

Evaluating the role of phage-shock protein A in *Burkholderia pseudomallei*

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The phage-shock protein (Psp) response is an extracytoplasmic response system that is vital for maintenance of the cytoplasmic membrane when the cell encounters stressful conditions. The paradigm of the Psp response has been established in *Escherichia coli*. The response has been shown to be important for survival during the stationary phase, maintenance of the proton motive force across membranes and implicated in virulence. In this study, we identified a putative PspA homologue in *Burkholderia pseudomallei*, annotated as BPSL2105. Similar to the induction of PspA in *E. coli*, the expression of *B. pseudomallei* BPSL2105 was induced by heat shock. Deletion of BPSL2105 resulted in a survival defect in the late stationary phase coincident with dramatic changes in the pH of the culture medium. The *B. pseudomallei* BPSL2105 deletion mutant also displayed reduced survival in macrophage infection – the first indication that the Psp response plays a role during intracellular pathogenesis in this species. The purified protein formed large oligomeric structures similar to those observed for the PspA protein of *E. coli*, and PspA homologues in *Bacillus*, cyanobacteria and higher plants, providing further evidence to support the identification of BPSL2105 as a PspA-like protein in *B. pseudomallei*.

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INTRODUCTION

Burkholderia pseudomallei is a Gram-negative bacterium and the causative agent of the disease melioidosis (White, 2003). Melioidosis is endemic to regions of Southeast Asia and Northern Australia where the bacterium is widely distributed in the soil (Currie *et al.*, 2008; Dance, 2000). It is a common cause of community-acquired bacteraemic pneumonia (Chaowagul *et al.*, 1989; Currie *et al.*, 2010), but the disease can manifest in different forms depending on the route of exposure (Cheng & Currie, 2005). Transmission via the aerosol route is thought to be a significant risk and, consequently, *B. pseudomallei* has been classified as a Category B biological threat agent by the Centers for Disease Control and Prevention in the USA.

B. pseudomallei is an extremely persistent bacterium, able to survive in a diverse range of environments. Studies have shown that it is able to withstand such conditions as low pH, high salt concentrations and high temperatures, and has been recovered from distilled water several years after initial inoculation (reviewed by Inglis & Sagripanti, 2006). It has a large genome, consisting of two chromosomes which encode many genes associated with adaptation to different environmental conditions and resistance to a range of niche-related stresses (Holden *et al.*, 2004). This adaptability has allowed the primarily environmental bacterium to colonize a further niche – the mammalian host. As an intracellular pathogen, *B. pseudomallei* can persist inside both phagocytic and non-phagocytic cells, where it must resist a novel panoply of stresses (Allwood *et al.*, 2011; Jones *et al.*, 1996). It is thought that, in cases of latency, bacteria reside intracellularly in a non-replicating form (Gan, 2005), only causing symptoms of disease later in life when the host immune system has been compromised (Ngauy *et al.*, 2005).

The phage-shock protein (Psp) response is an extracytoplasmic stress response in bacteria that functions to maintain cell membrane integrity during stress (Darwin, 2005).

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Abbreviations: PMF, proton motive force; Psp, phage-shock protein; RT, reverse transcription; TEM, transmission electron microscopy.

Six supplementary figures are available with the online Supplementary Material.

It was initially observed in *Escherichia coli*, with first reports describing a protein produced at a high concentration during filamentous phage infection, subsequently termed PspA (Brissette *et al.*, 1990). The Psp response is induced by a number of different stresses, many of which have a detrimental effect on the proton motive force (PMF) and therefore the Psp response is thought to have an important physiological role in maintaining the PMF across the cytoplasmic membrane (Kleerebezem *et al.*, 1996). The Psp response is thought to maintain the integrity of the cytoplasmic membrane in times of stress, and has been shown to be important in survival and virulence-related processes in several species of bacteria (Darwin, 2013; Darwin & Miller, 2001; Joly *et al.*, 2010). Its importance for survival during stationary phase growth has been demonstrated previously in *E. coli* (Weiner & Model, 1994).

The Psp response has been most studied in *E. coli* and *Yersinia enterocolitica*, where it has been shown to involve the products of the *pspABC* operon, regulated by PspF, the activity of which is in turn regulated by PspA (Dworkin *et al.*, 2000; Elderkin *et al.*, 2002; Jovanovic *et al.*, 1996; 1999). When the Psp response is induced, the concentration of PspA increases, whereupon it is recruited to the cytoplasmic membrane (Yamaguchi *et al.*, 2010). *In vitro*, PspA is able to form large oligomeric rings (Hankamer *et al.*, 2004), which are able to bind to membrane phospholipids and reduce proton leakage through the damaged membrane (Kobayashi *et al.*, 2007). Although the Psp systems of *E. coli* and *Y. enterocolitica* are considered the paradigm, there are a number of bacteria which possess isolated PspA homologues but lack other members of the *psp* operon. For example, *Streptomyces lividans* and *Bacillus subtilis* both possess a PspA homologue which is upregulated by known Psp-inducing conditions (Vrancken *et al.*, 2008; Wolf *et al.*, 2010).

In this study, we identified a putative PspA homologue in *B. pseudomallei* which responds to similar stresses as the Psp response in *E. coli*. We demonstrated its importance for survival during stationary phase growth and for intracellular survival in a macrophage cell line. In addition, the *B. pseudomallei* PspA-like protein was expressed and analysed by transmission electron microscopy (TEM), revealing the presence of a higher-order PspA-like protein species which assembled into ring-shaped oligomers.

METHODS

Bacterial strains, culture conditions and mutant construction.

E. coli and *B. pseudomallei* were cultured in Luria–Bertani (LB) broth at 37 °C with agitation, unless otherwise stated. Bacterial strains used are listed in Table 1. Antibiotics (chloramphenicol and kanamycin) were used at 50 µg ml⁻¹ final concentration.

Primers used for DNA amplification are listed in Table 2. The *B. pseudomallei* unmarked deletion mutant was made by homologous recombination using the pDM4 suicide vector by the method outlined in Logue *et al.* (2009). The pDM4.Δ*pspA* plasmid was constructed by ligation of *Bgl*II-linearized pDM4 with the truncated *BPSL2105*.

Table 1. Strains and plasmids used

Strain or plasmid	Characteristics	Reference or source
<i>E. coli</i>		
BL21(DE3)	F ⁻ <i>ompT</i> <i>hsdSB</i> (r _B , m _B) <i>gal dcm</i> BL21(DE3)	Invitrogen
DH5α	F ⁻ φ80 <i>lacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) <i>U169 recA1 endA1 hsdR17</i> (r _K , m _K) <i>phoA supE44 λ-thi-1 gyrA96 relA1</i>	<i>E. coli</i> genetic stock centre Laboratory collection
S17-λ <i>pir</i>	λ <i>pir recA thi pro hsdR</i> - <i>RP4-2</i> (Km :: Tn7 Tc :: Mu) <i>Tp</i> ^r <i>Sm</i> ^r	
<i>B. pseudomallei</i>		
K96243	Clinical isolate from Thailand, sequenced strain	Holden <i>et al.</i> (2004)
Δ <i>pspA</i>	K96243 derivative; unmarked deletion Δ <i>BPSL2105</i>	This study
Δ <i>pspA</i> /pBHR4. <i>pspA</i>	K96243 derivative; unmarked deletion Δ <i>BPSL2105</i> ; pBHR4 :: <i>BPSL2105</i> , Cm ^r	This study
Plasmids		
pCR-Blunt II-TOPO	Cloning vector for blunt-end PCR products, Km ^r	Invitrogen
pDM4	pNQ705 derivative, <i>oriR6K</i> , <i>mobRRP4</i> , <i>sacBR</i> , Cm ^r	Milton <i>et al.</i> (1996)
pDM4.Δ <i>pspA</i>	pDM4 derivative with <i>BPSL2105</i> flanking regions for mutagenesis, Cm ^r	This study
pBHR4-groS-RFP	pBHR1 derivative, <i>turboFP635</i> , P _{groES} <i>rrnB</i> , Cm ^r	Wand <i>et al.</i> (2011)
pBHR4. <i>pspA</i>	pBHR4-groS-RFP derivative with P _{BPSL2105} , <i>BPSL2105</i> , Cm ^r	This study
pET28a	<i>E. coli</i> expression vector, T7 <i>lac</i> promoter, Km ^r	Novagen
pET28- <i>His₆</i> - <i>pspA</i>	pET28a derivative, N-terminal His ₆ -tagged <i>BPSL2105</i> , Km ^r	This study

Table 2. Primers used

Primer	Sequence (5'→3')	Restriction site
BPSL2105 LF F	<u>AGATCT</u> TGAACGCGTGCATGGAATCG	<i>Bgl</i> II
BPSL2105 LF R	CATATGTTT <u>GATCGT</u> GCGCGAAATAG	<i>Nde</i> I
BPSL2105 RF F	<u>CATATG</u> GACCGCTCGAAGCGCTGAA	<i>Nde</i> I
BPSL2105 RF R	<u>AGATCT</u> CGAGCATGCCGCCGAGGTC	<i>Bgl</i> II
BPSL2105 prom F	<u>CGATCG</u> GCGCTGAACGCGTGCATGGA	<i>Pvu</i> I
BPSL2105 comp R	<u>GGATCC</u> TTACTGCGCCGGCGTGTTC	<i>Bam</i> HI
RT PspA F	CGCGCACGATCAAAGGTCTG	–
RT PspA R	GCCGCTGTTCTGATCGGCTG	–
16S rRNA F	GATGACGGTACCGGAAGAATAAGC	–
16S rRNA R	CCATGTCAAGGGTAGGTAAGGTTT	–
PspA F	GCCGCCGGATCCATGTCGCTTTTCGACTCTATTTC	<i>Bam</i> HI
PspA R	CGGCGGGAGCTCTTACTGCGCCGGCGTGTTCAG	<i>Sac</i> I
BPSL2105-6 F	GTCGGTCGACAAGCTCAAAG	–
BPSL2105-6 R	GGATCGGTGAAGCGCAGTTG	–

Restriction sites are underlined.

The truncated gene was produced by amplifying upstream and downstream flanking regions of *BPSL2105* from *B. pseudomallei* K96243 genomic DNA using primers BPSL2105 LF F/BPSL2105 LF R (upstream) and BPSL2105 RF F/BPSL2105 RF R (downstream). *Nde*I and *Bgl*II restriction sites were incorporated into either end of the flanks to aid manipulation. The resulting flanks were cloned into pCR-Blunt II-TOPO (Invitrogen) followed by digestion with *Nde*I and *Bgl*II for ligation with the pDM4 suicide vector. To construct *B. pseudomallei*Δ*pspA*, the pDM4.Δ*pspA* plasmid was transformed into *E. coli* S17-λ *pir* cells before conjugation with *B. pseudomallei* K96243. The merodiploid strain was screened for chloramphenicol resistance before sucrose selection was carried out to select for the second recombination event. Unmarked *BPSL2105* deletion mutants were verified by PCR and Southern blot.

To construct pBHR4.*pspA*, the *BPSL2105* gene and promoter were amplified from *B. pseudomallei* genomic DNA using primers BPSL2105 prom F/BPSL2105 comp R. This introduced *Pvu*I and *Bam*HI restriction sites onto either end of the fragment. The PCR product was cloned into pCR-Blunt II-TOPO before digestion and ligation with *Pvu*I/*Bam*HI-digested pBHR4-groS-RFP (Wand *et al.*, 2011). The pBHR4.*pspA* plasmid was transferred by conjugation into *B. pseudomallei*Δ*pspA*. The complemented strain *B. pseudomallei*Δ*pspA*/pBHR4.*pspA* was then selected for by plating onto LB agar containing 50 µg chloramphenicol ml⁻¹ and the presence of the plasmid confirmed by colony PCR.

To investigate bacterial survival, overnight cultures were diluted in 100 ml LB broth and grown to the stationary phase by continuous incubation at 37 °C with agitation. The cultures were titrated for viable cells daily by plating on LB agar with or without appropriate antibiotics. pH was measured using a Hanna Piccolo Plus pH meter (Sigma).

RNA isolation and reverse transcription (RT)-PCR. Overnight cultures of *B. pseudomallei* K96243 were diluted to OD₅₉₀ 0.1 and grown at 37 °C for 6 h until the mid-exponential phase was reached. Cultures were divided into 1 ml aliquots and incubated at 37 °C and 50 °C. RNA samples were collected at selected time points for up to 30 min by addition of 2 ml RNAprotect (Qiagen). RNA was recovered using a RNeasy Mini kit (Qiagen) as instructed by the manufacturer. This resulted in RNA at a concentration of 100–400 ng µl⁻¹, quantified using a NanoDrop 1000 spectrophotometer. Residual DNA was

removed by treating the RNA with TURBO DNA-free DNase (Ambion). During this step the RNA was standardized to a concentration of 125 ng µl⁻¹. Following this, the samples were reverse transcribed using Enhanced Avian Reverse Transcriptase (Sigma) according to the manufacturer's instructions. The resulting cDNA was amplified by PCR using Herculase II fusion DNA polymerase (Agilent Technologies) in a standard PCR. For each PCR, the appropriate controls with water and RNA in the absence of reverse transcriptase were included to ensure that amplifications were of cDNA and not contaminating genomic DNA. Transcripts of *BPSL2105* were amplified using RT PspA F/RT PspA R primers, and 16S rRNA was amplified as a positive control using 16S rRNA F/16S rRNA R primers. PCR was also performed using primers (BPSL2105-6 F/BPSL2105-6 R) complementary to sequences overlapping both *BPSL2105* and *BPSL2106* to determine whether the genes were co-transcribed.

Intracellular survival assays. J774A.1 cells (ECACC) were seeded in a 24-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 % L-glutamine and 10 % FCS at a concentration of 4 × 10⁵ cells ml⁻¹. The cells were incubated at 37 °C with 5 % CO₂ overnight until they had reached a density of 1 × 10⁶ cells ml⁻¹. A stationary phase culture was diluted in Leibovitz L-15 medium supplemented with 10 % FCS to OD₅₉₀ 0.35–0.4, equivalent to ~1 × 10⁸ c.f.u. ml⁻¹. This was serially diluted to 1 × 10⁷ c.f.u. ml⁻¹. The DMEM was removed from the J774A.1 cells and replaced with L-15 media containing 1 × 10⁷ c.f.u. ml⁻¹ bacteria. The cells were incubated at 37 °C for 30 min, and then the media was removed and the cells washed three times with PBS. To kill any extracellular bacteria, 1 ml L-15 supplemented with 1 mg kanamycin ml⁻¹ was added to each well and incubated at 37 °C. After 1 h, the antibiotic medium was removed and replaced with 1 ml L-15 supplemented with 250 µg kanamycin ml⁻¹, and incubated at 37 °C for 24 h. At selected time points the antibiotic medium was removed and the cells lysed by addition of 1 ml water. The lysate was serially diluted and cultured on LB agar to enumerate viable bacteria.

MIC determination. MIC determinations were carried out according to the method of Lambert & Pearson (2000) with some modifications. Briefly, stock solutions of H₂O₂, HCl, NaOH, NaCl, lysozyme and deferoxamine were prepared in LB broth at a concentration of 256 µg ml⁻¹. A 100 µl aliquot of the stock solution was added to 100 µl LB

broth in the first column of a 96-well plate and a twofold dilution carried out across the plate. The bacterial inoculum was prepared by growing a stationary phase culture of *B. pseudomallei*, adjusting to OD₅₉₀ 0.35–0.40 in LB broth and then serially diluting to a concentration of 1×10^6 c.f.u. ml⁻¹. A 100 µl aliquot of this culture was added to each compound dilution that had been dispensed into the test wells. This provided a final compound dilution range of 64–0.03 µg ml⁻¹. NaOH was tested at a concentration of 0.5–1024 µg ml⁻¹. The 96-well plates were incubated at 37 °C for 18 h before the optical density was recorded for each well.

Expression and purification of a His₆-tagged PspA-like protein.

BPSL2105 was amplified by PCR using the primers PspA F/PspA R and cloned into the corresponding sites in the pET28a vector (Novagen). The resulting pET28-*His₆-pspA* plasmid encoded N-terminal His₆-tagged *BPSL2105* (His₆-PspA), verified by DNA sequencing (Eurofins MWG), with a molecular mass of 27.876 kDa. His₆-PspA was expressed in *E. coli* BL21(DE3) as described previously (Elderkin *et al.*, 2002), with or without 1.1 % CHAPS. Briefly, cultures were grown in LB broth to OD₆₀₀ ~0.6, following which protein expression was induced by addition of 1 mM IPTG. The induced cultures were incubated overnight at 18 °C before harvesting the cells by centrifugation. To extract the protein, the cells were resuspended in lysis buffer (100 mM Tris/HCl, pH 7.5, 50 mM NaCl, 75 mM NaSCN) and sonicated. The insoluble and soluble fractions were separated by centrifugation and the soluble fraction directly purified by Ni²⁺-affinity chromatography, according to the manufacturer's instructions. Size exclusion chromatography was performed using a Superdex 200 gel filtration column (GE Healthcare) calibrated with the following molecular mass standards: blue dextran (2000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa). Gel filtration buffer (20 mM Tris/HCl, pH 7.5, 200 mM NaCl, 75 mM NaSCN) was passed through the column at a flow rate of 1 ml min⁻¹. The 1 ml fractions were collected between 35 and 55 ml. The yield after gel filtration was 7.6 mg when purified from a 500 ml culture. For purification under denaturing conditions, the cells were resuspended in denaturing lysis buffer (20 mM Tris/HCl, pH 7.5, 500 mM NaCl, 1 mM β-mercaptoethanol and 6 M guanidine hydrochloride). Purification was carried out by Ni²⁺-affinity chromatography, according to the manufacturer's instructions, using a wash and elution buffer containing 20 mM Tris/HCl, pH 7.5, 500 mM NaCl, 1 mM β-mercaptoethanol, 8 M urea and 10/500 mM imidazole. Protein mass was confirmed by MS.

Western blotting. Proteins were separated by SDS-PAGE on a 12 % (w/v) acrylamide gel and transferred onto nitrocellulose transfer membranes (Protran; Whatman). After blocking for 1 h with 5 % milk powder in PBS and 0.05 % Tween, the blots were incubated with anti-His (27-4710-01; GE Healthcare) at 1 : 1000 dilution, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (NA931; GE Healthcare) at 1 : 5000 dilution. Secondary antibody was detected using an enhanced chemiluminescence reagent (Western C; Bio-Rad).

TEM. PspA was negatively stained with 2 % (w/v) uranyl acetate on 50 % carbonyl Formvar grids and examined on a Hitachi H7000 transmission electron microscope operated at 80 kV with magnification ranging from $\times 50\,000$ to $\times 200\,000$. A protein-free control was used, whereby a negatively stained sample in the absence of any purified recombinant protein was imaged, to verify that the images observed were not due to staining or sample handling during image acquisition.

Statistical analysis. All data are reported as mean \pm SEM. Results were statistically analysed using a two-way ANOVA and Bonferroni post-tests. $P < 0.05$ was considered statistically significant.

RESULTS

BPSL2105 from *B. pseudomallei* is a putative PspA homologue

Several putative PspA homologues (annotated as PspA family proteins) from a number of *Burkholderia* species were compared with the *B. pseudomallei* K96243 sequenced genome. Although it is currently annotated as a hypothetical protein, *BPSL2105* in *B. pseudomallei* K96243 has 99–100 % identity to many of these proteins, including proteins from both the less pathogenic *Burkholderia thailandensis* and the close relative *Burkholderia mallei* (Fig. S1, available in the online Supplementary Material; Cole *et al.*, 2008; Gautier *et al.*, 2008; Jovanovic *et al.*, 2014a). The amino acid sequence of *BPSL2105* was further compared with known PspAs in species such as *E. coli* and *Y. enterocolitica* using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Fig. S1). This comparison showed low identity compared with the *Enterobacteriaceae*, e.g. *BPSL2105* had 22 % identity to *Y. enterocolitica* PspA and 21 % overall identity to *E. coli*. Similarly, less well characterized PspA homologues in *B. subtilis* and *S. lividans* showed 28 and 31 % identity, respectively. However, PspAs in the *Enterobacteriaceae* are known to be coiled-coil proteins (Dworkin *et al.*, 2000) and *BPSL2105* is predicted to contain several α-helices as well as an amphipathic helix sequence in the initial 20 amino acids, comparable to PspA in *E. coli* (Jovanovic *et al.*, 2014a) (see also Fig. S1). Despite this, no other members of the Psp regulon were found to be present in *B. pseudomallei* K96243. Instead, *BPSL2105* is predicted to form an operon with *BPSL2106*, which is annotated as a putative membrane protein (Figs. 1a and S1). This gene order is observed in other close relatives of *B. pseudomallei* that possess PspA family proteins. RT-PCR was performed on RNA isolated from *B. pseudomallei*, verifying that *BPSL2105* and *BPSL2106* were co-transcribed (Fig. 1b).

In order to determine whether *BPSL2105* possessed PspA-like features, RT-PCR was carried out on mRNA isolated from *B. pseudomallei* grown under conditions known to cause upregulation of PspA. Previous studies have shown that heat shock at 50 °C causes a transient increase in the concentration of PspA in *E. coli* (Brissette *et al.*, 1990). To investigate the effect of temperature on expression of *BPSL2105* in *B. pseudomallei*, RNA was collected from cultures shocked at 50 °C and RT-PCR performed using primers RT PspA F/RT PspA R (Table 1) to amplify *BPSL2105* mRNA. The PCR product was visualized using gel electrophoresis to provide a semiquantitative result. The results showed a transient increase in *BPSL2105* expression at 50 °C compared with the incubation at 37 °C, with maximal induction at 10–15 min (Fig. 1c).

Another known inducer of *E. coli* PspA is hyperosmotic shock (Brissette *et al.*, 1990). Whereas expression of PspA during heat shock is independent of the PspBC sensors, not present in *B. pseudomallei*, expression of PspA under high-salt

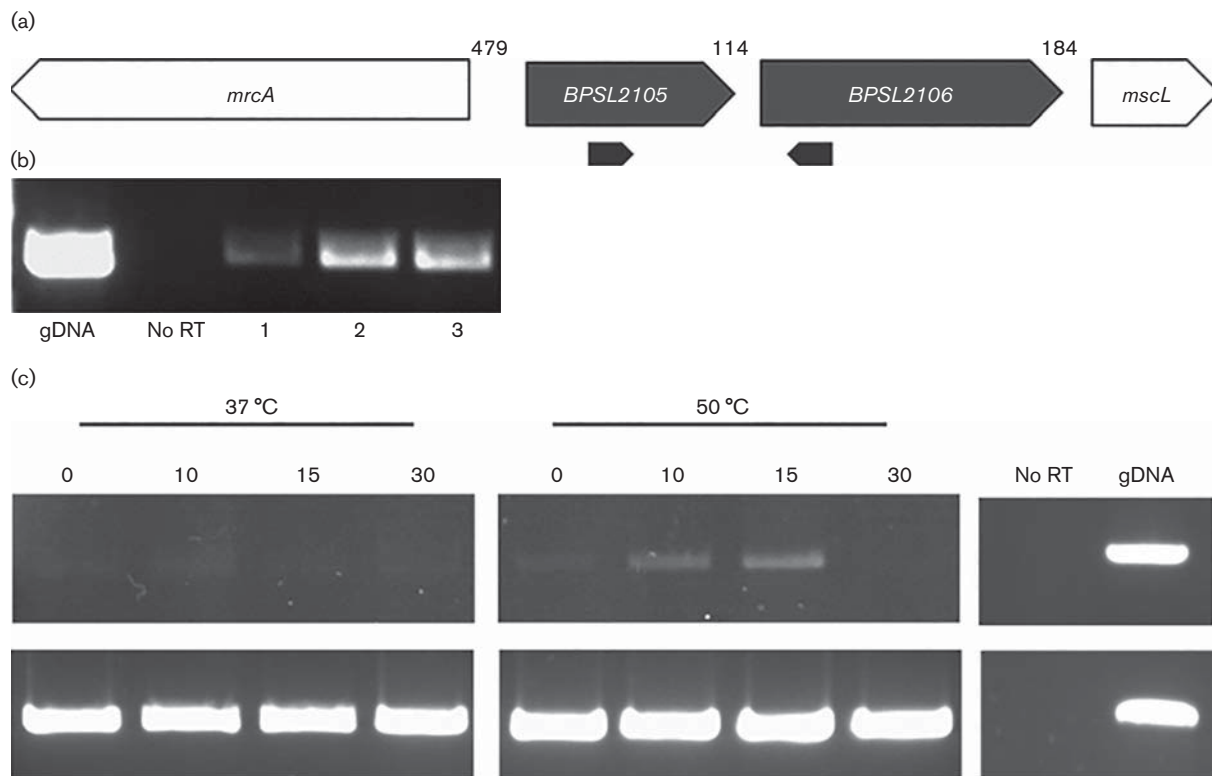


Fig. 1. *BPSL2105* organization and expression. (a) Predicted operon of *B. pseudomallei* *BPSL2105/2106*. (b) Demonstration of co-transcription of *BPSL2105* and *BPSL2106* by RT-PCR. RNA samples were collected from three separate cultures (1, 2 and 3). Primers used during RT-PCR are represented by the black arrows. (c) Expression of *BPSL2105* during extreme heat shock. RNA samples were collected at 0, 10, 15 and 30 min time points, purified and residual DNA removed. Following this, the samples were amplified using RT-PCR with primers amplifying a region either from *BPSL2105* or from 16S rRNA genes. Controls were carried out using RNA in the absence of reverse transcriptase. gDNA, genomic DNA.

conditions is partially dependent on these proteins (Weiner *et al.*, 1991). RT-PCR was used to measure the relative level of expression of *BPSL2105* in cultures exposed to 0.3 M NaCl compared with expression in unshocked samples. However, the results showed no increased expression of *BPSL2105* compared with controls (data not shown).

The data from these studies provided preliminary evidence that *BPSL2105* encodes a PspA-like protein in *B. pseudomallei*. *BPSL2105* has a secondary structure comparable to the α -helical structure of *E. coli* PspA, which is essential for its regulatory and effector functions (Elderkin *et al.*, 2005; Joly *et al.*, 2009), and a key amphipathic helix, thought to play an important role in inner membrane binding and signal transduction in *E. coli* (Jovanovic *et al.*, 2014a). In addition, *BPSL2105* expression is increased in response to heat shock at 50 °C, a known Psp-inducing condition, further indicating that *BPSL2105* is a PspA-like protein in this species.

Deletion of *BPSL2105* in *B. pseudomallei* results in a growth defect in the late stationary phase

A deletion mutant was constructed in *BPSL2105* using the pDM4 suicide vector and *sacB* counter-selection (Logue

et al., 2009). This mutant, *B. pseudomallei* Δ *pspA*, was evaluated for phenotypes known to induce PspA in other bacteria. The stationary phase of growth has been shown to be an important inducer of the Psp response in *E. coli* where PspA is rapidly accumulated in the cell after one day in the stationary phase (Weiner & Model, 1994). Further to this, growth of an *E. coli* Δ *pspABC* strain shows a sharp decline in viability after day 9 at the stationary phase compared with the WT.

B. pseudomallei Δ *pspA* was grown in LB broth at 37 °C with aeration for the duration of the experiment. Both *B. pseudomallei* WT and Δ *pspA* mutant maintained a density of $\sim 5 \times 10^9$ c.f.u. ml⁻¹ during the first 6 days (144 h). From day 7, *B. pseudomallei* Δ *pspA* began to decline in viability compared with the WT (Fig. 2a). The WT phenotype was restored in Δ *pspA* complemented with the plasmid expressing *BPSL2105*. The pH of the cultures was monitored daily and was found to have increased from 7.5 to ~ 8.5 over 192 h (8 days) in the *B. pseudomallei* Δ *pspA* culture, whereas the WT and complemented strain were able to maintain the pH between 6.5 and 7 over the course of the experiment (Fig. 2b).

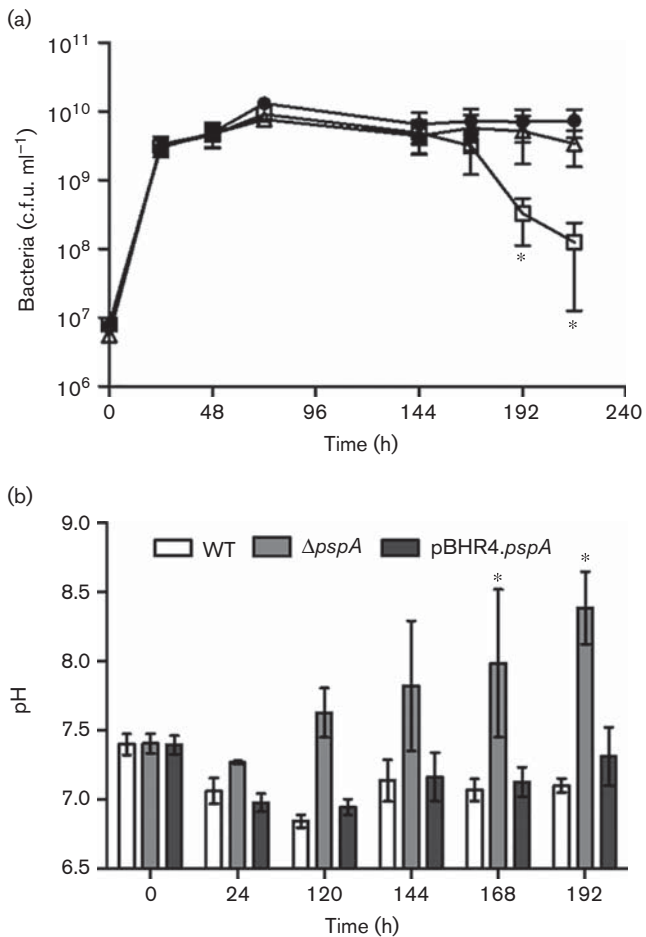


Fig. 2. BPSL2105 contributes to late stationary phase survival. (a) Growth of *B. pseudomallei* over 216 h in 100 ml LB broth cultures. Overnight cultures of WT *B. pseudomallei* K96243 (●), $\Delta pspA$ (□) and $\Delta pspA$ complemented with pBHR4.pspA (Δ) were diluted in 100 ml LB broth to OD₅₉₀ 0.1 and grown to the stationary phase by continuous incubation at 37 °C with agitation. Survival was measured by plating on LB agar with or without appropriate antibiotics. (b) pH of *B. pseudomallei* cultures measured over 192 h. Values are the mean \pm SEM from three independent experiments; * P <0.05.

***B. pseudomallei* $\Delta pspA$ is more susceptible to macrophage killing during the late stationary phase**

The Psp response is implicated in intracellular pathogenesis as *psp* genes are upregulated during macrophage infection in bacteria such as *Salmonella enterica*, *Shigella flexneri* and *M. tuberculosis* (Datta *et al.*, 2015, Eriksson *et al.*, 2003; Lucchini *et al.*, 2005). *B. pseudomallei* is an intracellular pathogen and the ability to multiply within macrophages is essential for virulence (Pilatz *et al.*, 2006; Stevens *et al.*, 2003, 2004). In order to investigate the ability of *B. pseudomallei* $\Delta pspA$ to survive intracellularly, an infection assay was performed using J774A.1 murine

macrophages. *B. pseudomallei* strains were grown for 6 days in 100 ml LB broth and used to infect a J774A.1 macrophage cell line at m.o.i. 10. The number of viable intracellular bacteria was measured by lysing the macrophages with water and culturing the bacteria. The WT bacteria were able to replicate intracellularly, but *B. pseudomallei* $\Delta pspA$ showed a reduction in the number of intracellular bacteria at 24 h (Fig. 3). Complementation of *B. pseudomallei* $\Delta pspA$ with the plasmid expressing BPSL2105 restored intracellular growth to WT levels. This result was divergent to that seen when macrophages were infected with exponential phase *B. pseudomallei* $\Delta pspA$, where there was no difference in intracellular survival compared with the WT strains at 24 h post-infection (data not shown).

B. pseudomallei-infected macrophages are prone to lysis by 24 h from high intracellular numbers of bacteria. This causes the internalized bacteria to be released into the kanamycin-containing media, killing any extracellular bacteria. Lactate dehydrogenase release was measured to verify that the reduction in intracellular bacteria at 24 h was not caused by increased cytotoxicity of the mutant. The lactate dehydrogenase levels in the media were similar for cells infected with *B. pseudomallei* WT, $\Delta pspA$ and the complemented strain (data not shown).

Macrophages infected with *B. pseudomallei* $\Delta pspA$ showed a decrease in intracellular bacteria to those of the original inoculum, indicating killing rather than just growth inhibition. Macrophages have a variety of mechanisms for killing phagocytosed bacteria. In an attempt to determine which of these mechanisms the mutant was more

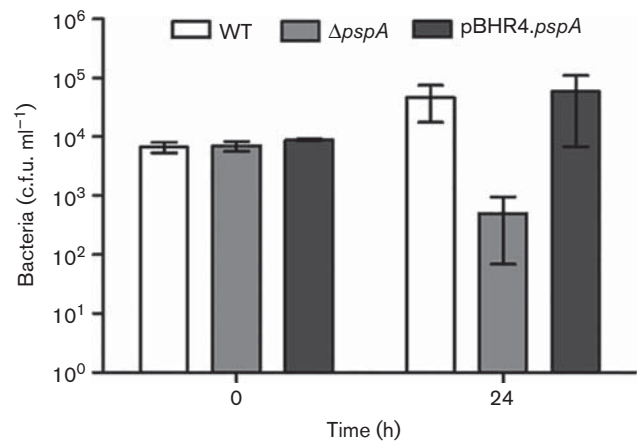


Fig. 3. Intracellular survival of stationary phase *B. pseudomallei* in J774A.1 macrophages. Macrophage cells were infected with 1×10^7 c.f.u. ml⁻¹ bacteria for 30 min, followed by incubation with 1 mg kanamycin ml⁻¹ for 1 h to kill any extracellular bacteria. The cells were maintained in the presence of 250 μ g kanamycin ml⁻¹ and periodically lysed in order to enumerate the number of intracellular bacteria. Values are the mean \pm SEM from at least three independent experiments; * P <0.05.

susceptible to, the bacteria were exposed to a range of conditions. Bacteria were grown to the stationary phase by incubation in 100 ml LB broth at 37 °C for 6 days. The level of growth of the stationary phase bacteria was measured under a range of conditions, including H₂O₂, NaCl, HCl, NaOH, lysozyme and an iron chelator, deferoxamine. Overall there was no difference between the susceptibility of either WT or the Δ pspA mutant to osmotic stress, pH, low iron or lysozyme. Similarly, both strains were highly susceptible to oxidative stress, with a MIC of 0.125–0.25 μ g H₂O₂ ml⁻¹ (Fig. S2).

B. pseudomallei BPSL2105 assembles into multimeric complexes

We have recently demonstrated that *E. coli* PspA assembles into higher-order, multimeric complexes (Male *et al.*, 2014). We therefore sought to establish whether *B. pseudomallei* BPSL2105 forms similar structures. His₆-tagged BPSL2105 (His₆-PspA) was recombinantly expressed and purified by Ni²⁺-affinity chromatography before being imaged by TEM. The purified His₆-PspA protein, purified in the absence of the detergent CHAPS, was analysed by gel filtration, resulting in two peaks that eluted at 49.5 and 83.0–109.0 ml (Fig. 4a). The wider peak between 83.0 and 109.0 ml may correspond to dimeric (81.9 ml) and monomeric (90.3 ml) PspA species, estimated by molecular mass standards (Fig. S3) and studying the elution of a denatured form of the protein at 88.1 ml (Fig. 4a).

The presence of His₆-PspA in both peaks and its absence elsewhere was confirmed by SDS-PAGE and Western blot (Fig. 4b, c). The peak at 49.5 ml (void volume fraction) showed the presence of His₆-PspA with expected molecular mass (Fig. S3) and corresponding to a species with a molecular mass >200 kDa. In other bacteria, the high-molecular-mass species of PspA and its homologues have been shown to include the putative 36-meric ring and other higher-order PspA complexes (Fuhrmann *et al.*, 2009; Hankamer *et al.* 2004). To investigate the higher-order species formed by *B. pseudomallei* PspA, the His₆-PspA eluted at 49.5 ml was negatively stained with uranyl acetate and imaged by TEM. Three different species were observed: ring-shaped complexes, rod-shaped species of varying lengths and aggregated PspA forming large mesh structures (Fig. 5). No such structures were observed in PspA-free controls.

The first type of PspA structure observed was a ring-shaped complex (Fig. 5c, d, f), similar to those previously observed in *E. coli* (Hankamer *et al.* 2004). The outer diameter of the *B. pseudomallei* PspA ring was ~40 nm, slightly larger than the *E. coli* PspA 36-mer (Hankamer *et al.* 2004) and more similar to the size of the clathrin-like species previously observed in *E. coli* (30–40 nm) (Standar *et al.*, 2008). Despite the difference in size, *B. pseudomallei* PspA appeared to form ring-like structures, visible as a ring of weak contrast with a black, stain-filled region in the

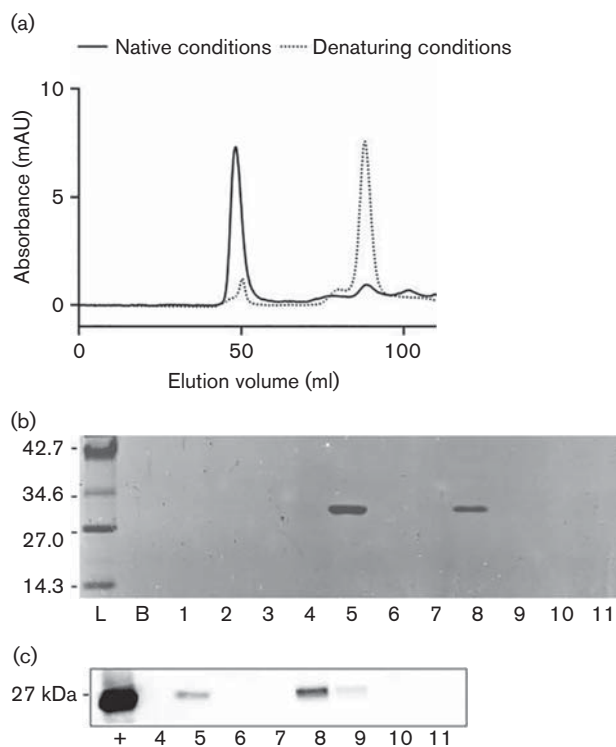


Fig. 4. Determining the oligomeric state of purified His₆-PspA protein. (a) Size exclusion chromatography trace of His₆-PspA showing a void volume at 49.5 ml containing the aggregated and higher-order PspA protein species, and a collection of peaks between 83.0 and 109.0 ml containing putative dimeric and monomeric His₆-PspA species. Included is an overlay of His₆-PspA protein purified under denaturing conditions. AU, absorbance units. (b) SDS-PAGE of His₆-PspA purified by gel filtration. Lanes represent pooled fractions from 0–10 (lane 1), 10–20 (lane 2), 20–30 (lane 3), 30–40 (lane 4), 40–50 (lane 5), 50–65 (lane 6), 65–80 (lane 7), 80–90 (lane 8), 90–100 (lane 9), 100–110 (lane 10) and 110–120 ml (lane 11). Values in kDa. (c) Western blot confirming the presence of His₆-PspA protein. Fractions are the same as those outlined in (b); +, His₆-PspA positive control.

middle, indicating the presence of a central hole with a diameter of ~10–12 nm (Fig. 5c, d, f).

The second species observed was analogous to the rod-like complexes formed by the PspA homologue Vipp1 in the cyanobacterium *Synechocystis* (Fuhrmann *et al.*, 2009). Slight variation in the diameter between the *B. pseudomallei* PspA rod-like complexes was observed, with a mean diameter between 40 and 45 nm (Fig. 5c, d). Along each rod-like complex, striations and indentations were visible, even at lower magnification, similar to those seen in the Vipp1 complexes (Fuhrmann *et al.*, 2009). These characteristics were uniform, suggestive of ring stacking and were ~15 nm apart (Fig. 5e). Some rod-like structures observed showed several structural anomalies, such as curved shapes and tapered ends (Fig. 5d, e), suggesting that the rings were

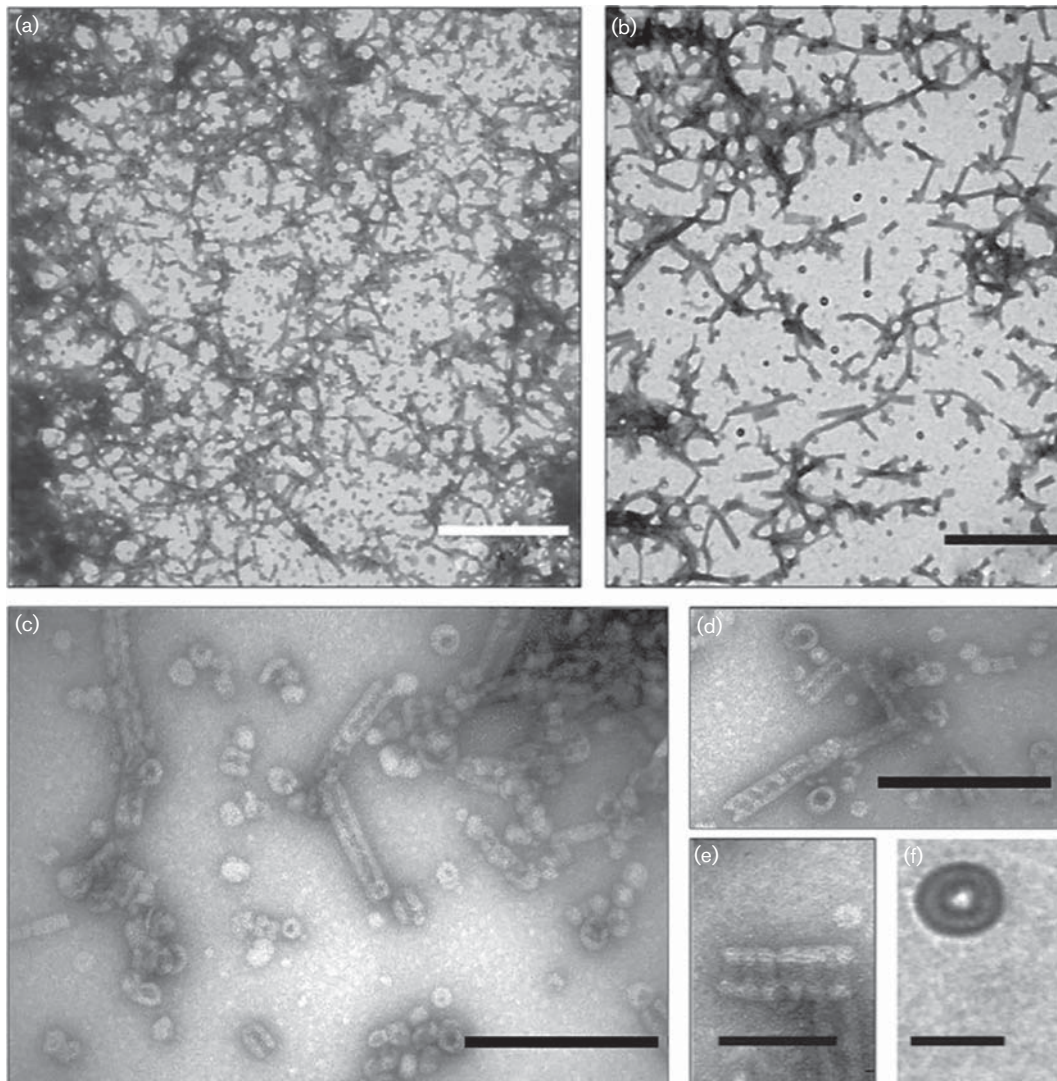


Fig. 5. Transmission electron micrographs of negatively stained PspA protein complexes. (a, b) Mixtures of rings, rod-like complexes and mesh-like structures are readily visible. Bar, 500 nm. (c, d) Both the ring structures and rod-like complexes are visible in this field of view. Bar, 200 nm. (e) Close-up of a rod-like structure, clearly showing the indentations and striations that indicate stacking of ring-like structures. This is also an example of the tapered end observed occasionally. Bar, 100 nm. (f) The putative 36-mer, ring-like PspA structure. Bar, 40 nm.

not necessarily stacked directly on top of one another during formation of the rods. The ring-like structures were observed in supercomplexes in the form of a mesh-like structure created by interactions between the rod-like species (Fig. 5a, b). These large complexes appeared to be ordered structures, unlike the aggregated protein observed in ~5–10 % of the sample (Fig. S4).

As the *B. pseudomallei* PspA higher-order structures observed above were purified in the absence of the detergent CHAPS, this soluble version of the protein may not be functional. As an additional control, the membrane-associated form of this protein was therefore purified with the addition of 1 % CHAPS to an extraction buffer

and eluted fractions (Fig. S5). The purified protein had similar properties to the protein purified in the absence of CHAPS. We also observed similar higher-order species forming for this protein as for PspA purified in the absence of CHAPS (Fig. S6), demonstrating that these higher-order structures are not an artefact of the purification conditions or indicative of inactive protein.

DISCUSSION

The Psp response is a poorly understood stress response system, expression of which is induced by changing conditions in the extracellular environment (Darwin, 2005;

Joly *et al.*, 2010). Its main function appears to be maintaining the integrity of the cytoplasmic membrane by generating a network of PspA complexes that are recruited to the cytoplasmic membrane during induction of the response (Yamaguchi *et al.*, 2010). Loss of a functional Psp response results in the dissipation of the PMF under certain inner membrane stress conditions (Kleerebezem *et al.*, 1996). This leads to defects in functions such as metal iron transport and biofilm formation (Beloin *et al.*, 2004; Karlinsey *et al.*, 2010).

The Psp response has been best characterized in the *Enterobacteriaceae* and few studies have been reported for other bacteria. We sought to determine whether *B. pseudomallei*, an opportunistic pathogen of medical importance, possessed a Psp response and whether it played a similar role in survival as reported for the enterobacterial paradigm. Bioinformatic analysis was carried out to identify whether Psp homologues were present in *B. pseudomallei*. Only a putative PspA homologue, BPSL2105, was identified, which showed low identity when compared with *Y. enterocolitica* or *E. coli* PspA amino acid sequences. However, the similarity in secondary structure to known PspAs from the enterobacteria supports the identification of BPSL2105 as a PspA homologue. PspA and its homologues are coiled-coil proteins containing four α -helical domains (Joly *et al.*, 2009). In *E. coli*, interactions between the N-terminal amphipathic helices are important for intra- and intermolecular signalling, which allows the protein to switch between its role as negative regulator and membrane-associated effector of stress response (Jovanovic *et al.*, 2014a). Although the primary sequence of this initial helix is not conserved in BPSL2105, it may nonetheless contribute to the ability to form similar protein interactions under conditions of stress.

The complete Psp regulon appears to be absent in *B. pseudomallei*; however, the presence of a predicted membrane protein that is co-transcribed with BPSL2105 bears resemblance to other species with only a single PspA homologue (Vrancken *et al.*, 2008; Wolf *et al.*, 2010). It is common with this response to find this arrangement as many of the *psp* genes appear to be dispensable (Darwin, 2005). For example, several Gram-positive bacteria, such as *B. subtilis* and *S. lividans*, have been reported to possess Psp-like responses without the accompanying regulatory function. The arrangement in *B. pseudomallei* could be similar to that reported for these species, which both contain a single PspA homologue in an operon with a predicted membrane protein, but lack a full *psp* operon (Vrancken *et al.*, 2008; Wolf *et al.*, 2010). Despite these differences to the accepted paradigm in enterobacteria, the response to heat stress in *B. pseudomallei* showed comparable induction of BPSL2105 to that of *E. coli* PspA, indicating a Psp-like response in this species. Conversely, there was no apparent change in the level of BPSL2105 expression in response to hyper osmotic shock, despite the proximity of the *mscL* gene encoding a mechanosensitive channel, involved in the cell's response

to osmotic pressure changes. In *E. coli*, the Psp response to hyperosmotic shock is partially dependent on the regulatory proteins PspB and PspC (Weiner *et al.*, 1991). The absence of these proteins in *B. pseudomallei* may account for the lack of BPSL2105 induction under similar conditions.

A PspA homologue is known to be expressed during the stationary phase in *B. pseudomallei* (Wongtrakoongate *et al.*, 2007), and it has been shown that PspA is important for stationary phase survival in *E. coli* as its loss results in a severe loss of fitness during this phase of growth (Weiner & Model, 1994). We have shown that the loss of BPSL2105 resulted in reduced viability of *B. pseudomallei* after several days of prolonged growth. As with the *E. coli* mutant, *B. pseudomallei* Δ *pspA* cultures showed an increase in pH over the course of the experiment, demonstrating a reduction in the ability of the bacteria to maintain the extracellular pH. Although not shown in this study, this lack of control strongly suggests dissipation of the PMF, which demonstrates a strong link to other PspA homologues which function to maintain the PMF in response to stress (Kleerebezem *et al.*, 1996). Whether the decrease in viability of the *B. pseudomallei* Δ *pspA* is linked to the change in its ability to maintain a tolerable extracellular pH has not been proven, but the *S. lividans* Δ *pspA* mutant showed a severe decrease in viability compared with the WT strain under alkaline conditions, indicating that this may be possible (Vrancken *et al.*, 2008). It has been proposed that the stationary phase is the normal state of affairs for bacteria, which rarely encounter conditions suitable for exponential growth in natural niches (Kolter *et al.*, 1993), indicating that BPSL2105 may play a more significant role under environmental survival conditions.

In *B. pseudomallei*, a PspA homologue has previously been shown to be downregulated at the stationary phase in an *rpoE* mutant, along with a number of other proteins important for responding to stress (Thongboonkerd *et al.*, 2007). This resulted in lower tolerance to osmotic and oxidative stress, and also in reduced viability in mammalian phagocytes. In order to identify a more specific role for PspA under these conditions, an intracellular survival assay was carried out using a mouse macrophage cell line. This demonstrated that *B. pseudomallei* Δ *pspA* was less able to survive when phagocytosed in the stationary phase and, as such, the loss of PspA at the stationary phase may be indicative of a possible role *in vivo*, where bacteria are more likely to be maintained at the stationary phase stage of growth. *B. pseudomallei* Δ *pspA* was able to survive initial uptake by macrophage cells but subsequently the number of intracellular bacteria declined. In addition to the Psp response, other extracytoplasmic stress responses are known to have an important role in intracellular survival and resisting the diverse stresses encountered within phagocytic cells (reviewed by Rowley *et al.*, 2006). The survival of *B. pseudomallei* Δ *pspA* exposed to selected stresses *in vitro* to mimic macrophage killing mechanisms failed to identify the specific cause for the reduced intracellular survival of the

mutant: there was no difference in survival between the WT and mutant strains under any of the conditions tested. This may indicate that it may not necessarily be a single mechanism killing the mutant. The Psp response may therefore have an important role in this complex and harsh environment, which has been implicated in macrophage infection in *S. enterica*, *S. flexneri* and *M. tuberculosis* (Datta *et al.*, 2015; Eriksson *et al.*, 2003; Lucchini *et al.*, 2005).

PspA is hypothesized to stabilize the cytoplasmic membrane in times of stress by forming large structures that associate with the membrane to prevent leakage, particularly of protons, to maintain the PMF essential for many key bacterial processes (Kobayashi *et al.*, 2007). Previous studies have observed the formation of 36-mers by *E. coli* PspA *in vitro* (Hankamer *et al.*, 2004), supported by further data showing that PspA forms up to 36-mers *in vivo* (Jovanovic *et al.*, 2014b). Similarly, in *B. subtilis* the Lia system contains a PspA homologue, LiaH, which has been shown to form large oligomeric rings (Wolf *et al.*, 2010). The ability to form large oligomeric rings is also observed for the Psp homologue Vipp1 in cyanobacteria (Fuhrmann *et al.*, 2009). It has been demonstrated that the *E. coli* PspA structures interact with the phospholipids when in an oligomeric form (Kobayashi *et al.*, 2007). This study has shown that, similar to PspA of *E. coli* and *B. subtilis*, *B. pseudomallei* BPSL2105 assembles into large multimeric complexes. The α -helical domains in other PspA and PspA-like proteins have been shown to be vital for complex formation (Aseeva *et al.*, 2004; Joly *et al.* 2009), and this may be the case in *B. pseudomallei* where the secondary structure of BPSL2105 resembles these proteins. It may be postulated that these large complexes are the physiologically relevant form of PspA, functioning as scaffolds to maintain membrane integrity in the face of membrane disruption caused by stress.

In conclusion, *B. pseudomallei* BPSL2105 encodes a PspA-like protein that is induced in response to extreme heat shock, similar to the PspA protein of *E. coli*. It is important for survival during stationary phase and during infection of macrophages. The purified protein is able to assemble into high-order oligomers. This behaviour may aid in the stabilization of the *B. pseudomallei* cytoplasmic membrane during induction of the Psp-like response as a result of stressful conditions. Recently, it has been proposed that in *E. coli* PspA is potentially targeted to areas of the inner membrane associated with peptidoglycan biosynthesis machinery (Jovanovic *et al.*, 2014b). In light of this, the presence of a gene encoding a penicillin-binding protein, *mrcA*, directly upstream of *BPSL2105* may be significant. Future studies are needed to elucidate the importance of this and the function of downstream genes, such as *BPSL2106*, in the *B. pseudomallei* Psp response.

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