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

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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 bpsl1528 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 saprophytic 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 \textit{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 \textit{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 \textit{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 \textit{B. pseudomallei} pathogenesis. However, much remains poorly understood and the vast majority of \textit{B. pseudomallei} virulence factors remain to be identified. One technique that has been highly successful at identifying genes that are required for the \textit{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 \textit{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 \textit{in vivo}. This method identified the \textit{B. pseudomallei} capsule and the branched chain amino acid synthase \textit{ilvE} as essential for \textit{in vivo} survival, which led to the development of an \textit{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 mM NaCl, 20 mM 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(pGATCGGAAGACCGGTTCAGCAGGAATGCCGAGACGGATCTC) were ligated
onto the samples. PCR was performed using primers
PE_PCR_V3.3(CAAGCAGAAGACGGCATACGAGATCGGTACACTCTTTCCCTACACGACG
CTCTTCCGATC) and
MnTn5_P5_3pr_3(AATGATACGGCGACCACCGAGATCTACACCTAGGCGCGGCGACTT
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
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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* MFDpir 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-sucreose 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 Itd, 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 log2 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 log2 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 log2 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 *Clostridium 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. *Bpsl1528*, 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, Δ*tex*, Δ*rpiR*, Δ*1728*, Δ*2248*, and Δ*bapA* 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
internalized differently than wild-type bacteria, suggesting that they do not have defects related to adhesion or invasion of host cells (Figure 3a).

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

To determine whether the attenuated phenotypes of the TraDIS mutants is due to an impaired intracellular life cycle, we analyzed the ability of each mutant to escape from the phagocytic vacuole, polymerize actin to become motile within the host cell cytoplasm, and form multinucleated giant cells (MNGCs) by fusing the infected host cell with neighboring cells. At six hours post infection we found that each of the TraDIS mutants was present in the host cell cytoplasm and could be seen to polymerize actin comet tails that allow the bacteria to extrude out of the host cell (Figure 4). Moreover, despite the decreased levels of bacteria within the host cells, each mutant was also able to form MNGCs, showing that they are capable of spreading from cell to cell and inducing cell fusion (data not shown). This suggests...
that none of the TraDIS mutants are blocked at any stage of the intracellular life cycle, but rather are less capable of surviving intracellularly and/or have a delayed life cycle.

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

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

**DISCUSSION**

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

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

Among the genes newly identified as negatively selected by TraDIS were multiple genes that been previously demonstrated to be involved in virulence in \textit{B. pseudomallei} and in other species of bacteria. These include the genes \textit{flgK} and \textit{fliN}, which are associated with flagella biosynthesis and function, the thiol peroxidase \textit{tpx}, which mediates resistance to oxidative stress the shikimate dehydrogenase \textit{aroE} (40-43). A number of metabolic genes and transcriptional regulators were also identified, suggesting that \textit{B. pseudomallei} K96243 must adapt its metabolic functions in an \textit{in vivo} environment in order to be a successful pathogen. In addition, multiple mutants in putative glycosyltransferases (\textit{bpss2167, bpss2148, bpss2167}, \textit{bpss2148}, \textit{bpss2167}, \textit{bpss2148}).
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
associated with the capsule, LPS, and flagella, which all play a role in extracellular, rather
than intracellular, survival (26, 38, 46). Moreover, the majority of \textit{B. pseudomallei} genes that
have been implicated in intracellular growth and survival, such as BimA and the Bsa Type III
secretion system, have been demonstrated to interfere with at least one stage of the
intracellular life cycle (13, 16). This suggests that the mutants described here represent a
class of virulence factors required for intracellular survival rather than subjugation of the host
cell to complete the bacterial life cycle. A similar class of virulence factors was identified in an
\textit{in vitro} screen for \textit{B. pseudomallei} mutants that failed to form plaques on cell monolayers by
Pilatz et al., and it is interesting to note that one of the 9 genes identified in their screen,
\textit{purM}, was also identified in our assay (Table S1)(47). It is likely that the TraDIS screen was
able to identify this class of mutants in an \textit{in vivo} model because this technique is capable of
following mild attenuation phenotypes that would otherwise be overlooked in screens that
focus on animal survival and/or host cell death.

Of the mutants characterized in this work, only \textit{Δtex} displayed a degree of attenuation
both \textit{in vivo} and \textit{in vitro} that is comparable to the mutants identified in our original STM
screen. It is likely that this mutant was missed in the STM screen only because it is less highly
represented in the input pool compared to the capsule mutants, making the difference
between input and output pools less obvious by microarray analysis (Table S1). Tex has been
shown to play an important role in virulence in both \textit{B. pertussis} and \textit{S. pneumoniae}, but the
exact nature of this role appears to differ between species as Tex regulates toxin expression
in \textit{B. pertussis} but not \textit{S. pneumoniae} (32, 33). As Tex is predicted to be a transcription factor
and has been shown to bind DNA in both \textit{S. pneumoniae} and \textit{P. aeruginosa} (33, 34), it will be
interesting to determine the transcriptome of this gene in \textit{B. pseudomallei} and determine if
Tex regulates toxin expression or other known virulence factors. Moreover, since the
protection provided by \textit{Δtex} mutants is comparable to other \textit{B. pseudomallei} mutants that
have been investigated as live vaccine candidates, so it will be interesting to further 512 investigate the potential of Δtex vaccine candidates (25). Transposon mutant screens have 513 historically been successful at identifying both major virulence factors and potential live 514 vaccine candidates, and the identification of B. pseudomallei Tex demonstrates that TraDIS 515 can be used to identify such genes that may have been missed in previous screening 516 methods, as well as to identify mutants with mild virulence phenotypes that can provide new 517 insight into aspects bacterial pathogenesis that would otherwise be overlooked.

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REFERENCES


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

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