseudomallei* immotile and reduces protease production. PPIases have been shown to assist in the 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 localized growth of 5.4 mm at the site of inoculation and significantly less bacterial spread (Fig. 5A and B) ($P < 0.001$). In addition, unlike *B. pseudomallei* AI, the Δ*BPSS1823* mutant did not produce flagella (Fig. 5B). Complementation of the Δ*BPSS1823* mutant strain fully restored bacterial motility and flagellum formation, resulting in significantly increased bacterial spread compared to that of both *B. pseudomallei* AI Δ*BPSS1823* and *B. pseudomallei* AI (Fig. 5A and 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 (Fig. 5C) ($P < 0.01$). This indicates that BPSS1823 is required for production of putative virulence mechanisms in *B. pseudomallei*, such as swarming motility and protease production.

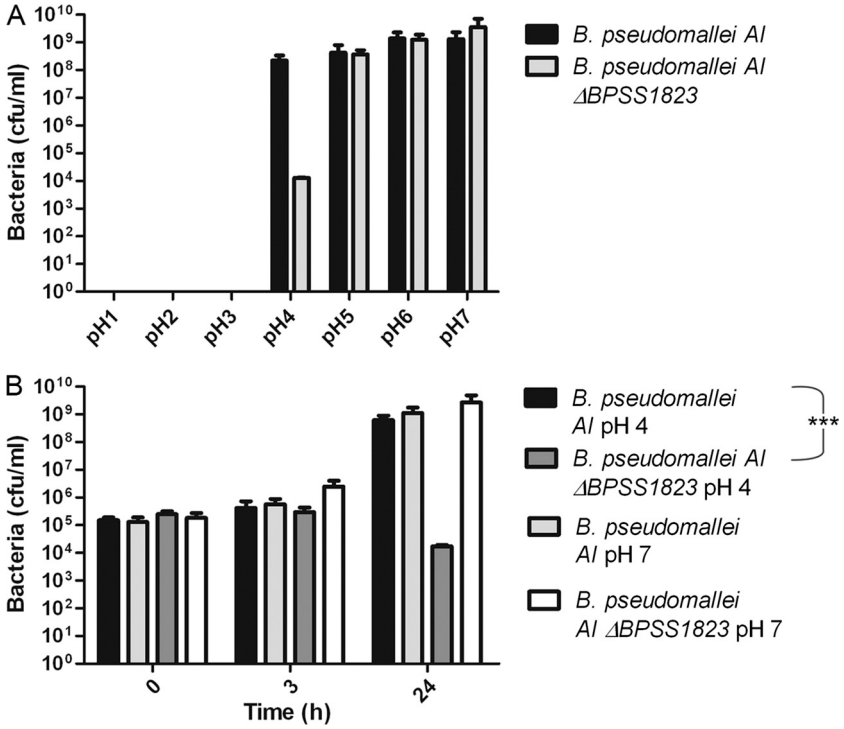

FIG. 4. Growth of *B. pseudomallei* AI and *B. pseudomallei* AI Δ*BPSS1823* at different pHs. (A) Bacteria grown in media adjusted to pH 1 to 7 for 24 h. Values are the means from duplicate experiments ± standard errors. (B) Bacteria grown at pH 4 or 7 at 0, 3, and 24 h postinoculation. Values are the means from triplicate experiments ± standard errors. *P* values are shown for the comparison of intracellular bacteria at 24 h postinfection.

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 6.2×10^6 CFU *B. pseudomallei* AI or 2.5×10^6 CFU *B. pseudomallei* AI Δ*BPSS1823*. All mice challenged with *B. pseudomallei* AI had succumbed to infection by 1 day postchallenge. In contrast, animals challenged with *B. pseudomallei* AI Δ*BPSS1823* had significantly increased survival, with a mean time to death of >35 days (Fig. 6) ($P < 0.001$). The mice were monitored for 5 weeks postchallenge, survivors culled, and spleens aseptically removed. Colonies showing morphology typical of *B. pseudomallei* were recovered from a spleen from one out of three surviving mice, with a bacterial burden of $<3 \times 10^2$ 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.

L. pneumophila Mip is a dimeric outer membrane lipoprotein containing an N-terminal dimerization and chaperone domain and a C-terminal PPIase domain (36). BPSS1823 shows significant sequence identity (>40%) to the *L. pneumophila* Mip PPIase domain. Three-dimensional modeling of BPSS1823 indicated that the structure is highly conserved (Fig. 1B) 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 nuclear magnetic resonance (NMR) and X-ray determination of the structure of BPSS1823 (34a). These observations strongly suggest that BPSS1823 is a functional orthologue of Mip.

The k_{cat}/K_m of *L. pneumophila* Mip is reported to be 1.2×10^6, (25). We have shown that recombinant BPSS1823 exhibits PPIase activity which is >5-fold higher than that of *L. pneumophila* Mip ($k_{cat}/K_m = 6.7 \times 10^6 \pm 0.4 \times 10^6$ M^{-1} s^{-1}). As the same substrate (Suc-Ala-Phe-Pro-Phe-*p*-nitroanilide) was used to analyze PPIase activity in both cases, this observation is unlikely to be due to a difference in substrate specificity; instead, these 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 *L. pneumophila* Mip because its variants showing a strongly reduced PPIase activity could complement *L. pneumophila* strains for intracellular survival in U937 cells and *Acantham-*

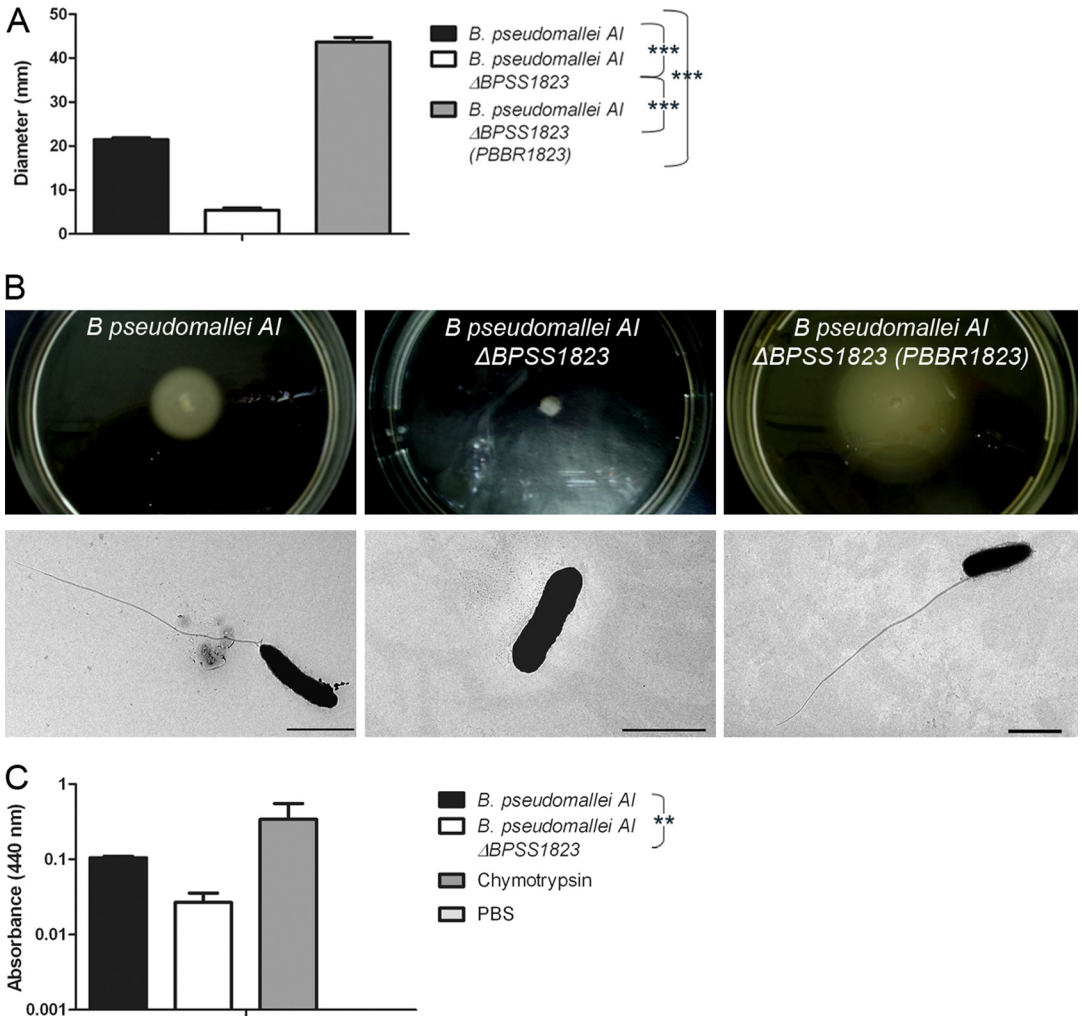

FIG. 5. Swarming motility and protease production of *B. pseudomallei* AI and *B. pseudomallei* AI Δ*BPSS1823.* (A) Diameters of bacterial spread through 0.3% agar. (B) Photographs of bacterial spread through 0.3% agar and representative electron micrographs showing flagella. Scale bar, 2 μm. (C) Protease 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.

oeba castellani (44). 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 *L. pneumophila* Mip with activity-neutralizing monoclonal antibodies inhibited *Legionella* infection of cells, and FK506 or rapamycin inhibited transmigration of *L. pneumophila* across NCl-H292 lung epithelial cells (19, 42). In addition, removal of the PPIase domain of *L. pneumophila* 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's role in virulence is required.

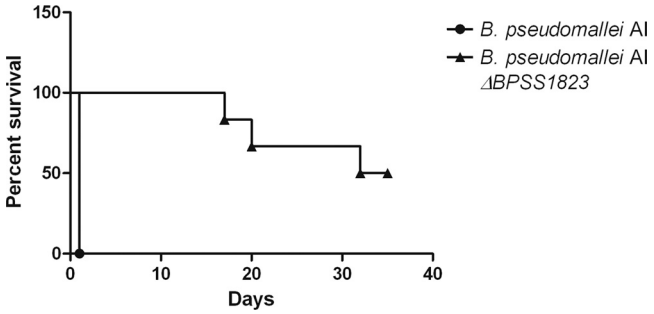

FIG. 6. *B. pseudomallei* AI Δ*BPSS1823* is significantly attenuated in a BALB/c mouse model of infection. Intraperitoneal infection of BALB/c mice ($n = 6$) with 6.2×10^6 CFU *B. pseudomallei* AI or 2.5×10^6 CFU *B. pseudomallei* AI Δ*BPSS1823* is shown.

Inactivation of *L. pneumophila* 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 or replication within eukaryotic cells and significant attenuation in a BALB/c mouse model of infection. Although intracellular growth of the mutant strain was fully restored *in vitro* by complementation (Fig. 3C and D), care must be taken when extrapolating these data to *in vivo*

studies. The defects in intracellular growth 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 and 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 have reported that flagella from *B. pseudomallei* are involved in invasion of cell lines and virulence in a BALB/c mouse model (5, 6, 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 hypothesized that BPSS1823 acts to fold or export proteins required for formation of the flagellar 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 indicate that the mutant exhibits defective rather than a lack of infection of cells and virulence mechanisms. Therefore, it could be suggested that BPSS1823 is required for acute infection in BALB/c mice. Additional studies are required to validate the association between BPSS1823 and *B. pseudomallei* virulence, in particular the contribution of PPIase activity to the protein function.

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* indicates its potential as a target for development of novel antimicrobials to treat melioidosis.

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REFERENCES

1. **Adler, N. R. L., et al.** 2009. The molecular and cellular basis of pathogenesis in melioidosis: how does *Burkholderia pseudomallei* cause disease? FEMS Microbiol. Rev. **33:**1079–1099.
2. **Brock, F. M., C. W. Forsberg, and J. G. Buchanansmith.** 1982. Proteolytic activity of rumen microorganisms and effects of proteinase-inhibitors. Appl. Environ. Microbiol. **44:**561–569.
3. **Ceymann, A., et al.** 2008. Solution structure of the *Legionella pneumophila* Mip-rapamycin complex. BMC Struct. Biol. **8:**17.
4. **Cheng, A. C., and B. J. Currie.** 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. **18:**383–416.
5. **Chua, K. L., Y. Y. Chan, and Y. H. Gan.** 2003. Flagella are virulence determinants of *Burkholderia pseudomallei*. Infect. Immun. **71:**1622–1629.
6. **Chuaygud, T., S. Tungpradabkul, S. Sirisinha, K. L. Chua, and P. Utaisincharoen.** 2008. A role of *Burkholderia pseudomallei* flagella as a virulent factor. Trans. R. Soc. Trop. Med. Hyg. **102**(Suppl. 1)**:**S140–S144.
7. **Cianciotto, N. P., B. I. Eisenstein, C. H. Mody, G. B. Toews, and N. C. Engleberg.** 1989. A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. Infect. Immun. **57:**1255–1262.
8. **Cianciotto, N. P., B. I. Eisenstein, C. H. Mody, and N. C. Engleberg.** 1990. A mutation in the *mip* gene results in an attenuation of *Legionella pneumophila* virulence. J. Infect. Dis. **162:**121–126.
9. **Cianciotto, N. P., and B. S. Fields.** 1992. *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. Proc. Natl. Acad. Sci. U. S. A. **89:**5188–5191.
10. **Currie, B. J., and S. P. Jacups.** 2003. Intensity of rainfall and severity of melioidosis, Australia. Emerg. Infect. Dis. **9:**1538–1542.
11. **Downey, G. P., et al.** 1999. Phagosomal maturation, acidification, and inhibition of bacterial growth in nonphagocytic cells transfected with Fc gamma RIIA receptors. J. Biol. Chem. **274:**28436–28444.
12. **Eswar, N., et al.** 2006. Comparative protein structure modeling using Modeller. Curr. Protoc. Bioinformatics **5:**5.6.
13. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. U. S. A. **76:**1648–1652.
14. **Fischer, G., H. Bang, and C. Mech.** 1984. Detection of enzyme catalysis for *cis-trans* isomerization of peptide-bonds using proline-containing peptides as substrates. Biomed. Biochim. Acta **43:**1101–1111.
15. **Gauthier, Y. P., F. M. Thibault, J. C. Paucod, and D. R. Vidal.** 2000. Protease production by *Burkholderia pseudomallei* and virulence in mice. Acta Trop. **74:**215–220.
16. **Gemmill, T. R., X. Y. Wu, and S. D. Hanes.** 2005. Vanishingly low levels of Ess1 prolyl-isomerase activity are sufficient for growth in *Saccharomyces cerevisiae*. J. Biol. Chem. **280:**15510–15517.
17. **Harley, V. S., D. A. B. Dance, G. Tovey, M. V. McCrossan, and B. S. Drasar.** 1998. An ultrastructural study of the phagocytosis of *Burkholderia pseudomallei*. Microbios **94:**35–45.
18. **Harrison, R. K., and R. L. Stein.** 1990. Substrate specificities of the peptidyl prolyl *cis-trans* isomerase activities of cyclophilin and FK-506 binding-protein—evidence for the existence of a family of distinct enzymes. Biochemistry **29:**3813–3816.
19. **Helbig, J. H., et al.** 2003. The PPIase active site of *Legionella pneumophila* Mip protein is involved in the infection of eukaryotic host cells. Biol. Chem. **384:**125–137.
20. **Horne, S. M., T. J. Kottom, L. K. Nolan, and K. D. Young.** 1997. Decreased intracellular survival of an *fkpA* mutant of *Salmonella typhimurium* Copenhagen. Infect. Immun. **65:**806–810.
21. **Ikura, T., and N. Ito.** 2007. Requirements for peptidyl-prolyl isomerization activity: a comprehensive mutational analysis of the substrate-binding cavity of FK506-binding protein 12. Protein Sci. **16:**2618–2625.
22. **Inglis, T. J. J., T. Robertson, D. E. Woods, N. Dutton, and B. J. Chang.** 2003. Flagellum-mediated adhesion by *Burkholderia pseudomallei* precedes invasion of *Acanthamoeba astronyxis*. Infect. Immun. **71:**2280–2282.
23. **Jones, A. L., T. J. Beveridge, and D. E. Woods.** 1996. Intracellular survival of *Burkholderia pseudomallei*. Infect. Immun. **64:**782–790.
24. **Kespichayawattana, W., S. Rattanachetkul, T. Wanun, P. Utaisincharoen, and S. Sirisinha.** 2000. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect. Immun. **68:**5377–5384.
25. **Kohler, R., et al.** 2003. Biochemical and functional analyses of the Mip protein: influence of the N-terminal half and of peptidylprolyl isomerase activity on the virulence of *Legionella pneumophila*. Infect. Immun. **71:**4389–4397.
26. **Leuzzi, R., et al.** 2005. Ng-MIP, a surface-exposed lipoprotein of Neisseria gonorrhoeae, has a peptidyl-prolyl *cis/trans* isomerase (PPIase) activity and is involved in persistence in macrophages. Mol. Microbiol. **58:**669–681.
27. **Logue, C. A., I. R. A. Peak, and I. R. Beacham.** 2009. Facile construction of unmarked deletion mutants in *Burkholderia pseudomallei* using sacB counter-selection in sucrose-resistant and sucrose-sensitive isolates. J. Microbiol. Methods **76:**320–323.
28. **Lovell, S. C., et al.** 2003. Structure validation by C alpha geometry: phi, psi and C beta deviation. Proteins **50:**437–450.
29. **Löw, C., et al.** 2010. Crystal structure determination and functional characterization of the metallochaperone SlyD from *Thermus thermophilus*. J. Mol. Biol. **398:**375–390.
30. **Lundemose, A. G., J. E. Kay, and J. H. Pearce.** 1993. *Chlamydia trachomatis* Mip-like protein has peptidyl-prolyl *cis-trans* isomerase activity that is inhibited by FK506 and rapamycin and is implicated in initiation of chlamydial infection. Mol. Microbiol. **7:**777–783.
31. **Lupyan, D., A. Leo-Macias, and A. R. Ortiz.** 2005. A new progressive-iterative algorithm for multiple structure alignment. Bioinformatics **21:**3255–3263.
32. **Mizuguchi, K., C. M. Deane, T. L. Blundell, M. S. Johnson, and J. P. Overington.** 1998. JOY: protein sequence-structure representation and analysis. Bioinformatics **14:**617–623.
33. **Moro, A., F. Ruizcabello, A. Fernandezcano, R. P. Stock, and A. Gonzalez.** 1995. Secretion by *Trypanosoma cruzi* of a peptidyl-prolyl *cis-trans* isomerase involved in cell infection. EMBO J. **14:**2483–2490.
34. **Mukhopadhyay, A., K. H. Lee, and P. A. Tambyah.** 2004. Bacteraemic melioidosis pneumonia: impact on outcome, clinical and radiological features. J. Infect. **48:**334–338.

34a. **Norville, I. H., et al.** 2011. The structure of a *Burkholderia pseudomallei* immunophilin-inhibitor complex reveals new approaches to antimicrobial development. Biochem. J. **437:**413–422.

35. **Penfold, R. J., and J. M. Pemberton.** 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene **118:**145–146.
36. **Riboldi-Tunnicliffe, A., et al.** 2001. Crystal structure of Mip, a prolylisomerase from *Legionella pneumophila*. Nat. Struct. Biol. **8:**779–783.
37. **Rotz, L. D., A. S. Khan, S. R. Lillibridge, S. M. Ostroff, and J. M. Hughes.** 2002. Public health assessment of potential biological terrorism agents. Emerg. Infect. Dis. **8:**225–230.
38. **Schreiber, S. L.** 1991. Chemistry and biology of the immunophilins and their immunosuppressive ligands. Science **251:**283–287.
39. **Sexton, M. M., A. L. Jones, W. Chaowagul, and D. E. Woods.** 1994. Purification and characterization of a protease from *Pseudomonas pseudomallei*. Can. J. Microbiol. **40:**903–910.
40. **Shi, J. Y., T. L. Blundell, and K. Mizuguchi.** 2001. FUGUE: sequence-structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. J. Mol. Biol. **310:**243–257.
41. **Vertommen, D., N. Ruiz, P. Leverrier, T. J. Silhavy, and J. F. Collet.** 2009. Characterization of the role of the *Escherichia coli* periplasmic chaperone SurA using differential proteomics. Proteomics **9:**2432–2443.
42. **Wagner, C., et al.** 2007. Collagen binding protein Mip enables *Legionella pneumophila* to transmigrate through a barrier of NCI-H292 lung epithelial cells and extracellular matrix. Cell. Microbiol. **9:**450–462.
43. **Williams, J. W., and J. F. Morrison.** 1979. The kinetics of reversible tight-binding inhibition. Methods Enzymol. **63:**437–467.
44. **Wintermeyer, E., et al.** 1995. Influence of site-specifically altered Mip proteins on intracellular survival of *Legionella pneumophila* in eukaryotic cells. Infect. Immun. **63:**4576–4583.

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