Supplemental Material

Membrane Bound PenA β-lactamase of *Burkholderia pseudomallei*

Linnell B. Randall, Karen Dobos-Elder, Krisztina M. Papp-Wallace, Robert A. Bonomo and Herbert P. Schweizer

1. Evaluation of methods for extraction of periplasmic proteins

Our previous studies were hampered by lack of a well-controlled method for extraction of periplasmic proteins from *B. pseudomallei*. Although several studies used either osmotic shock (1) or chloroform extraction (2) for release of periplasmic proteins from *B. pseudomallei*, a major limitation of judging the efficacy of these methods is lack of a reliable periplasmic marker protein. *E. coli* alkaline phosphatase (PhoA) is a well-characterized periplasmic protein (3), whose activity in the periplasm has been utilized in various reporter systems (4-6). In addition, alkaline phosphatase activity can easily be detected and quantified using the colorimetric substrate *p*-nitrophenylphosphate (*p*NPP). A *B. pseudomallei* control strain was generated that constitutively expresses the *E. coli phoA* gene from the constitutive *B. thailandensis* ribosomal S12 gene promoter (P_{S12})(7), for the purpose of comparing different methods of extracting periplasmic proteins from *B. pseudomallei*. Extraction efficiency was assessed by measuring alkaline phosphatase activity in each of the extracted fractions. Strain Bp82 was used as a negative control.

1.1 Construction of a Bp82 reporter strain expressing *E. coli* PhoA

The *phoA* gene was PCR amplified on a 1,435-bp DNA fragment from *E. coli* MC4100 (8, 9) genomic DNA using primers 2390 (5'-CATGGAGAAAATAAAGTGAAACAAAGC) and 2391 (5'-GGTTTTATTTCAGCCCCAGAG) and cloned into pGEM-T Easy (Promega, Madison, WI) to form pPS2921. Next, a 1,525 bp *Zra*I-*Sac*I fragment from pPS2921 containing *phoA* was ligated into *Sma*I+*Sac*I-linearized pUC18T-mini-Tn7T-FKM-P_{S12} (Schweizer laboratory collection) to form pUC18T-mini-Tn*7*T-FKM-P_{S12}-*phoA* (or pPS2669). The mini-Tn*7*T-FKM-P_{S12}-*phoA* construct was transposed to the Bp82 *glmS2*-associated *att*Tn*7* site as previously described (7), to generate Bp82.75 (Bp82::P_{S12}-*phoA*). Constitutive PhoA expression in this strain was confirmed by whole cell enzyme assay (**Fig. S1**) and Western blot analysis (**Fig. S2**). The low-level phosphatase activity in Bp82 is likely due to an endogenous phosphatase, perhaps *acpA* (2). A capsule deficient Δ(*wcbR-A*) Bp82.75 derivative, Bp82.101, was used to assess possible effects of capsule on periplasmic protein extraction efficiency.

1.2 Periplasmic fractionation methods

1.2.1 Cold osmotic shock

Cold osmotic shock was performed according to the method of Higgins and Hardie (10). 0.5 ml of 0.5 M Tris (pH 7.8) was added to 5 ml of overnight cultures and the mixture was incubated for 10 min at room temperature. Cells were collected by centrifugation at 2,500xg for 10 min at room temperature. Pellets were resuspended in 0.8 ml of sucrose solution (30 mM Tris [pH 7.8]; 40% sucrose; 2 mM EDTA), incubated with shaking for 10 min at 30°C, and centrifuged for

30 s at 14,000xg at room temperature. Pellets were rapidly resuspended in 0.5 ml ice cold water, incubated on ice for 10 min, and the suspensions were centrifuged at 14,000xg for 5 min at 4°C. Supernatants (periplasmic fractions) were removed and stored at -20°C.

1.2.2 PeriPreps Periplasting Kit

The PeriPreps Periplasting Kit from EpiCentre Biotechnologies (Madison, WI) is a commercial version of the cold osmotic shock procedure and was used according to the manufacturer's instructions. Cells from a 1 ml bacterial culture grown overnight were harvested by centrifugation at room temperature for 30 s at 14,000xg, resuspended in 50 µl PeriPreps Periplasting Buffer, and incubated for 5 min at room temperature. 50 µl ice cold water was added and mixed by inversion. The suspension was incubated for 5 min on ice, and then centrifuged for 2 min at 14,000xg at room temperature. Supernatants, containing periplasmic fractions, were removed to clean tubes and stored at -20^{o}C.

1.2.3. Chloroform shock treatment

This method was first described by Ames *et al.* (11). Cells were collected from 2 ml of overnight cultures by centrifugation for 30 s at 14,000xg at room temperature. Pellets were resuspended in approximately 20 µl of residual medium, 20 µl of chloroform was added, and the cell suspensions were vortexed periodically while incubating at room temperature for 15 min. 200 µl of 10 mM Tris (pH 8) was added, followed by centrifugation at 6,000xg for 20 min at room temperature. The top, aqueous layers were removed as periplasmic fractions and stored at -20°C.

1.2.4 Alkaline phosphatase assays

Alkaline phosphatase activity was detected using the colorimetric substrate *p*-nitrophenyl-phosphate (*p*NPP). In microtitre plates, 20 µl of cellular fractions were diluted in 180 µl 10 mM Tris (pH 8.0), and 4 µl of 40 mM *p*NPP (Sigma-Aldrich, Saint Louis, MO) was added and mixed by pipette. Samples were incubated 30 min at 37°C, and the A_{410nm} was read on a Multiskan Spectrum plate reader (Thermo Fisher Scientific, Waltham, MA). Samples were assayed in duplicate, and alkaline phosphatase activity was calculated as (average sample A_{410nm} - average blank A_{410nm})/(A_{600nm} starting culture x ml culture used). Each assay was repeated in three separate technical replicates.

1.2.5 Chloroform treatment is more efficacious than cold osmotic shock in releasing periplasmic proteins from *B. pseudomallei*

As shown in **Fig. S1**, chloroform shock treatment extracted more alkaline phosphatase activity from Bp82.75 (Bp82::P_{S12}-*phoA*) than either the PeriPreps or cold osmotic shock methods, though none of the methods extracted all of the activity seen in whole cells, indicating that periplasmic protein extraction was not complete. Presence of capsule had no effect on alkaline phosphatase extraction efficacy since no difference in activity was seen in extracts derived either from capsule containing Bp82.75 (Bp82::P_{S12}-*phoA*) or Bp82.101 (Bp82 Δ[*wcbR-A*]::P_{S12}-*phoA*) lacking the capsule synthesis genes.

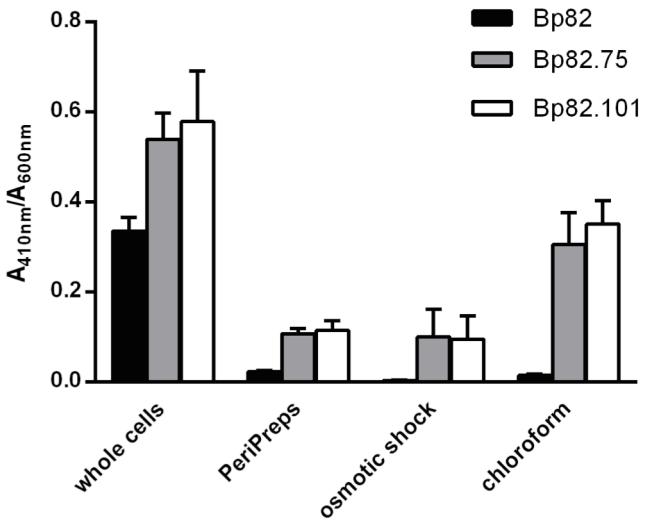

Figure S1. Alkaline phosphatase activity in Bp82, Bp82.75 (Bp82::P_{S12}-*phoA*) and Bp82.101 (Bp82 Δ(*wcbR-A*)::P_{S12}-*phoA*). Periplasmic fractions were prepared using the PeriPreps, cold osmotic shock, and chloroform shock methods. Alkaline phosphatase activities were measured and are expressed as (average sample A_{410nm} - average blank A_{410nm})/(A_{600nm} starting culture x ml culture used). Each assay was performed in duplicate, and values shown are the averages of two separate assays. Error bars represent the standard error of the mean.

Table S1. Antibodies used for Western blot analyses

Antigen	Antibody Type	Dilution	Source
E. coli PhoA	Mouse monoclonal	1:1,000	Life Technologies, Carlsbad, CA
B. pseudomallei PenA	Rabbit polyclonal	1:1,000	(12)
E. coli RpoB	Mouse monoclonal	1:2,000	Neoclone, Madison, WI
B. pseudomallei Omp85	Mouse polyclonal	1:500	(13)
Mouse IgG	Goat polyclonal, HRP conjugate	1:2,500	Promega, Madison, WI
Rabbit IgG	Goat polyclonal, HRP conjugate	1:2,500	Promega, Madison, WI

2. *E. coli* PhoA, but not PenA, is localized in the *B. pseudomallei* periplasm

Upon establishment of a successful periplasmic extraction method in *B. pseudomallei*, defined whole cell and periplasmic fractions obtained by chloroform extraction were examined alongside whole cell fractions for the presence of PhoA and PenA by Western blot to detect proteins and enzyme assays to determine enzyme activities, respectively. PhoA (approximately 50 kDa) was present in substantial amounts in both whole cell and periplasmic fractions from Bp82.75 (Bp82::P_{S12}-*phoA*), which is in agreement with alkaline phosphatase enzyme activity in both whole cells and periplasmic fractions. No PhoA protein or activity was detectable in extracts derived from Bp82 and Bp82 Δ*penA*. The 85 kDa outer membrane protein Omp85 (13) and the 150 kDa cytoplasmic RpoB protein were present in whole cell fractions from all three strains, but absent from any of the periplasmic fractions, indicating that periplasmic fractions are free of outer membrane and cytoplasmic contamination.

Whole cell fractions derived from both strains with the wild-type *penA* gene (Bp82 and Bp82.75 [Bp82::P_{S12}-*phoA*]) contain a strong ~27 kDa PenA band that is absent in Bp82Δ*penA*. However, periplasmic fractions from these strains contain very little PenA protein. Furthermore, while β-lactamase activity from PenA is present in wild-type Bp82 whole cell fractions (as compared with Bp82Δ*penA* [Bp82.11]), essentially no β-lactamase activity could be detected in chloroform extracted periplasmic fractions (**Fig. 1** in main paper).

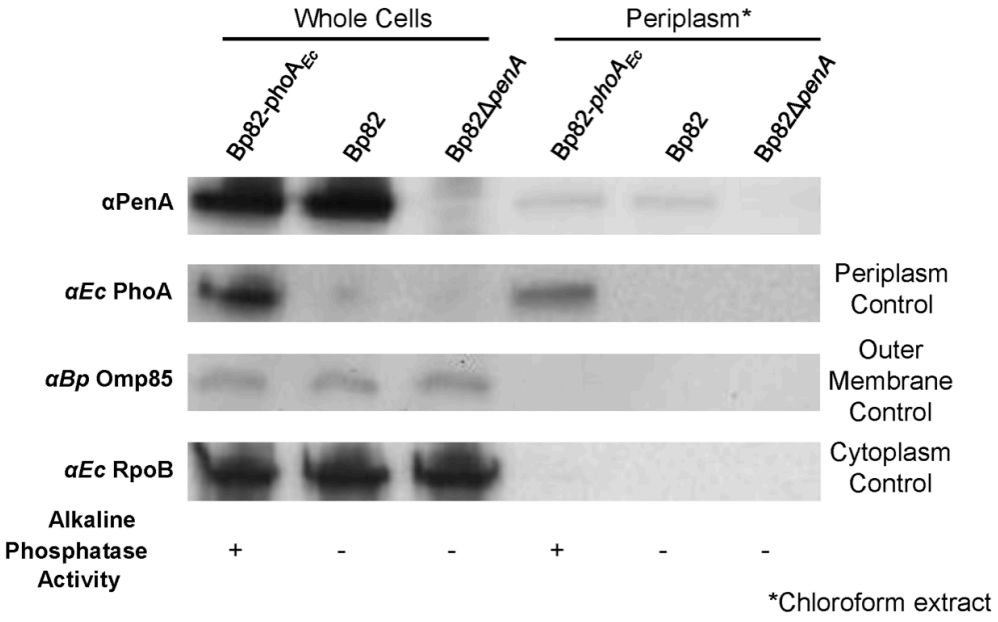

Figure S2. Cellular localization of PenA assessed by Western blot analysis. Whole cell and chloroform-extracted periplasmic fractions derived from Bp82 expressing E. coli *phoA* (Bp82-*phoA*$_{EC}$ or Bp82.75), Bp82, and Bp82Δ*penA* (Bp82.11) were separated by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against PenA, PhoA, Omp85, and RpoB. The panels shown represent four separate blots. Presence or absence of alkaline phosphatase activity in the respective fractions is indicted at the bottom by + and – symbols.

3. Reverse transcriptase quantitative real time PCR (RT-qPCR) to assess transcription of wild-type *penA* and *penA*$_{C23S}$

Overnight cultures were subcultured into LB + 80 μg/ml adenine, and upon reaching log phase (OD_{600} between 0.6 and 0.8), RNA was extracted from 1 ml of culture using the Qiagen RNeasy RNA Protect Mini Bacteria kit according to manufacturer's instructions (Qiagen, Germantown, MD). RNA was quantified with a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA), and 1 μg was treated with DNaseI (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized using the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA). qPCR was then performed on an iCycler iQ instrument (Bio-Rad Laboratories, Hercules, CA) with SYBR GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) with primers P2077 (5'-GTTCTGCAGCA-CATCCAAGA) and P2078 (5'-CGGTGTTGTCGCTGTACTGA)(12) to amplify *penA* derived

cDNA and Bp23S-F (P1516; 5'-GTAGACCCGAAACCAGGTGA) and Bp23S-R (P1517; 5'-CACCCCTATCCACAGCTCAT) (14) to amplify 23S ribosomal RNA derived cDNA. Each sample was assayed in technical triplicate, melt curve analysis showed clean amplification, and control reactions with no template, or template untreated with reverse transcriptase showed no amplification. *penA* transcript levels were normalized to the 23S rRNA gene, and expressed as fold change relative to Bp82.

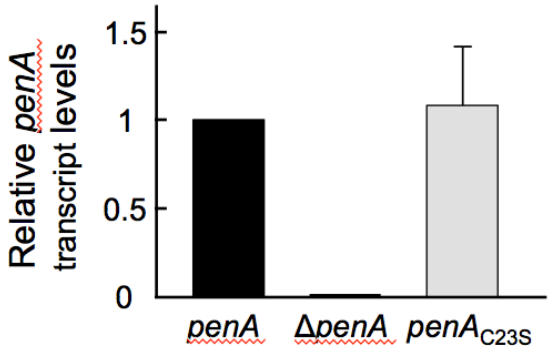

Figure S3. The *penA*$_{C23S}$ mutant gene is transcribed at wild-type levels. qRT-PCR was employed to determine *penA* transcripts levels in Bp82 (wild-type *penA*, Bp82 Δ*penA* (Bp82.11), and Bp82 *penA*$_{C23S}$ (Bp82.143). Data shown represent the average expression from three biological replicates, relative to Bp82. The error bar represents one standard deviation from the mean.

References

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