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*.

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 both phagocytic and non-phagocytic stresses, 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).
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 (Brisette 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., 2001; 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* which responds to similar stresses as the Psp systems of *E. coli* and *Y. enterocolitica*.

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 assemble 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 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::AppA plasmid was constructed by ligation of BglII-linearized pDM4 with the truncated BPSL2105.
Table 2. Primers used

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPSL2105 LF F</td>
<td>AGATCTTTGAGGCTGGTCAATGGAATCG</td>
<td>Bgl II</td>
</tr>
<tr>
<td>BPSL2105 LF R</td>
<td>CATATGTTGTATGCTGGAATATAG</td>
<td>Nde I</td>
</tr>
<tr>
<td>BPSL2105 RF F</td>
<td>CATATGGAAACGCTGATGCGTCGA</td>
<td>Nde I</td>
</tr>
<tr>
<td>BPSL2105 RF R</td>
<td>AGATCTGGAGCTGCGCGCGAGGTTC</td>
<td>Bgl II</td>
</tr>
<tr>
<td>BPSL2105 prom F</td>
<td>CGATCCGCGGTGAACCGGCTGATGGA</td>
<td>Pvu I</td>
</tr>
<tr>
<td>BPSL2105 comp R</td>
<td>GATCTGGTATCGGCGGGCGGGTGGTCA</td>
<td>Bam HI</td>
</tr>
<tr>
<td>RT PspA F</td>
<td>CGCCGACGATCAAGATGTTCTG</td>
<td>–</td>
</tr>
<tr>
<td>RT PspA R</td>
<td>GCGGCTGTTCGTGATACGGCTG</td>
<td>–</td>
</tr>
<tr>
<td>16S rRNA F</td>
<td>GATGACGGTACCGGAAAGAATAAGC</td>
<td>–</td>
</tr>
<tr>
<td>16S rRNA R</td>
<td>CCGTGTCAAGGCGGATGTAAGGTT</td>
<td>–</td>
</tr>
<tr>
<td>PspA F</td>
<td>GCGCGCCGATCCATGTCGCTTTTCGACTCTATTTTC</td>
<td>Bam HI</td>
</tr>
<tr>
<td>PspA R</td>
<td>CGCGCGGACGACCTTCGACCGCGACGGCGCGGTTCAG</td>
<td>Sac I</td>
</tr>
<tr>
<td>BPSL2105-6 F</td>
<td>GTGGTGAGACAGGCTCAAG</td>
<td>–</td>
</tr>
<tr>
<td>BPSL2105-6 R</td>
<td>GGATCGGTGAACGGCGAGTTG</td>
<td>–</td>
</tr>
</tbody>
</table>

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 ΔpspA, the pDM4 ΔpspA plasmid was transformed into E. coli S17-Δpir cells before conjugation with B. pseudomallei K96243. The merodiploid strain was screened for chloramphenicol resistance before sucrase 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 ΔpspA, 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-groSR-RFP (Wand et al., 2011). The pBHR4 ΔpspA plasmid was transferred by conjugation into B. pseudomallei ΔpspA. The complemented strain B. pseudomallei ΔpspA/ pBHR4 ΔpspA 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 5% CO₂. 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 ~1×10⁶ c.f.u. ml⁻¹. This was serially diluted to 1×10⁴ c.f.u. ml⁻¹. The DMEM was removed from the J774A.1 cells and replaced with L-15 media containing 1×10⁶ 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 250 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 \textit{B. pseudomallei}, adjusting to OD_{590} 0.35–0.40 in LB broth and then serially diluting to a concentration of $1 \times 10^8$ 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 His$_6$-tagged PspA-like protein.**

BPSL2105 was amplified by PCR using the primers PspA F/PspA R and cloned into the corresponding sites in the pET28 vector (Novagen). The resulting pET28-His$_6$-PspA plasmid encoded N-terminal His$_6$-tagged BPSL2105 (His$_6$-PspA), verified by DNA sequencing (Eurofins MWG), with a molecular mass of 27,876 kDa. His$_6$-PspA was expressed in \textit{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 OD$_{600}$ ~ 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 Ni$^{2+}$-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), carbonic anhydrase (29 kDa) and cytochrome $b_5$ (12.4 kDa).

**RESULTS**

**BPSL2105 from \textit{B. pseudomallei} is a putative PspA homologue**

Several putative PspA homologues (annotated as PspA family proteins) from a number of \textit{Burkholderia} species were compared with the \textit{B. pseudomallei} K96243 sequenced genome. Although it is currently annotated as a hypothetical protein, BPSL2105 in \textit{B. pseudomallei} K96243 has 99–100 % identity to many of these proteins, including proteins from both the less pathogenic \textit{Burkholderia thailandensis} and the close relative \textit{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 \textit{E. coli} and \textit{Y. enterocolitica} using \texttt{BLAST} (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Fig. S1). This comparison showed low identity compared with the \textit{Enterobacteriaceae}, e.g. BPSL2105 had 22 % identity to \textit{Y. enterocolitica} PspA and 21 % overall identity to \textit{E. coli}. Similarly, less well characterized PspA homologues in \textit{B. subtilis} and \textit{S. lividans} showed 28 and 31 % identity, respectively. However, PspAs in the \textit{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 \textit{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 \textit{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 \textit{B. pseudomallei} that possess PspA family proteins. RT-PCR was performed on RNA isolated from \textit{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 \textit{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 \textit{E. coli} (Brissette et al., 1990). To investigate the effect of temperature on expression of BPSL2105 in \textit{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 \textit{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 \textit{B. pseudomallei}, expression of PspA under high-salt conditions requires the PspBC system.

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 × 50 000 to × 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±SEM. Results were statistically analysed using a two-way ANOVA and Bonferroni post-tests. P<0.05 was considered statistically significant.
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 a-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. pseudomalleiΔpspA, 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 ΔpspABC strain shows a sharp decline in viability after day 9 at the stationary phase compared with the WT.

B. pseudomalleiΔpspA was grown in LB broth at 37 °C with aeration for the duration of the experiment. Both B. pseudomallei WT and ΔpspA mutant maintained a density of ~5 × 10⁹ c.f.u. ml⁻¹ during the first 6 days (144 h). From day 7, B. pseudomalleiΔpspA began to decline in viability compared with the WT (Fig. 2a). The WT phenotype was restored in ΔpspA 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. pseudomalleiΔpspA 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).
**B. pseudomallei**

DpsA 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*ΔpSpA 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*ΔpSpA showed a reduction in the number of intracellular bacteria at 24 h (Fig. 3). Complementation of *B. pseudomallei*ΔpSpA 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*ΔpSpA, 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, ΔpSpA and the complemented strain (data not shown).

Macrophages infected with *B. pseudomallei*ΔpSpA 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

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**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 (●), ΔpSpA (□) and ΔpSpA complemented with pBHR4.pSpA (Δ) were diluted in 100 ml LB broth to OD_590 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±SEM from three independent experiments; *P<0.05.

**Fig. 3.** Intracellular survival of stationary phase *B. pseudomallei* in J774A.1 macrophages. Macrophage cells were infected with 1×10⁷ 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 μg kanamycin ml⁻¹ and periodically lysed in order to enumerate the number of intracellular bacteria. Values are the mean±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 ΔpspA 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 μg 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 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
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 \( \sim 5\)–\(10\)\% 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 PspA proteins 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 S. 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 hyperosmotic 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 (Wongtrakooongate 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. pseudomalleiΔpspA 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. pseudomalleiΔpspA is linked to the change in its ability to maintain a tolerable extracellular pH has not been proven, but the S. lividans ΔpspA 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. pseudomalleiΔpspA 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. pseudomalleiΔpspA 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. pseudomalleiΔpspA exposed to selected stresses in vitro to mimic macrophage killing mechanisms failed to identify the specific cause for the reduced intracellular survival of the.
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.

REFERENCES


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