IAI Accepted Manuscript Posted Online 28 December 2015
Infect. Immun. doi:10.1128/IAI.01102-15

Characterization of new virulence factors involved in the intracellular growth and survival of *Burkholderia pseudomallei*

Madeleine G. Moule[1], Natasha Spink[2], Sam Willcocks[1], Jiali Lim[1], José Afonso Guerra-Assunção[1], Felipe Cia[2], Olivia Champion[3], Nicola Senior[3], Helen S. Atkins[4], Taane Clark[1], Gregory J. Bancroft[2], Jon Cuccui[1], Brendan W Wren[1#]

[1] Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, Keppel St., London, WC1E 7HT, United Kingdom.

[2] Department of Infection and Immunity, London School of Hygiene and Tropical Medicine, Keppel St., London, WC1E 7HT, United Kingdom.

[3] School of Biosciences, University of Exeter, Devon, United Kingdom.

[4] Defence Science and Technology Laboratory, Porton Down, Salisbury, Wiltshire, SP4 0JQ, United Kingdom.

[#] Corresponding author. Mailing address: London School of Hygiene and Tropical Medicine, Keppel St., London, WC1E 7HT, United Kingdom. Phone: +44 (0) 20 7927 2288. Email: bwren@lshtm.ac.uk

ABSTRACT

Burkholderia pseudomallei, the causative agent of melioidosis, has a complex and poorly understood extracellular and intracellular lifestyle. We used transposon insertion-site sequencing (TraDIS) to retrospectively analyze a transposon library that had previously been screened through a Balb/c mouse model to identify genes important for growth and survival *in vivo*. This allowed us to identify the insertion sites and phenotypes of negatively selected mutants that were previously overlooked due to technical constraints. All 23 unique genes identified in the original screen were confirmed by TraDIS and an additional 105 mutants were identified with varying degrees of attenuation *in vivo*. Five of the newly identified genes were chosen for further characterization and clean, unmarked deletion mutants of *bpsl2248, tex, rpiR, bpsl1728* and *bpss1528* were constructed in the wild-type strain K96243. Each of these mutants was tested *in vitro* and *in vivo* to confirm their attenuated phenotypes and investigate the nature of the attenuation. Our results confirm that we have identified new genes important to *in vivo* virulence with roles in different stages of *B. pseudomallei* pathogenesis including extracellular and intracellular survival. Of particular interest, deletion of the transcription accessory protein Tex was shown to be highly attenuating and the *tex* mutant was capable of providing protective immunity against challenge with wild-type *B. pseudomallei*, suggesting that the genes identified in our TraDIS screen have the potential to be investigated as live vaccine candidates.

INTRODUCTION

Burkholderia pseudomallei is a gram-negative, motile saphrophytic bacterium that is the causative agent of melioidosis. This emerging human pathogen is endemic to the soil and water of tropical areas including Thailand, Singapore, and northern Australia and can cause infection through contact with broken skin or through ingestion or inhalation of the bacterium (1). The resulting disease can manifest as a localized skin ulcer or can progress to a systemic infection that is associated with mortality rates as high as 50% in some endemic regions (2)There is currently no licensed vaccine available against *B. pseudomallei* and it is highly resistant to most antibiotics, severely limiting treatment options (3). Due to the virulent nature of the pathogen, potential for aerosol transmission, and lack of therapeutic options, *B. pseudomallei* is listed as a Tier 1 bioterrorism threat by the Centers for Disease Control and Prevention (4).

B. pseudomallei is a facultative intracellular pathogen capable of invading and replicating within both epithelial cells and macrophages (5) While *B. pseudomallei* is capable of extracellular growth and survival and is highly resistant to complement-mediated killing in human sera, intracellular growth is essential for virulence (2, 6) When *B. pseudomallei* enters the host cell, either through phagocytosis or by inducing its own uptake into non-phagocytic cells, it is able to escape from the phagosome or endocytic vacuole into the cell cytoplasm (7). There, *B. pseudomallei* is able to exploit the host cell cytoskeleton by inducing actin polymerization at one pole of the bacterium, forming actin comet tails which propel the bacteria through the cytoplasm and forming membrane protrusions into adjacent cells, facilitating cell-to-cell spread (8). Unique among bacterial pathogens that polymerize actin for motility, *B. pseudomallei* is capable of inducing cell fusion upon contact with neighboring cells, resulting in the formation of multinucleated giant cells (MNGCs) that can contain up to hundreds of nuclei (9).

This complex intracellular lifestyle is regulated by a number of virulence factors encoded within the large 7.25 megabase *B. pseudomallei* genome including three type III secretion systems (T3SS), six type VI secretion systems (T6SS), multiple polysaccharide loci, and a number of secreted effectors (10). The *B. pseudomallei* polysaccharide capsule and lipopolysaccharide (LPS) help the bacteria survive extracellularly and resist complement deposition(2, 11, 12),while the *Bsa* T3SS has been implicated in helping *B. pseudomallei* induce uptake into non-phagocytic cells, escape the vacuole, and resist killing by autophagy (13, 14). In addition, actin polymerization has been shown to be mediated by the autotransporter BimA, which is expressed on one pole of the bacteria and stimulates the formation of new actin filaments (15, 16) Finally, the T6SS-1 is required for cell fusion and the formation of MNGCs (17, 18)

The identification and characterization of these important virulence factors has greatly improved our understanding of *B. pseudomallei* pathogenesis. However, much remains poorly understood and the vast majority of *B. pseudomallei* virulence factors remain to be identified. One technique that has been highly successful at identifying genes that are required for the *in vivo* virulence of many bacterial species has been the application of large-scale forward genetic screens using libraries of bacterial transposon insertion mutants (19-24). We have previously successfully applied this strategy to the study of *B. pseudomallei* using an approach known as signature tagged mutagenesis (STM) in which pools of mutants each containing a unique tag are used to infect an animal model (25, 26). By comparing the population of mutants present in infected animals (output pools) to the original pool of mutants used to infect the animals (input pools), it is possible to identify mutants that are unable to survive and grow *in vivo*. Rhis method identified the *B. pseudomallei* capsule and the branched chain amino acid synthase *ilvE* as essential for *in vivo* survival, which led to the development of an *ilvE* mutant as a live attenuated vaccine candidate. A number of additional

virulence factors have also been identified by this method, the majority of which are predicted to be involved in metabolism and replication (25, 26). However, these studies were constrained by technical limitations regarding library size and lacked the sensitivity to distinguish mild attenuation phenotypes. While microarray technology was used to identify mutants negatively selected in the output pools, the insertion site of each mutant needed to be identified individually using a difficult and time-consuming PCR approach. As a result, only the most strongly attenuated mutants were followed up to determine the gene of interest.

More recently, the development of next-generation sequencing technology has resulted in the development of transposon library sequencing techniques known as transposon insertion-site sequencing (TraDIS) and tn-seq that allow entire libraries to be screened and all insertion sites identified quickly and easily. This technique has been applied to large bacterial libraries to identify every essential gene within the genome and to identify new *in vivo* virulence factors (27, 28). It can also be retrospectively applied to previously screened STM libraries to identify the insertion sites and phenotypes of mutants that were previously overlooked due to technical constraints, allowing the identification of new virulence factors without undertaking further animal experiments (29) Here we describe the retroactive sequencing of a *B. pseudomallei* K96243 STM library that we previously screened through an *in vivo* mouse model (26). Using this improved technique we were able to identify many new potential virulence factors and overcome biases that had constrained the original screen without the requirement for further animal experiments. Moreover, we were able to identify mutants with intermediate phenotypes that would otherwise have been overlooked. We selected five of these newly-identified mutants for additional characterization and created clean unmarked deletion mutants for each gene of interest. We then confirmed the *in vivo* growth and survival defect identified in our screen and examined the ability of each mutant to enter and replicate within epithelial cells and macrophages and complete the *B. pseudomallei*

intracellular lifecycle as well as survive extracellular sera killing. We found that each of these mutants was attenuated to varying degrees, confirming that we have identified new genes with important roles in different stages of *B. pseudomallei* pathogenesis and increasing our understanding of this important human pathogen.

MATERIALS AND METHODS

Bacterial strains and culture conditions

B. pseudomallei strain K96243, a clinical isolate from Thailand was used for the construction of the STM library and for each of the individual mutants . *Escherichia coli* 19851 (*pir*+) was used for direct conjugation in the construction of the STM library, and *E. coli* MFD*pir* was used for conjugation in the construction of individual mutants (30). All experiments were performed in Luria-Bertani (LB) broth or agar at 37°C, and *E. coli* MFD*pir* cells were also supplemented with 0.3 mM diaminopimelic acid (DAP). When necessary plates and cultures were supplemented with antibiotics at the following concentrations: 100 µg/mL Zeocin (Life Technologies), 400 µg /mL kanamycin, 100 µg /mL ampicillin.

Genomic DNA Extraction

10 mL of overnight shaken cultures was spun down at 4000 RPM in a bench top centrifuge and resuspended in 10 mL of lysis buffer (100 µg/mL proteinase K, 10 mL NaCL, 20 mL Tris HCl pH8, 1 mM EDTA, 0.5% SDS). 3 mL of sodium perchlorate was added to the solution and incubated for 1 hour at room temperature. Genomic DNA was isolated using a phenol:chloroform:isoamyl alcohol extraction (25:24:1), precipitated with ethanol and spooled into deionised water.

Illumina Sequencing

Approximately 5 µg of genomic DNA from each of the input, lung and spleen samples was fragmented to ~300 bp by sonication in a BioRupter. The fragmented DNA was end repaired

and A-tailed using the NEBNext DNA library preparation reagent kit for Illumina (NEB). Annealed adapters Ind_Ad_T(ACACTCTTTCCCTACACGACGCTCTTCCGATC*T, * indicates phosphorothioate) and Ind_Ad_B(pGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTC) were ligated onto the samples. PCR was performed using primers PE_PCR_V3.3(CAAGCAGAAGACGGCATACGAGATCGGTACACTCTTTCCCTACACGACG CTCTTCCGATC) and MnTn*5*_P5_3pr_3(AATGATACGGCGACCACCGAGATCTACACCTAGGCtGCGGCtGCACTT GTG), which include flow cell binding sites. The PCR program used was 2 minutes at 94°C, 22 cycles of (30 s at 94°C, 20 s at 65°C and 30 s at 72°C), and 10 minutes at 72°C. They were then size selected to between 200-400 bp in a 2% agarose gel made up with 1xTBE buffer, with purification by Qiagen Gel Extraction kit. The final concentration of the samples were checked by both BioAnalyzer and qPCR. Preparation products were sequenced on an Illumina Hi-Seq 2000 as 36 bp single-end reads. Concentration of the samples was established using qPCR with the primers Syb_FP5(ATGATACGGCGACCACCGAG) and Syb_RP7(CAAGCAGAAGACGGCATACGAG). They were then size selected to between 300-500 bp in a 2% agarose gel made up with 1xTBE buffer, with purification by Qiagen Gel Extraction kit. The final concentration of the samples were checked by both BioAnalyzer and qPCR. Preparation products were sequenced on an Illumina Hi-Seq 2000 as 100 bp single-end reads.

Bioinformatic and statistical analysis

Raw reads that passed quality control filters and contained the transposon were mapped onto the *B. pseudomallei* K96243 reference genome (version 6) using *bowtie* (version 2-1.0) allowing for zero mismatches, and excluding non-uniquely mapped reads. The SAMtools toolkit (*samtools.sourceforge.net*) was applied to the alignment files to determine insertion

sites and coverage. For differential expression analysis, the coverage values were variance-stabilized using an arcsine-root transformation , and log_2 ratios between the input pools and the lung and spleen samples were calculated. Minimum starting values of 200 sequencing reads within the input pool were used to ensure sufficient starting quantities for negative selection analysis and avoid background. To define negative selection, cut-offs of the lowest 2.5% of log_2 ratios within the spleen pool comparisons and the lowest 5% of ratios within the lung pool comparisons were set based on the mean distribution of the log_2 fold change.

Generation of clean deletion mutants

Unmarked deletion mutants were constructed as has been previously described using the suicide vector pDM4 (31). Briefly, 600-1000 bp regions flanking each gene of interest were amplified with an XbaI restriction site on the 5' end and overlapping sequences on the 3' end of the PCR product using Phusion High-Fidelity PCR master mix (ThermoScientific). The resulting products were then spliced together using splicing by overlapping extension PCR (SOE PCR) to generate a full-length product consisting of the upstream and downstream flanks lacking the target gene. This product was then cloned into the intermediate plasmid pGEM-T and then subcloned into pDM4 using XbaI. The resulting mutagenesis construct was then introduced into *E. coli* MFD*pir* cultured in LB media containing 0.3 mM DAP, and then transferred into *B. pseudomallei* K96243 by direct mating. Merodiploids containing the integrated plasmid were selected for on LB agar containing 30 µg/ml chloroamphenicol, and screened using primers designed against the gene of interest. Successful clones were then plated onto high-sucrose agar (10 g/L tryptone, 5 g/L yeast extract, 100 g/L sucrose) and grown for 48-72 hours at 24°C. Colonies were screened for sensitivity to chloroamphenicol due to loss of the pDM4 cassette, as well as by PCR using primers designed against the gene of interest and across the deletion junction. The resulting mutants were confirmed by full genome sequencing using an Illumina MiSeq sequencer to confirm the loss of pDM4 and the

null mutation. The primers used for mutagenesis and screening the resulting clones are listed in Supplemental Table 2.

Mouse infections

Female BALB/c mice (Charles Rivers Laboratories International, INC, Kent, UK) aged between 6-8 weeks were used. Mice were housed under specific pathogen-free conditions, with free access to food and water. All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act of 1986 and the local Ethical Review Committee, under animal biohazard Containment Level 3 conditions (CL3). For infections, aliquots of *B. pseudomallei* K96243 mutants were thawed from frozen stocks, diluted to the desired concentration in pyrogen-free saline (PFS), and administered via the intranasal route (i.n.). A sample of the inoculum was diluted appropriately, plated out on TSA and incubated overnight at 37°C to confirm the actual inoculation dose. For each infection, mice were anaesthetised intraperitoneally (i.p.) with a combination of Ketamine (50 mg/kg; Ketalar, Pfizer ltd, Kent, UK) and Xylazine (10 mg/kg; Rompun; Berkshire, UK) diluted in PFS. Each mouse was weighed and the volume of anaesthetic given was adjusted accordingly. Once mice were anaesthetised, the inoculum was administered by slowly pipetting a total of 50 µl into both nostrils. Mice were then held upright for 30 sec to ensure the liquid had passed into the lungs and were monitored until they had fully recovered from the anaesthetic. In all cases mice were checked at least daily for signs of illness, and if determined to have reached the humane end point specified in the Project Licence, were culled.

Tissue culture infections

A549 human lung epithelial cells were grown in F12-K tissue culture medium supplemented with 10% fetal bovine serum (FBS), and J774 mouse macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. For invasion and intracellular growth assays, $2x10^5$ cells were seeded into 24-well tissue cultures dishes and

allowed to adhere for 16 hours. The cells were then washed with PBS and inoculated with $2x10^6$ CFU of wild-type *B. pseudomallei* or one of the TraDIS mutants in 1 mL of DMEM. The infection was allowed to proceed for one hour, at which point the media was removed, the cells washed with PBS, and 1 mL fresh DMEM or F12-K media containing 200 μg/mL of kanamycin was added to the wells. The cells were incubated with antibiotics for 2 hours at 37°C. For invasion assays, the cells were then lysed immediately with 0.1% Triton-X100 and 10-fold dilutions were plated out onto LB agar to determine how many cells were internalized. For intracellular growth assays, the infections were allowed to proceed for 6-24 hours, at which point the cells were lysed and CFUs plated as described.

Immunofluorescence

J774A mouse macrophages were seeded onto glass coverslips in 6-well tissue cultures plates at a concentration of 10^5 CFU/mL and infected with Burkholderia strains as described above. At 24 hours post-infection, the cells were washed twice with PBS, and fixed with 4 % paraformaldehyde overnight at 4°C. The fixed cells were then washed again with PBS, permeabilized with 0.5 % Triton X-100, and blocked for 1 hour at 37°C with 5 % FBS. The cover slips were then incubated with a 1:1000 dilution of MAb CC6 (Jones et al., 2002) for 1 h at 37°C, washed 3 times in PBS for 5 minutes each, and then incubated again with a 1:10000 dilution of Alexafluor488-conjugated anti-mouse secondary antibody antibody (Molecular Probes) and Alexafluor555-phalloidin conjugate solution (Molecular Probes) for 1 hr at 37°C. The cells were then again washed 3 times for 5 minutes in PBS to remove unbound antibodies and stained with DAPI (Molecular Probes) according to manufacturer's instructions before the coverslips were mounted onto glass slides using DPX mounting medium. Samples were analyzed using a CCD fluorescence microscope (Axioplan 2 upright microscope).

Serum survival assays

Wild-type *B. pseudomallei* and the TraDIS mutant strains were incubated with 30% pooled NHS or heat-inactivated (HI) serum in PBS at 37°C for 2 hours. HI serum was prepared by incubating the NHS at 56°C for 1 hour. Following serum exposure, the samples were serially diluted and plated onto LB agar to determine viable bacteria counts.

RESULTS

Identification of novel *B. pseudomallei* K96243 genes important for growth and survival *in vivo*

We previously identified 39 *B. pseudomallei* mutants that were unable to grow and/or disseminate in an *in vivo* murine infection model using a signature tagged mutagenesis (STM) screen (26). Pools of 96 mutants were used to infect BALB/c mice via the intranasal route and mutants that were negatively selected in lungs and spleens were identified using microarrays directed against the unique tag on each mutant. However, due to the difficulty of identifying the transposon insertion site of each mutant with this method, only the most strongly attenuated mutants as visualized by microarray were selected to determine the nature of the mutation and verify the attenuated phenotype. We hypothesized that by applying the recently developed TraDIS sequencing technique we could quickly and easily extract additional information regarding *B. pseudomallei* pathogenesis from the archived bacterial genomic DNA samples from this STM screen without the requirement to undertake additional animal infections. We predicted that this method could identify additional mutants involved in pathogenesis, including those with more subtle effects acting at different stages of infection. To prepare TraDIS sequencing libraries, we pooled the archived genomic DNA samples from each input pool to create an input sample representing the entire library. As each original pool of 96 mutants was assayed through two mice, one mouse from each pool was combined to produce biological duplicate lung and spleen output pools. We then applied the TraDIS

sequencing technique and compared the input and output pools using a fold-change analysis. This improved method allowed us to gather information on every individual mutant within the library and determine whether they were negatively selected, positively selected, or unchanged between input and output pools.

To identify mutants that were negatively selected in the mouse lung and spleen samples, we used a previously described quantification method (29). The total number of sequencing reads matched to each gene in the library were converted using an arcsine-root transformation and $\log_2$ fold change values between input and lung and input and spleen pools were calculated to determine the fitness of each mutant in terms of its ability to colonize within lung tissue and disseminate to and colonize the spleen. To define attenuation, we set a cut-off of the 2.5% most attenuated mutants in the spleen and the 5% most attenuated mutants in the lung based on the mean distribution of the $\log_2$ fold change. This resulted in a list of 129 mutants that were negatively selected in mouse spleen samples representing approximately 10% of the library of 1248 mutants screened (Table S1). Nine of these mutants were also strongly negatively selected in the mouse lung despite being inoculated through an intranasal route, indicating an inability to survive in that tissue. None of the mutants screened in our experiment were positively selected by our statistical cut-offs. The original 39 mutants previously identified using STM mapped to 23 different genes, all of which were also identified as negatively selected by the TraDIS method. The majority of these genes were among the most strongly negatively selected, with 20 of the 23 genes found within the cut-off we selected of the top 2.5% of $\log_2$ ratios. The three remaining transposon mutations mapped either to intergenic regions or to sequences that matched more than one gene, and thus were unable to be confirmed. Six of the genes identified by STM, *wcbC, wcbJ, wcbN, gmhA, aroB,* and *vacJ* had previously been independently confirmed to be attenuated for growth and survival in individual intranasal infections of BALB/c mice *(J. Lim et al., unpublished data).* This confirms

the ability of our screen to identify attenuated *B. pseudomallei* mutants and serves as further proof of principle of the TraDIS assay

Confirmation of attenuated TraDIS mutant phenotypes with unmarked deletion mutants

Five genes identified as negatively selected in the spleen output pools, some of which were also negatively selected in the lungs, were selected for further characterization based on strength of phenotype and predicted functional domains. *Bpsl1527*, which encodes the transcription accessory protein Tex, was selected because this mutant demonstrated one of the strongest attenuated phenotypes in both lung and spleen output pools. Tex is required for toxin regulation in *Bordetella pertussis* and *Clostridum perfringens* and has been shown to play a role in virulence in *Pseudomonas aeruginosa* and *Streptococcus pneumoniae (32-35).* Moreover, the structure of the *P. aeruginosa* homolog has been solved and shown to bind DNA, suggesting that this gene is likely to function as a transcriptional regulator. Another strongly negatively selected putative transcriptional regulator, RpiR, is encoded by *bpsl0629.* RpiR has been demonstrated to regulate various virulence factors of *Staphylococcus aureus,* suggesting that this could be another conserved regulatory gene required for *in vivo* virulence (36). *Bpsl1728* and *bpss1528* both encode predicted secreted proteins, with *bpss1528* being a type III secretion system secreted protein and *bpsl1728* showing homology to be a secreted outer membrane porin from *Bordetella pertussis*. *Bpsl1728* was also of interest to us for technical reasons because it is present just above the predicted threshold of detection in our input pool, which allowed us to use this gene as an indicator of the sensitivity and accuracy of our TraDIS screen. *Bpss1528*, which encodes the putative Type III secretion system effector protein BapA was selected because although the *B. pseudomallei* T3SS-3 is known to be required for virulence, studies with this mutant in a hamster model did not display any survival phenotype, suggesting that our TraDIS assay may be able to pick up moderately attenuated mutants that would be missed in other screening methods (37) Finally, *bpsl2248* was selected

for further characterization because it encodes a putative glycosyltransferase that is not associated with any of the previously characterized polysaccharide loci in *B. pseudomallei.* Our previous STM screen and a number of additional studies have indicated the importance of polysaccharides to *B. pseudomallei* virulence (26, 38).

To absolutely confirm the attenuated phenotypes of each of these mutants and address the possibility of polar effects, clean unmarked deletion mutants were constructed for each gene as has been described previously (Logue et al., 2009). Briefly, a suicide plasmid containing a null allele consisting of the upstream and downstream flanking regions of the gene of interest was introduced via homologous recombination with chloramphenicol selection. A second recombination event was then selected for with *sacB*-mediated counter selection against sucrose sensitivity, and the resulting colonies were screened by PCR for loss of the wild-type allele. Each mutant was then verified by Illumina whole genome sequencing to confirm the expected deletion of each gene of interest and to ascertain that no secondary mutations had occurred. Three of the genes selected for mutagenesis, *bapA, rpiR,* and *bpss2248* are located within predicted operons, while *tex* and *bspl1728* do not have any downstream genes located within the same reading frame (39). Due to the nature of our mutagenesis strategy, we did not expect to see polar effects from any of the mutants we constructed, including those for genes within operons. However, to be certain that transcription of downstream genes was not affected by mutagenesis, we performed RT-PCR analysis of each gene within the operons of our genes of interest and the nearest genes to *tex* and *bspl1728* and found that transcription was not affected for any of the genes tested (Supplemental Figure 1).

The resulting deletion mutants, *Δtex*, *ΔrpiR, Δ1728, Δ2248,* and *ΔbapA* were each used to infect Balb/c mice via an intranasal route alongside five mice infected with wild type *B. pseudomallei* K96243. Colony forming units (CFUs) were plated from the inocula to determine

the exact infectious dose, and the infections were allowed to proceed for 48 hours to match the time point of the original screen. At this point lungs and spleens from each mouse were harvested, homogenized, and plated for CFUs. Four of the mutants, *Δtex*, *ΔrpiR, Δ1728,* and *Δ2248,* demonstrated significantly reduced CFUs in mouse spleens compared to wild-type *B. pseudomallei*. The *Δtex* mutants displayed the strongest attenuation within the spleen and also displayed strong attenuation within the lung, consistent with the TraDIS screen predictions. The remaining mutant, *ΔbapA* showed slightly reduced CFUs compared to wild-type, but this decrease was not statistically significant (Figure 1). These results showed that the TraDIS screen was not only able to identify genes important for growth and survival in a mouse model, but was able to do so in a semi-quantitative manner and predict the relative strength of phenotype.

B. pseudomallei Δtex mutants are highly attenuated and protect against challenge with wild-type _B. pseudomallei_

We next tested each TraDIS mutant in a survival assay to determine if the reduced CFUs seen in lung and spleen tissues correlated with decreased virulence. Interestingly, *ΔrpiR, Δ1728, Δ2248*, and *ΔbapA* demonstrated similar survival phenotypes to wild-type *B. pseudomallei* at an infectious dose of approximately 10^3 CFU despite significant reduction of bacterial CFUs in the spleen. This suggests that the sensitivity of our TraDIS assay allowed the identification of mildly attenuated phenotypes below the threshold of attenuation that would lead to a decrease in virulence as defined by survival. Supporting this hypothesis, the mutant with the strongest TraDIS phenotype, *Δtex,* showed increased mouse survival compared to wild-type bacteria, with over 80% long-term survival (Figure 2a). To determine if the surviving animals had completely cleared the infection with the *Δtex* mutant, we plated CFUs from four of the remaining mice at 60 days post-infection. We found that all four mice

retained *Δtex* CFUs within the spleen, while only half of the mice had CFUs above the level of detection within the lungs (Figure 2b).

Since *Δtex* proved to be attenuated in the acute model of infection, we sought to examine whether it is able to confer protection against subsequent challenge with virulent wild-type *B. pseudomallei*. Five weeks after intranasal challenge with either saline or *Δtex*, Balb/c mice were challenged with approximately 1000 CFU of *B. pseudomallei* K96243 and survival was monitored. Our data indicate that *Δtex* is able to provide protection in the acute model of infection (Figure 2c), resulting in significantly increased time to death. Analysis of organ CFU from surviving mice revealed the retention of wild-type bacteria in both the lung and spleen (Figure 2d) and splenomegaly in a minority of cases (data not shown). However, in contrast to challenge with *Δtex*, none of the surviving mice demonstrated retention of the *Δtex* mutant in lung or spleen (data not shown).

Δtex, _ΔrpiR_, _Δ1728,_ and _ΔbapA_ display decreased intracellular survival, but are able to complete the intracellular life cycle

B. pseudomallei is considered a facultative intracellular pathogen, but is highly resistant to killing by human sera and is able to survive and replicate extracellularly. We were interested in determining how large a role, if any, intracellular survival and replication played in the attenuated phenotypes of the TraDIS mutants. As *B. pseudomallei* is able to induce its own uptake into epithelial cells (9), we first analyzed invasion of A549 human lung epithelial cells by infecting a monolayer of cells with a multiplicity of infection (MOI) of 10 CFU of *Δtex*, *ΔrpiR, Δ1728, Δ2248, ΔbapA,* or wild type *B. pseudomallei* K96243 per cell and allowed the infection to proceed for one hour. The cells were then gently washed and kanamycin was added to the media to kill any remaining extracellular bacteria. At 2 hours post-infection, the cells were lysed and plated to determine the number of intracellular CFU. Intracellular bacteria were present for every condition tested, and none of the mutants appeared to be

388 internalized differently than wild-type bacteria, suggesting that they do not have defects
389 related to adhesion or invasion of host cells (Figure 3a).

390 We next analyzed whether the TraDIS mutants were able to survive and replicate
391 within A549 lung epithelial cells. We found that at 18 hours post-infection *Δ1728* and *Δ2248*
392 replicated to similar levels as wild-type *B. pseudomallei*, while *ΔrpiR* and *ΔbapA* showed
393 reduced intracellular CFUs. The most highly attenuated mutant, *Δtex*, demonstrated
394 significantly reduced levels of intracellular bacteria, suggesting that this mutant is either killed
395 by intracellular immune responses such as autophagy or is not capable of completing the
396 intracellular life cycle (Figure 3b). As *B. pseudomallei* is also capable of replicating within
397 professional phagocytes suchas macrophages, we also analyzed intracellular survival within
398 J774 mouse macrophage cells. We found that at 16 hours post-infection all of the mutants
399 with decreased CFUs within A549 cells were also attenuated within J774 cells. Interestingly,
400 the mutant *Δ1728,* which showed comparable intracellular growth and survival to wild-type *B.*
401 *pseudomallei* in A549 cells demonstrated reduced bacterial load in J774 cells, suggesting
402 susceptibility to innate immune killing mechanisms rather than intracellular survival (Figure
403 3c).

404 To determine whether the attenuated phenotypes of the TraDIS mutants is due to an
405 impaired intracellular life cycle, we analyzed the ability of each mutant to escape from the
406 phagocytic vacuole, polymerize actin to become motile within the host cell cytoplasm, and
407 form multinucleated giant cells (MNGCs) by fusing the infected host cell with neighboring
408 cells. At six hours post infection we found that each of the TraDIS mutants was present in the
409 host cell cytoplasm and could be seen to polymerize actin comet tails that allow the bacteria
410 to extrude out of the host cell (Figure 4). Moreover, despite the decreased levels of bacteria
411 within the host cells, each mutant was also able to form MNGCs, showing that they are
412 capable of spreading from cell to cell and inducing cell fusion (data not shown). This suggests

413 that none of the TraDIS mutants are blocked at any stage of the intracellular life cycle, but
414 rather are less capable of surviving intracellularly and/or have a delayed life cycle.

415 ***Δ1728* and *Δ2248* are sensitive to killing by human sera**

416 We next tested if the TraDIS mutants are resistant to killing by human serum. *B.*
417 *pseudomallei* has been shown to be highly resistant to complement-mediated killing and
418 complement deposition, and is capable of surviving within human sera. We found that while
419 *ΔTex*, *ΔrpiR,* and *ΔbapA* are also fully resistant to human sera, both *Δ1728* and *Δ2248* show
420 reduced survival in 30% pooled normal human sera (NHS) compared to PBS. This suggests
421 that extracellular survival may play a role in the attenuation of at least two of the TraDIS
422 mutants, and that our TraDIS screen is capable of identifying attenuated mutants with more
423 than one phenotype.

424

425 **DISCUSSION**

426 TraDIS sequencing technology has previously been demonstrated to be useful for
427 mining new data from archived experimental samples . While the microarray-based method
428 used in STM screens relies on hybridization of fluorescent probes and is thus only semi-
429 quantitative, TraDIS can quantitate the number of sequencing reads that match to each gene
430 in every pool, allowing a statistical comparison (Table S1)(27, 29). We were able to re-
431 analyze our archived STM samples using TraDIS and identify over 100 new attenuated
432 mutants as well as provide fitness information for every mutant screened without the need for
433 additional animal experiments. This demonstrated the sensitivity and value of the TraDIS
434 technology over other screening techniques and identified novel virulence factors for future
435 characterization. By comparing the TraDIS and STM data we also noticed that the STM
436 analysis was biased towards identifying genes which were heavily represented in the input

437 pool, while the TraDIS analysis gave us information on every mutant regardless of how
438 abundant they were in the library.

439 Our TraDIS screening method successfully identified the 23 genes previously
440 determined to be attenuated in our STM screen, providing a proof of principle for the TraDIS
441 screening method and validating our STM data. In both screens, the majority of the
442 attenuation mutants were negatively selected only in mouse spleens, while a minority were
443 attenuated in both spleens and lungs. This is most likely a consequence of the intranasal
444 route of infection used for these experiments, as dissemination to other tissue types
445 represents a more extreme selection than survival and replication within the tissue that was
446 directly inoculated. In addition to those described in this manuscript, a number of the mutants
447 identified in both our STM and TraDIS screens have since been individually tested and
448 confirmed to be attenuated following the initial screen, which further validates both screening
449 methods. These include mutants in multiple genes within the bacterial capsule locus which
450 have since been further characterized to clarify their role in capsule biosynthesis . In addition,
451 both *aroB* (*bpsl3168*) and *vacJ* (*bpsl3147*) mutants have been independently confirmed to
452 have delayed mean time-to-death and decreased CFU phenotypes compared to wild-type *B.*
453 *pseudomallei* (*J. Lim et al., unpublished data*).

454 Among the genes newly identified as negatively selected by TraDIS were multiple
455 genes that been previously demonstrated to be involved in virulence in *B. pseudomallei* and
456 in other species of bacteria. These include the genes *flgK* and *fliN,* which are associated with
457 flagella biosynthesis and function, the thiol peroxidase *tpx,* which mediates resistance to
458 oxidative stress the shikimate dehydrogenase *aroE (40-43)*. A number of metabolic genes
459 and transcriptional regulators were also identified, suggesting that *B. pseudomallei* K96243
460 must adapt its metabolic functions in an *in vivo* environment in order to be a successful
461 pathogen. In addition, multiple mutants in putative glycosyltransferases (*bpss2167, bpss2148,*

bpsl1444) were identified as negatively selected, suggesting a role for polysaccharides other than the capsule in virulence. Furthermore, many of the novel *B. pseudomallei* genes that were identified in our negative selection screen have been shown to play a role in virulence in the closely related species *P. aeruginosa*, including the tryptophan synthesis genes *trpB, trpE,* and *trpF* as well as the methyltransferase *hemK(44, 45)*. Many of the other genes identified were hypothetical proteins or genes that have not yet been shown to play a role in bacterial virulence.

A number of the newly identified attenuated mutants are putative polysaccharide biosynthesis genes including *bpss2167, bpsl1444*, and *bpss2248.* The *B. pseudomallei* genome encodes four large polysaccharide loci all of which have been demonstrated to play a role in virulence *in vivo*; the type I O-PS capsule, the type II O-PS LPS, and two additional clusters defined as type III O-PS and type IV O-PS . However, the genes identified in our screen do not belong to any of these clusters, which suggest that the role of polysaccharides in *B. pseudomallei* infections is even more complex than has been previously described. *Bpss2167* and *bpss2248* both encode predicted glycosyltransferases belonging to glycosyltransferase family 2, but their specific roles are unknown. *Bpsl1444* shows similarity to the glycotransferase *waaG*. This is notable because many of the other *waa* genes, which are involved in the biosynthesis and construction of the core sugar of the *B. pseudomallei* LPS, were identified as essential genes (28). It would be of interest in future experiments to determine if *bpsl1444* plays a role in virulence due to being important to the structural integrity of the bacterium or if this phenotype is due to compromised LPS.

It is interesting to note that of the five mutants characterized in this study, all but one displayed some level of intracellular attenuation in at least one cell line. This is not unexpected as *B. pseudomallei* is considered to be a facultative intracellular pathogen, but is notable because many of the best-studied *B. pseudomallei* virulence factors are genes

487 associated with the capsule, LPS, and flagella, which all play a role in extracellular, rather
488 than intracellular, survival (26, 38, 46). Moreover, the majority of *B. pseudomallei* genes that
489 have been implicated in intracellular growth and survival, such as BimA and the Bsa Type III
490 secretion system, have been demonstrated to interfere with at least one stage of the
491 intracellular life cycle (13, 16). This suggests that the mutants described here represent a
492 class of virulence factors required for intracellular survival rather than subjugation of the host
493 cell to complete the bacterial life cycle. A similar class of virulence factors was identified in an
494 *in vitro* screen for *B. pseudomallei* mutants that failed to form plaques on cell monolayers by
495 Pilatz et al., and it is interesting to note that one of the 9 genes identified in their screen,
496 *purM,* was also identified in our assay (Table S1)(47). It is likely that the TraDIS screen was
497 able to identify this class of mutants in an *in* vivo model because this technique is capable of
498 following mild attenuation phenotypes that would otherwise be overlooked in screens that
499 focus on animal survival and/or host cell death.

500 Of the mutants characterized in this work, only *Δtex* displayed a degree of attenuation
501 both *in vivo* and *in vitro* that is comparable to the mutants identified in our original STM
502 screen. It is likely that this mutant was missed in the STM screen only because it is less highly
503 represented in the input pool compared to the capsule mutants, making the difference
504 between input and output pools less obvious by microarray analysis (Table S1). Tex has been
505 shown to play an important role in virulence in both *B. pertussis* and *S. pneumoniae*, but the
506 exact nature of this role appears to differ between species as Tex regulates toxin expression
507 in *B. pertussis* but not *S. pneumoniae (32, 33).* As Tex is predicted to be a transcription factor
508 and has been shown to bind DNA in both *S. pneumoniae* and *P. aeruginosa* (33, 34), it will be
509 interesting to determine the transcriptome of this gene in *B. pseudomallei* and determine if
510 Tex regulates toxin expression or other known virulence factors. Moreover, since the
511 protection provided by *Δtex* mutants is comparable to other *B. pseudomallei* mutants that

have been investigated as live vaccine candidates, so it will be interesting to further investigate the potential of *Δtex* vaccine candidates *(25)*. Transposon mutant screens have historically been successful at identifying both major virulence factors and potential live vaccine candidates, and the identification of *B. pseudomallei* Tex demonstrates that TraDIS can be used to identify such genes that may have been missed in previous screening methods, as well as to identify mutants with mild virulence phenotypes that can provide new insight into aspects bacterial pathogenesis that would otherwise be overlooked.

ACKNOWLEDGEMENTS

We thank Robert Gilbert and all of the members of the London School of Hygiene and Tropical Medicine Biological Services Facility for animal husbandry. We thank Dr. Konrad Paszkiewicz and Dr. Karen Moore of the University of Exeter Sequencing service for sequencing the TraDIS libraries.

REFERENCES

1. **Wiersinga WJ, Currie BJ, Peacock SJ.** 2012. Melioidosis. N Engl J Med **367:**1035-1044.
2. **Reckseidler-Zenteno SL, DeVinney R, Woods DE.** 2005. The capsular polysaccharide of Burkholderia pseudomallei contributes to survival in serum by reducing complement factor C3b deposition. Infection and immunity **73:**1106-1115.
3. **Limmathurotsakul D, Funnell SG, Torres AG, Morici LA, Brett PJ, Dunachie S, Atkins T, Altmann DM, Bancroft G, Peacock SJ, Steering Group on Melioidosis Vaccine D.** 2015. Consensus on the development of vaccines against naturally acquired melioidosis. Emerg Infect Dis **21**.
4. **Centers for Disease C, Prevention DoH, Human S.** 2012. Possession, use, and transfer of select agents and toxins; biennial review. Final rule. Fed Regist **77:**61083-61115.
5. **Jones AL, Beveridge TJ, Woods DE.** 1996. Intracellular survival of Burkholderia pseudomallei. Infect Immun **64:**782-790.
6. **Pilatz S, Breitbach K, Hein N, Fehlhaber B, Schulze J, Brenneke B, Eberl L, Steinmetz I.** 2006. Identification of Burkholderia pseudomallei genes required for the intracellular life cycle and in vivo virulence. Infection and immunity **74:**3576-3586.
7. **Harley VS, Dance DA, Tovey G, McCrossan MV, Drasar BS.** 1998. An ultrastructural study of the phagocytosis of Burkholderia pseudomallei. Microbios **94:**35-45.

8. **Kespichayawattana W, Rattanachetkul S, Wanun T, Utaisincharoen P, Sirisinha S.** 2000. Burkholderia pseudomallei induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect Immun **68:**5377-5384.
9. **Harley VS, Dance DA, Drasar BS, Tovey G.** 1998. Effects of Burkholderia pseudomallei and other Burkholderia species on eukaryotic cells in tissue culture. Microbios **96:**71-93.
10. **Holden MT, Titball RW, Peacock SJ, Cerdeno-Tarraga AM, Atkins T, Crossman LC, Pitt T, Churcher C, Mungall K, Bentley SD, Sebaihia M, Thomson NR, Bason N, Beacham IR, Brooks K, Brown KA, Brown NF, Challis GL, Cherevach I, Chillingworth T, Cronin A, Crossett B, Davis P, DeShazer D, Feltwell T, Fraser A, Hance Z, Hauser H, Holroyd S, Jagels K, Keith KE, Maddison M, Moule S, Price C, Quail MA, Rabbinowitsch E, Rutherford K, Sanders M, Simmonds M, Songsivilai S, Stevens K, Tumapa S, Vesaratchavest M, Whitehead S, Yeats C, Barrell BG, Oyston PC, Parkhill J.** 2004. Genomic plasticity of the causative agent of melioidosis, Burkholderia pseudomallei. Proc Natl Acad Sci U S A **101:**14240-14245.
11. **DeShazer D, Brett PJ, Woods DE.** 1998. The type II O-antigenic polysaccharide moiety of Burkholderia pseudomallei lipopolysaccharide is required for serum resistance and virulence. Molecular microbiology **30:**1081-1100.
12. **Reckseidler SL, DeShazer D, Sokol PA, Woods DE.** 2001. Detection of bacterial virulence genes by subtractive hybridization: identification of capsular polysaccharide of Burkholderia pseudomallei as a major virulence determinant. Infect Immun **69:**34-44.
13. **Stevens MP, Wood MW, Taylor LA, Monaghan P, Hawes P, Jones PW, Wallis TS, Galyov EE.** 2002. An Inv/Mxi-Spa-like type III protein secretion system in Burkholderia pseudomallei modulates intracellular behaviour of the pathogen. Mol Microbiol **46:**649-659.
14. **Gong L, Cullinane M, Treerat P, Ramm G, Prescott M, Adler B, Boyce JD, Devenish RJ.** 2011. The Burkholderia pseudomallei type III secretion system and BopA are required for evasion of LC3-associated phagocytosis. PLoS One **6:**e17852.
15. **Sitthidet C, Stevens JM, Field TR, Layton AN, Korbsrisate S, Stevens MP.** 2010. Actin-based motility of Burkholderia thailandensis requires a central acidic domain of BimA that recruits and activates the cellular Arp2/3 complex. J Bacteriol **192:**5249-5252.
16. **Stevens MP, Stevens JM, Jeng RL, Taylor LA, Wood MW, Hawes P, Monaghan P, Welch MD, Galyov EE.** 2005. Identification of a bacterial factor required for actin-based motility of Burkholderia pseudomallei. Mol Microbiol **56:**40-53.
17. **French CT, Toesca IJ, Wu TH, Teslaa T, Beaty SM, Wong W, Liu M, Schroder I, Chiou PY, Teitell MA, Miller JF.** 2011. Dissection of the Burkholderia intracellular life cycle using a photothermal nanoblade. Proc Natl Acad Sci U S A **108:**12095-12100.
18. **Schwarz S, Singh P, Robertson JD, LeRoux M, Skerrett SJ, Goodlett DR, West TE, Mougous JD.** 2014. VgrG-5 is a Burkholderia type VI secretion system-exported protein required for multinucleated giant cell formation and virulence. Infect Immun **82:**1445-1452.
19. **Mei JM, Nourbakhsh F, Ford CW, Holden DW.** 1997. Identification of Staphylococcus aureus virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. Mol Microbiol **26:**399-407.
20. **Shea JE, Hensel M, Gleeson C, Holden DW.** 1996. Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. Proc Natl Acad Sci U S A **93:**2593-2597.

21. **Polissi A, Pontiggia A, Feger G, Altieri M, Mottl H, Ferrari L, Simon D.** 1998. Large-scale identification of virulence genes from Streptococcus pneumoniae. Infect Immun **66:**5620-5629.
22. **Sassetti CM, Rubin EJ.** 2003. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A **100:**12989-12994.
23. **Cox JS, Chen B, McNeil M, Jacobs WR, Jr.** 1999. Complex lipid determines tissue-specific replication of Mycobacterium tuberculosis in mice. Nature **402:**79-83.
24. **Moule MG, Monack DM, Schneider DS.** 2010. Reciprocal analysis of Francisella novicida infections of a Drosophila melanogaster model reveal host-pathogen conflicts mediated by reactive oxygen and imd-regulated innate immune response. PLoS Pathog **6:**e1001065.
25. **Atkins T, Prior R, Mack K, Russell P, Nelson M, Prior J, Ellis J, Oyston PC, Dougan G, Titball RW.** 2002. Characterisation of an acapsular mutant of Burkholderia pseudomallei identified by signature tagged mutagenesis. Journal of medical microbiology **51:**539-547.
26. **Cuccui J, Easton A, Chu KK, Bancroft GJ, Oyston PC, Titball RW, Wren BW.** 2007. Development of signature-tagged mutagenesis in Burkholderia pseudomallei to identify genes important in survival and pathogenesis. Infection and immunity **75:**1186-1195.
27. **Langridge GC, Phan MD, Turner DJ, Perkins TT, Parts L, Haase J, Charles I, Maskell DJ, Peters SE, Dougan G, Wain J, Parkhill J, Turner AK.** 2009. Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants. Genome research **19:**2308-2316.
28. **Moule MG, Hemsley CM, Seet Q, Guerra-Assuncao JA, Lim J, Sarkar-Tyson M, Clark TG, Tan PB, Titball RW, Cuccui J, Wren BW.** 2014. Genome-wide saturation mutagenesis of Burkholderia pseudomallei K96243 predicts essential genes and novel targets for antimicrobial development. MBio **5:**e00926-00913.
29. **Eckert SE, Dziva F, Chaudhuri RR, Langridge GC, Turner DJ, Pickard DJ, Maskell DJ, Thomson NR, Stevens MP.** 2011. Retrospective application of transposon-directed insertion site sequencing to a library of signature-tagged mini-Tn5Km2 mutants of Escherichia coli O157:H7 screened in cattle. Journal of bacteriology **193:**1771-1776.
30. **Ferrieres L, Hemery G, Nham T, Guerout AM, Mazel D, Beloin C, Ghigo JM.** 2010. Silent mischief: bacteriophage Mu insertions contaminate products of Escherichia coli random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. J Bacteriol **192:**6418-6427.
31. **Logue CA, Peak IR, Beacham IR.** 2009. Facile construction of unmarked deletion mutants in Burkholderia pseudomallei using sacB counter-selection in sucrose-resistant and sucrose-sensitive isolates. J Microbiol Methods **76:**320-323.
32. **Fuchs TM, Deppisch H, Scarlato V, Gross R.** 1996. A new gene locus of Bordetella pertussis defines a novel family of prokaryotic transcriptional accessory proteins. J Bacteriol **178:**4445-4452.
33. **He X, Thornton J, Carmicle-Davis S, McDaniel LS.** 2006. Tex, a putative transcriptional accessory factor, is involved in pathogen fitness in Streptococcus pneumoniae. Microb Pathog **41:**199-206.
34. **Johnson SJ, Close D, Robinson H, Vallet-Gely I, Dove SL, Hill CP.** 2008. Crystal structure and RNA binding of the Tex protein from Pseudomonas aeruginosa. J Mol Biol **377:**1460-1473.
35. **Abe K, Obana N, Nakamura K.** 2010. Effects of depletion of RNA-binding protein Tex on the expression of toxin genes in Clostridium perfringens. Biosci Biotechnol Biochem **74:**1564-1571.

36. **Zhu Y, Nandakumar R, Sadykov MR, Madayiputhiya N, Luong TT, Gaupp R, Lee CY, Somerville GA.** 2011. RpiR homologues may link Staphylococcus aureus RNAIII synthesis and pentose phosphate pathway regulation. J Bacteriol **193:**6187-6196.
37. **Warawa J, Woods DE.** 2005. Type III secretion system cluster 3 is required for maximal virulence of Burkholderia pseudomallei in a hamster infection model. FEMS Microbiol Lett **242:**101-108.
38. **Sarkar-Tyson M, Thwaite JE, Harding SV, Smither SJ, Oyston PC, Atkins TP, Titball RW.** 2007. Polysaccharides and virulence of Burkholderia pseudomallei. Journal of medical microbiology **56:**1005-1010.
39. **Taboada B, Ciria R, Martinez-Guerrero CE, Merino E.** 2012. ProOpDB: Prokaryotic Operon DataBase. Nucleic Acids Res **40:**D627-631.
40. **Gourlay LJ, Thomas RJ, Peri C, Conchillo-Sole O, Ferrer-Navarro M, Nithichanon A, Vila J, Daura X, Lertmemongkolchai G, Titball R, Colombo G, Bolognesi M.** 2015. From crystal structure to in silico epitope discovery in the Burkholderia pseudomallei flagellar hook-associated protein FlgK. FEBS J **282:**1319-1333.
41. **Stritzker J, Janda J, Schoen C, Taupp M, Pilgrim S, Gentschev I, Schreier P, Geginat G, Goebel W.** 2004. Growth, virulence, and immunogenicity of Listeria monocytogenes aro mutants. Infect Immun **72:**5622-5629.
42. **Simpson DA, Ramphal R, Lory S.** 1995. Characterization of Pseudomonas aeruginosa fliO, a gene involved in flagellar biosynthesis and adherence. Infect Immun **63:**2950-2957.
43. **La Carbona S, Sauvageot N, Giard JC, Benachour A, Posteraro B, Auffray Y, Sanguinetti M, Hartke A.** 2007. Comparative study of the physiological roles of three peroxidases (NADH peroxidase, Alkyl hydroperoxide reductase and Thiol peroxidase) in oxidative stress response, survival inside macrophages and virulence of Enterococcus faecalis. Mol Microbiol **66:**1148-1163.
44. **Palmer GC, Jorth PA, Whiteley M.** 2013. The role of two Pseudomonas aeruginosa anthranilate synthases in tryptophan and quorum signal production. Microbiology **159:**959-969.
45. **Pustelny C, Brouwer S, Musken M, Bielecka A, Dotsch A, Nimtz M, Haussler S.** 2013. The peptide chain release factor methyltransferase PrmC is essential for pathogenicity and environmental adaptation of Pseudomonas aeruginosa PA14. Environ Microbiol **15:**597-609.
46. **Wikraiphat C, Charoensap J, Utaisincharoen P, Wongratanacheewin S, Taweechaisupapong S, Woods DE, Bolscher JG, Sirisinha S.** 2009. Comparative in vivo and in vitro analyses of putative virulence factors of Burkholderia pseudomallei using lipopolysaccharide, capsule and flagellin mutants. FEMS immunology and medical microbiology **56:**253-259.
47. **Pilatz S, Breitbach K, Hein N, Fehlhaber B, Schulze J, Brenneke B, Eberl L, Steinmetz I.** 2006. Identification of Burkholderia pseudomallei genes required for the intracellular life cycle and in vivo virulence. Infect Immun **74:**3576-3586.

Figure 1. TraDIS mutants show reduced bacterial burdens in infected Balb/c mice

Balb/C mice (n = 5) were infected intranasally with either *B. pseudomallei* K96243 or the individual deletion mutant indicated. At 48 hours post-infection spleens and lungs were

harvested from the infected animals and bacterial loads were determined. Lines indicated mean and standard error for each sample. Statistical significance was determined using the Mann-Whitney test with p values indicated above each sample. *ns* = not significant. Mice infected with (A) 500 CFU of K96243 or ΔRpiR (spleen, p=0.0079) (B) 500 CFU of K96243 or 800 CFU of ΔBapA (ns) (C) $1x10^3$ CFU of K96243 or Δ1728 (lungs, p=0.0317; spleen p=0.0159) (D) $1x10^3$ CFU of K96243 or Δtex (lungs, p=0.0079; spleen p=0.0079), and (E) $2x10^3$ CFU of K96243 or Δ2248 (lungs ns, spleen, p=0.0317)

Figure 2. Survival of Balb/C mice following infection with TraDIS mutants

(A) Balb/C mice (n=5) were infected with 10^3 CFU of K96243, Δ1728, or ΔTex. The median survival for K96243 was 2.5 days post infection, while the median survival for Δ1728 was 3 days post infection 4 out of 5 mice infected with Δtex were still alive when the experiment was terminated at 60 days post-infection. The survival of both mutants was statistically significantly different from wild-type as determined by Log-rank (Mantle-Cox) test with p values of 0.0449 and 0.0009 respectively. (B) The bacterial load in mice infected with Δtex was determined in surviving mice at 60 days post infection. All four mice displayed detectable levels of *B. pseudomallei* Δtex in the spleen, while only two animals had detectable CFUs in the lungs. (C) Balb/C mice were vaccinated with either 10^3 CFU Δtex or a saline control and challenged intranasally with 10^3 CFU wild-type *B. pseudomallei* at 5 weeks post-vaccination. Survival up to 80 days post-challenge is shown, with the Δtex vaccinated animals showing a statistically significantly different mean time-to-death of 31 days compared to 6 days for saline vaccinated animals (p<0.001). (D) At 80 days post-challenge, surviving Δtex-vaccinated mice were sacrificed and lungs and spleens were harvested and plated to determine if the bacteria had been cleared from the animals. All colonies isolated from both organs were determined to be wild-type *B. pseudomallei* by PCR screening.

Figure 3. Internalization, growth, and survival of TraDIS mutants in cultured cells

(A) The *B. pseudomallei* TraDIS mutants are all able to induce their own uptake into A549 human lung epithelial cells. Cells were infected at a MOI of 10 for 1 hr, then washes and overlayed with 400 μg/mL kanamycin. At 2 hours post-infection cells were lysed and the bacterial loads determined. None of the mutants displayed statistically significantly different bacterial CFU compared to wild-type as determined by ANOVA. (B) The *B. pseudomallei* TraDIS mutants show variable growth and survival in A549 cells. Cells were infected with a MOI of 1, and the infection was allowed to proceed for 18 hours. ΔRpiR, ΔBapA, and Δtex all had statistically significantly decreased bacterial loads as determined by Mann-Whitney test with p values of p=0.0005, p<0.00001, and p<0.00001 respectively. (C) The *B. pseudomallei* TraDIS mutants show variable growth and survival in J774 murine macrophages. Cells were infected with a MOI of 1, and the infection was allowed to proceed for 16 h. ΔRpiR, ΔBapA, and Δtex all had statistically significantly decreased bacterial loads as determined by Mann-Whitney test with p values of p=0.0045, p=0.0078, and p=0.0002 respectively. Interestingly, in this cell line Δ1728 also showed reduced bacterial load compared to wild-type (p=0.0019).

Figure 4. All *B. pseudomallei* TraDIS mutants can polymerize actin

J774 murine macrophages were infected with either (A) *B. pseudomallei* K96243, (B) ΔRpiR, (C) ΔBapA, (D) Δ1728, (E) Δtex and (F) Δ2248 at an MOI of 10. After four hours, cells were fixed and stained with the CC6 monoclonal antibody against *B. pseudomallei* LPS (green) and phalloidin (red) which stains actin filaments. Actin comet tails (blue arrows) were visible in all samples, indicating that the *B. pseudomallei* mutants are capable of entering cells and escaping into the cytoplasm where they are able to polymerize actin to spread cell-to-cell.

733 **Figure 5. Sensitivity of *B. pseudomallei* TraDIS mutants to human sera**

734 10^6 CFU of *B. pseudomallei* K96243 wild-type, ΔRpiR, ΔBapA, Δ1728, Δtex, and Δ2248 were
735 incubated with either 30% natural human serum (NHS), 30% heat inactivated NHS, or PBS
736 control for 2 hr at 37° C. While wild-type, ΔRpiR, ΔBapA, and Δtex were resistant to killing by
737 human sera as has been previously reported for *B. pseudomallei* K96243, Δ1728 and Δ2248
738 were both sensitive to complement killing by human sera (p value 0.0029 and 0.0000056
739 respectively).
740
741