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

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19

20 **ABSTRACT**

21 *Burkholderia pseudomallei*, the causative agent of melioidosis, has a complex and poorly
22 understood extracellular and intracellular lifestyle. We used transposon insertion-site
23 sequencing (TraDIS) to retrospectively analyze a transposon library that had previously been
24 screened through a Balb/c mouse model to identify genes important for growth and survival *in*
25 *vivo*. This allowed us to identify the insertion sites and phenotypes of negatively selected
26 mutants that were previously overlooked due to technical constraints. All 23 unique genes
27 identified in the original screen were confirmed by TraDIS and an additional 105 mutants were
28 identified with varying degrees of attenuation *in vivo*. Five of the newly identified genes were
29 chosen for further characterization and clean, unmarked deletion mutants of *bpsi2248*, *tex*,
30 *rpiR*, *bpsi1728* and *bpss1528* were constructed in the wild-type strain K96243. Each of these
31 mutants was tested *in vitro* and *in vivo* to confirm their attenuated phenotypes and investigate
32 the nature of the attenuation. Our results confirm that we have identified new genes important
33 to *in vivo* virulence with roles in different stages of *B. pseudomallei* pathogenesis including
34 extracellular and intracellular survival. Of particular interest, deletion of the transcription
35 accessory protein Tex was shown to be highly attenuating and the *tex* mutant was capable of
36 providing protective immunity against challenge with wild-type *B. pseudomallei*, suggesting
37 that the genes identified in our TraDIS screen have the potential to be investigated as live
38 vaccine candidates.

39

40 INTRODUCTION

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

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

Page 3

65 This complex intracellular lifestyle is regulated by a number of virulence factors
66 encoded within the large 7.25 megabase *B. pseudomallei* genome including three type III
67 secretion systems (T3SS), six type VI secretion systems (T6SS), multiple polysaccharide loci,
68 and a number of secreted effectors (10). The *B. pseudomallei* polysaccharide capsule and
69 lipopolysaccharide (LPS) help the bacteria survive extracellularly and resist complement
70 deposition(2, 11, 12), while the *Bsa* T3SS has been implicated in helping *B. pseudomallei*
71 induce uptake into non-phagocytic cells, escape the vacuole, and resist killing by autophagy
72 (13, 14). In addition, actin polymerization has been shown to be mediated by the
73 autotransporter BimA, which is expressed on one pole of the bacteria and stimulates the
74 formation of new actin filaments (15, 16) Finally, the T6SS-1 is required for cell fusion and the
75 formation of MNGCs (17, 18)

76 The identification and characterization of these important virulence factors has greatly
77 improved our understanding of *B. pseudomallei* pathogenesis. However, much remains poorly
78 understood and the vast majority of *B. pseudomallei* virulence factors remain to be identified.
79 One technique that has been highly successful at identifying genes that are required for the *in*
80 *vivo* virulence of many bacterial species has been the application of large-scale forward
81 genetic screens using libraries of bacterial transposon insertion mutants (19-24). We have
82 previously successfully applied this strategy to the study of *B. pseudomallei* using an
83 approach known as signature tagged mutagenesis (STM) in which pools of mutants each
84 containing a unique tag are used to infect an animal model (25, 26). By comparing the
85 population of mutants present in infected animals (output pools) to the original pool of mutants
86 used to infect the animals (input pools), it is possible to identify mutants that are unable to
87 survive and grow *in vivo*. Rhis method identified the *B. pseudomallei* capsule and the
88 branched chain amino acid synthase *ilvE* as essential for *in vivo* survival, which led to the
89 development of an *ilvE* mutant as a live attenuated vaccine candidate. A number of additional
Page 4

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

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

Page 5

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

119

120 **MATERIALS AND METHODS**

121 **Bacterial strains and culture conditions**

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

130 **Genomic DNA Extraction**

131 10 mL of overnight shaken cultures was spun down at 4000 RPM in a bench top centrifuge
132 and resuspended in 10 mL of lysis buffer (100 µg/mL proteinase K, 10 mL NaCl, 20 mL Tris
133 HCl pH8, 1 mM EDTA, 0.5% SDS). 3 mL of sodium perchlorate was added to the solution and
134 incubated for 1 hour at room temperature. Genomic DNA was isolated using a
135 phenol:chloroform:isoamyl alcohol extraction (25:24:1), precipitated with ethanol and spooled
136 into deionised water.

137 **Illumina Sequencing**

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

140 and A-tailed using the NEBNext DNA library preparation reagent kit for Illumina (NEB).
141 Annealed adapters Ind_Ad_T(ACACTCTTTCCCTACACGACGCTCTTCCGATC*T,
142 * indicates phosphorothioate) and
143 Ind_Ad_B(pGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTC) were ligated
144 onto the samples. PCR was performed using primers
145 PE_PCR_V3.3(CAAGCAGAAGACGGCATACGAGATCGGTACACTCTTTCCCTACACGACG
146 CTCTTCCGATC) and
147 MnTn5_P5_3pr_3(AATGATACGGCGACCACCGAGATCTACACCTAGGctGCGGctGCACTT
148 GTG), which include flow cell binding sites. The PCR program used was 2 minutes at 94°C,
149 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
150 were then size selected to between 200-400 bp in a 2% agarose gel made up with 1xTBE
151 buffer, with purification by Qiagen Gel Extraction kit. The final concentration of the samples
152 were checked by both BioAnalyzer and qPCR. Preparation products were sequenced on an
153 Illumina Hi-Seq 2000 as 36 bp single-end reads. Concentration of the samples was
154 established using qPCR with the primers Syb_FP5(ATGATACGGCGACCACCGAG) and
155 Syb_RP7(CAAGCAGAAGACGGCATACGAG). They were then size selected to between 300-
156 500 bp in a 2% agarose gel made up with 1xTBE buffer, with purification by Qiagen Gel
157 Extraction kit. The final concentration of the samples were checked by both BioAnalyzer and
158 qPCR. Preparation products were sequenced on an Illumina Hi-Seq 2000 as 100 bp single-
159 end reads.

160 **Bioinformatic and statistical analysis**

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

165 sites and coverage. For differential expression analysis, the coverage values were variance-
166 stabilized using an arcsine-root transformation , and \log_2 ratios between the input pools and
167 the lung and spleen samples were calculated. Minimum starting values of 200 sequencing
168 reads within the input pool were used to ensure sufficient starting quantities for negative
169 selection analysis and avoid background. To define negative selection, cut-offs of the lowest
170 2.5% of \log_2 ratios within the spleen pool comparisons and the lowest 5% of ratios within the
171 lung pool comparisons were set based on the mean distribution of the \log_2 fold change.

172 **Generation of clean deletion mutants**

173 Unmarked deletion mutants were constructed as has been previously described using the
174 suicide vector pDM4 (31). Briefly, 600-1000 bp regions flanking each gene of interest were
175 amplified with an XbaI restriction site on the 5' end and overlapping sequences on the 3' end
176 of the PCR product using Phusion High-Fidelity PCR master mix (ThermoScientific). The
177 resulting products were then spliced together using splicing by overlapping extension PCR
178 (SOE PCR) to generate a full-length product consisting of the upstream and downstream
179 flanks lacking the target gene. This product was then cloned into the intermediate plasmid
180 pGEM-T and then subcloned into pDM4 using XbaI. The resulting mutagenesis construct was
181 then introduced into *E. coli* MFD π cultured in LB media containing 0.3 mM DAP, and then
182 transferred into *B. pseudomallei* K96243 by direct mating. Merodiploids containing the
183 integrated plasmid were selected for on LB agar containing 30 μ g/ml chloroamphenicol, and
184 screened using primers designed against the gene of interest. Successful clones were then
185 plated onto high-sucrose agar (10 g/L tryptone, 5 g/L yeast extract, 100 g/L sucrose) and
186 grown for 48-72 hours at 24°C. Colonies were screened for sensitivity to chloroamphenicol
187 due to loss of the pDM4 cassette, as well as by PCR using primers designed against the gene
188 of interest and across the deletion junction. The resulting mutants were confirmed by full
189 genome sequencing using an Illumina MiSeq sequencer to confirm the loss of pDM4 and the
Page 8

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

192 **Mouse infections**

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

210 **Tissue culture infections**

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

Page 9

215 allowed to adhere for 16 hours. The cells were then washed with PBS and inoculated with
216 2×10^6 CFU of wild-type *B. pseudomallei* or one of the TraDIS mutants in 1 mL of DMEM. The
217 infection was allowed to proceed for one hour, at which point the media was removed, the
218 cells washed with PBS, and 1 mL fresh DMEM or F12-K media containing 200 μ g/mL of
219 kanamycin was added to the wells. The cells were incubated with antibiotics for 2 hours at
220 37°C. For invasion assays, the cells were then lysed immediately with 0.1% Triton-X100 and
221 10-fold dilutions were plated out onto LB agar to determine how many cells were internalized.
222 For intracellular growth assays, the infections were allowed to proceed for 6-24 hours, at
223 which point the cells were lysed and CFUs plated as described.

224 **Immunofluorescence**

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

238 **Serum survival assays**

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

243

244 **RESULTS**

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

246 ***in vivo***

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

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

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

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

291 **Confirmation of attenuated TraDIS mutant phenotypes with unmarked deletion mutants**

292 Five genes identified as negatively selected in the spleen output pools, some of which
293 were also negatively selected in the lungs, were selected for further characterization based on
294 strength of phenotype and predicted functional domains. *BpsI1527*, which encodes the
295 transcription accessory protein Tex, was selected because this mutant demonstrated one of
296 the strongest attenuated phenotypes in both lung and spleen output pools. Tex is required for
297 toxin regulation in *Bordetella pertussis* and *Clostridium perfringens* and has been shown to
298 play a role in virulence in *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* (32-35).
299 Moreover, the structure of the *P. aeruginosa* homolog has been solved and shown to bind
300 DNA, suggesting that this gene is likely to function as a transcriptional regulator. Another
301 strongly negatively selected putative transcriptional regulator, RpiR, is encoded by *bpsI0629*.
302 RpiR has been demonstrated to regulate various virulence factors of *Staphylococcus aureus*,
303 suggesting that this could be another conserved regulatory gene required for *in vivo* virulence
304 (36). *BpsI1728* and *bpps1528* both encode predicted secreted proteins, with *bpps1528* being
305 a type III secretion system secreted protein and *bpsI1728* showing homology to be a secreted
306 outer membrane porin from *Bordetella pertussis*. *BpsI1728* was also of interest to us for
307 technical reasons because it is present just above the predicted threshold of detection in our
308 input pool, which allowed us to use this gene as an indicator of the sensitivity and accuracy of
309 our TraDIS screen. *Bpps1528*, which encodes the putative Type III secretion system effector
310 protein BapA was selected because although the *B. pseudomallei* T3SS-3 is known to be
311 required for virulence, studies with this mutant in a hamster model did not display any survival
312 phenotype, suggesting that our TraDIS assay may be able to pick up moderately attenuated
313 mutants that would be missed in other screening methods (37) Finally, *bpsI2248* was selected

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

318 To absolutely confirm the attenuated phenotypes of each of these mutants and
319 address the possibility of polar effects, clean unmarked deletion mutants were constructed for
320 each gene as has been described previously (Logue et al., 2009). Briefly, a suicide plasmid
321 containing a null allele consisting of the upstream and downstream flanking regions of the
322 gene of interest was introduced via homologous recombination with chloramphenicol
323 selection. A second recombination event was then selected for with *sacB*-mediated counter
324 selection against sucrose sensitivity, and the resulting colonies were screened by PCR for
325 loss of the wild-type allele. Each mutant was then verified by Illumina whole genome
326 sequencing to confirm the expected deletion of each gene of interest and to ascertain that no
327 secondary mutations had occurred. Three of the genes selected for mutagenesis, *bapA*, *rpiR*,
328 and *bps2248* are located within predicted operons, while *tex* and *bsp1728* do not have any
329 downstream genes located within the same reading frame (39). Due to the nature of our
330 mutagenesis strategy, we did not expect to see polar effects from any of the mutants we
331 constructed, including those for genes within operons. However, to be certain that
332 transcription of downstream genes was not affected by mutagenesis, we performed RT-PCR
333 analysis of each gene within the operons of our genes of interest and the nearest genes to *tex*
334 and *bsp1728* and found that transcription was not affected for any of the genes tested
335 (Supplemental Figure 1).

336 The resulting deletion mutants, Δtex , $\Delta rpiR$, $\Delta 1728$, $\Delta 2248$, and $\Delta bapA$ were each
337 used to infect Balb/c mice via an intranasal route alongside five mice infected with wild type *B.*
338 *pseudomallei* K96243. Colony forming units (CFUs) were plated from the inocula to determine

339 the exact infectious dose, and the infections were allowed to proceed for 48 hours to match
340 the time point of the original screen. At this point lungs and spleens from each mouse were
341 harvested, homogenized, and plated for CFUs. Four of the mutants, Δtex , $\Delta rpiR$, $\Delta 1728$, and
342 $\Delta 2248$, demonstrated significantly reduced CFUs in mouse spleens compared to wild-type *B.*
343 *pseudomallei*. The Δtex mutants displayed the strongest attenuation within the spleen and
344 also displayed strong attenuation within the lung, consistent with the TraDIS screen
345 predictions. The remaining mutant, $\Delta bapA$ showed slightly reduced CFUs compared to wild-
346 type, but this decrease was not statistically significant (Figure 1). These results showed that
347 the TraDIS screen was not only able to identify genes important for growth and survival in a
348 mouse model, but was able to do so in a semi-quantitative manner and predict the relative
349 strength of phenotype.

350 ***B. pseudomallei* Δtex mutants are highly attenuated and protect against challenge with**
351 **wild-type *B. pseudomallei***

352 We next tested each TraDIS mutant in a survival assay to determine if the reduced
353 CFUs seen in lung and spleen tissues correlated with decreased virulence. Interestingly,
354 $\Delta rpiR$, $\Delta 1728$, $\Delta 2248$, and $\Delta bapA$ demonstrated similar survival phenotypes to wild-type *B.*
355 *pseudomallei* at an infectious dose of approximately 10^3 CFU despite significant reduction of
356 bacterial CFUs in the spleen. This suggests that the sensitivity of our TraDIS assay allowed
357 the identification of mildly attenuated phenotypes below the threshold of attenuation that
358 would lead to a decrease in virulence as defined by survival. Supporting this hypothesis, the
359 mutant with the strongest TraDIS phenotype, Δtex , showed increased mouse survival
360 compared to wild-type bacteria, with over 80% long-term survival (Figure 2a). To determine if
361 the surviving animals had completely cleared the infection with the Δtex mutant, we plated
362 CFUs from four of the remaining mice at 60 days post-infection. We found that all four mice

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

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

375 **Δtex , $\Delta rpiR$, $\Delta 1728$, and $\Delta bapA$ display decreased intracellular survival, but are able to**
376 **complete the intracellular life cycle**

377 *B. pseudomallei* is considered a facultative intracellular pathogen, but is highly
378 resistant to killing by human sera and is able to survive and replicate extracellularly. We were
379 interested in determining how large a role, if any, intracellular survival and replication played
380 in the attenuated phenotypes of the TraDIS mutants. As *B. pseudomallei* is able to induce its
381 own uptake into epithelial cells (9), we first analyzed invasion of A549 human lung epithelial
382 cells by infecting a monolayer of cells with a multiplicity of infection (MOI) of 10 CFU of Δtex ,
383 $\Delta rpiR$, $\Delta 1728$, $\Delta 2248$, $\Delta bapA$, or wild type *B. pseudomallei* K96243 per cell and allowed the
384 infection to proceed for one hour. The cells were then gently washed and kanamycin was
385 added to the media to kill any remaining extracellular bacteria. At 2 hours post-infection, the
386 cells were lysed and plated to determine the number of intracellular CFU. Intracellular
387 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 $\Delta 1728$ and $\Delta 2248$
392 replicated to similar levels as wild-type *B. pseudomallei*, while $\Delta rpiR$ and $\Delta bapA$ 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 such as 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 $\Delta 1728$, 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 **$\Delta 1728$ and $\Delta 2248$ 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 , $\Delta rpiR$, and $\Delta bapA$ are also fully resistant to human sera, both $\Delta 1728$ and $\Delta 2248$ 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* (*bpsI3168*) and *vacJ* (*bpsI3147*) 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*,

462 *bps1444*) were identified as negatively selected, suggesting a role for polysaccharides other
463 than the capsule in virulence. Furthermore, many of the novel *B. pseudomallei* genes that
464 were identified in our negative selection screen have been shown to play a role in virulence in
465 the closely related species *P. aeruginosa*, including the tryptophan synthesis genes *trpB*,
466 *trpE*, and *trpF* as well as the methyltransferase *hemK*(44, 45). Many of the other genes
467 identified were hypothetical proteins or genes that have not yet been shown to play a role in
468 bacterial virulence.

469 A number of the newly identified attenuated mutants are putative polysaccharide
470 biosynthesis genes including *bpss2167*, *bps1444*, and *bpss2248*. The *B. pseudomallei*
471 genome encodes four large polysaccharide loci all of which have been demonstrated to play a
472 role in virulence *in vivo*; the type I O-PS capsule, the type II O-PS LPS, and two additional
473 clusters defined as type III O-PS and type IV O-PS . However, the genes identified in our
474 screen do not belong to any of these clusters, which suggest that the role of polysaccharides
475 in *B. pseudomallei* infections is even more complex than has been previously described.
476 *Bpss2167* and *bpss2248* both encode predicted glycosyltransferases belonging to
477 glycosyltransferase family 2, but their specific roles are unknown. *Bps1444* shows similarity
478 to the glycotransferase *waaG*. This is notable because many of the other *waa* genes, which
479 are involved in the biosynthesis and construction of the core sugar of the *B. pseudomallei*
480 LPS, were identified as essential genes (28). It would be of interest in future experiments to
481 determine if *bps1444* plays a role in virulence due to being important to the structural integrity
482 of the bacterium or if this phenotype is due to compromised LPS.

483 It is interesting to note that of the five mutants characterized in this study, all but one
484 displayed some level of intracellular attenuation in at least one cell line. This is not
485 unexpected as *B. pseudomallei* is considered to be a facultative intracellular pathogen, but is
486 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

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

519

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525

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- 678

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

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

683 harvested from the infected animals and bacterial loads were determined. Lines indicated
684 mean and standard error for each sample. Statistical significance was determined using the
685 Mann-Whitney test with p values indicated above each sample. *ns* = not significant. Mice
686 infected with (A) 500 CFU of K96243 or Δ RpiR (spleen, $p=0.0079$) (B) 500 CFU of K96243 or
687 800 CFU of Δ BapA (*ns*) (C) 1×10^3 CFU of K96243 or Δ 1728 (lungs, $p=0.0317$; spleen
688 $p=0.0159$) (D) 1×10^3 CFU of K96243 or Δ tex (lungs, $p=0.0079$; spleen $p=0.0079$), and (E)
689 2×10^3 CFU of K96243 or Δ 2248 (lungs *ns*, spleen, $p=0.0317$)

690

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

692 (A) Balb/C mice ($n=5$) were infected with 10^3 CFU of K96243, Δ 1728, or Δ Tex. The median
693 survival for K96243 was 2.5 days post infection, while the median survival for Δ 1728 was 3
694 days post infection 4 out of 5 mice infected with Δ tex were still alive when the experiment was
695 terminated at 60 days post-infection. The survival of both mutants was statistically
696 significantly different from wild-type as determined by Log-rank (Mantle-Cox) test with p
697 values of 0.0449 and 0.0009 respectively. (B) The bacterial load in mice infected with Δ tex
698 was determined in surviving mice at 60 days post infection. All four mice displayed detectable
699 levels of *B. pseudomallei* Δ tex in the spleen, while only two animals had detectable CFUs in
700 the lungs. (C) Balb/C mice were vaccinated with either 10^3 CFU Δ tex or a saline control and
701 challenged intranasally with 10^3 CFU wild-type *B. pseudomallei* at 5 weeks post-vaccination.
702 Survival up to 80 days post-challenge is shown, with the Δ tex vaccinated animals showing a
703 statistically significantly different mean time-to-death of 31 days compared to 6 days for saline
704 vaccinated animals ($p<0.001$). (D) At 80 days post-challenge, surviving Δ tex-vaccinated mice
705 were sacrificed and lungs and spleens were harvested and plated to determine if the bacteria
706 had been cleared from the animals. All colonies isolated from both organs were determined to
707 be wild-type *B. pseudomallei* by PCR screening.

Page 26

708

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

710 (A) The *B. pseudomallei* TraDIS mutants are all able to induce their own uptake into A549
711 human lung epithelial cells. Cells were infected at a MOI of 10 for 1 hr, then washed and
712 overlaid with 400 µg/mL kanamycin. At 2 hours post-infection cells were lysed and the
713 bacterial loads determined. None of the mutants displayed statistically significantly different
714 bacterial CFU compared to wild-type as determined by ANOVA. (B) The *B. pseudomallei*
715 TraDIS mutants show variable growth and survival in A549 cells. Cells were infected with a
716 MOI of 1, and the infection was allowed to proceed for 18 hours. Δ RpiR, Δ BapA, and Δ tex all
717 had statistically significantly decreased bacterial loads as determined by Mann-Whitney test
718 with p values of $p=0.0005$, $p<0.00001$, and $p<0.00001$ respectively. (C) The *B. pseudomallei*
719 TraDIS mutants show variable growth and survival in J774 murine macrophages. Cells were
720 infected with a MOI of 1, and the infection was allowed to proceed for 16 h. Δ RpiR, Δ BapA,
721 and Δ tex all had statistically significantly decreased bacterial loads as determined by Mann-
722 Whitney test with p values of $p=0.0045$, $p=0.0078$, and $p=0.0002$ respectively. Interestingly, in
723 this cell line Δ 1728 also showed reduced bacterial load compared to wild-type ($p=0.0019$).

724

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

726 J774 murine macrophages were infected with either (A) *B. pseudomallei* K96243, (B) Δ RpiR,
727 (C) Δ BapA, (D) Δ 1728, (E) Δ tex and (F) Δ 2248 at an MOI of 10. After four hours, cells were
728 fixed and stained with the CC6 monoclonal antibody against *B. pseudomallei* LPS (green) and
729 phalloidin (red) which stains actin filaments. Actin comet tails (blue arrows) were visible in all
730 samples, indicating that the *B. pseudomallei* mutants are capable of entering cells and
731 escaping into the cytoplasm where they are able to polymerize actin to spread cell-to-cell.

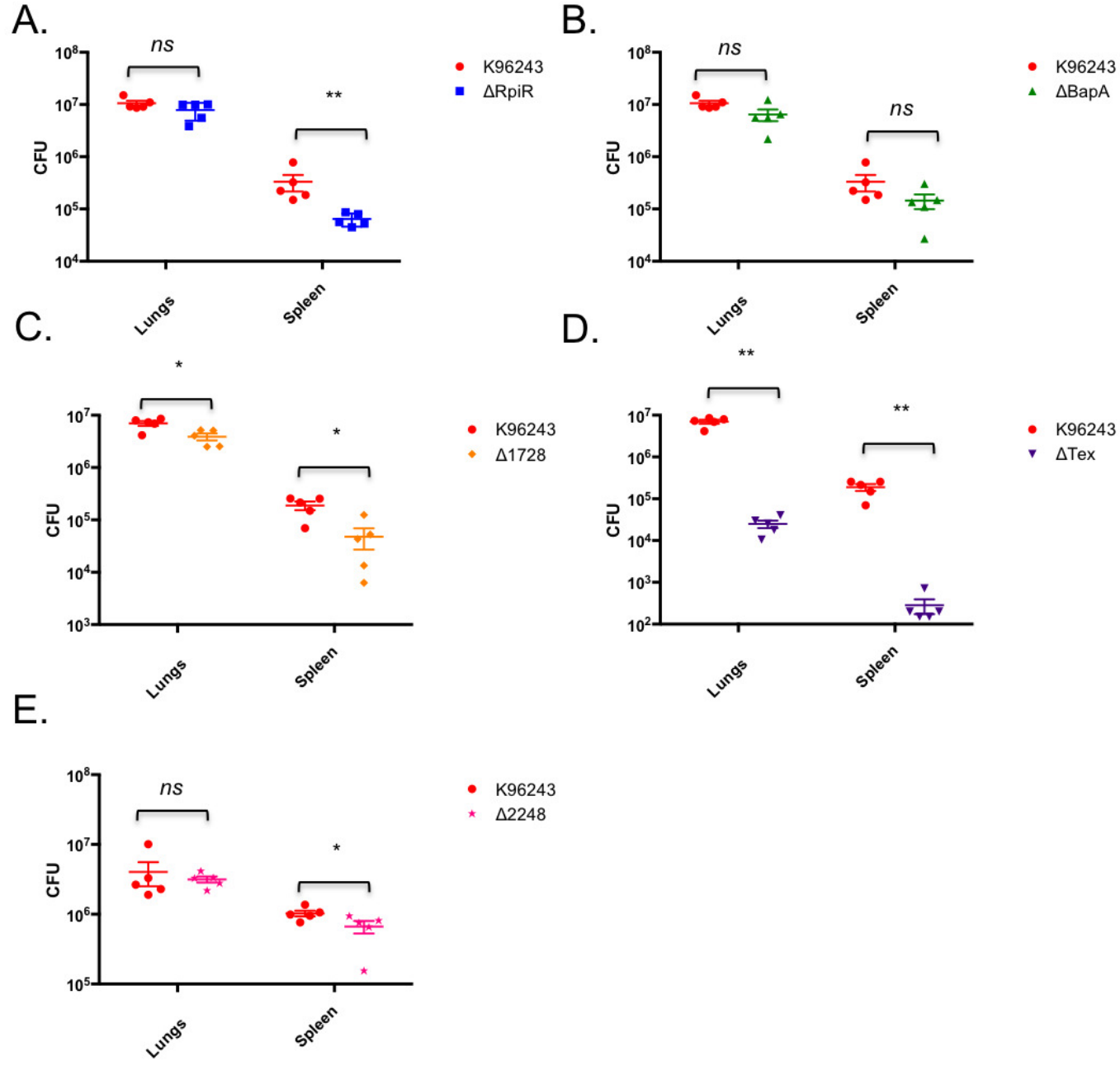
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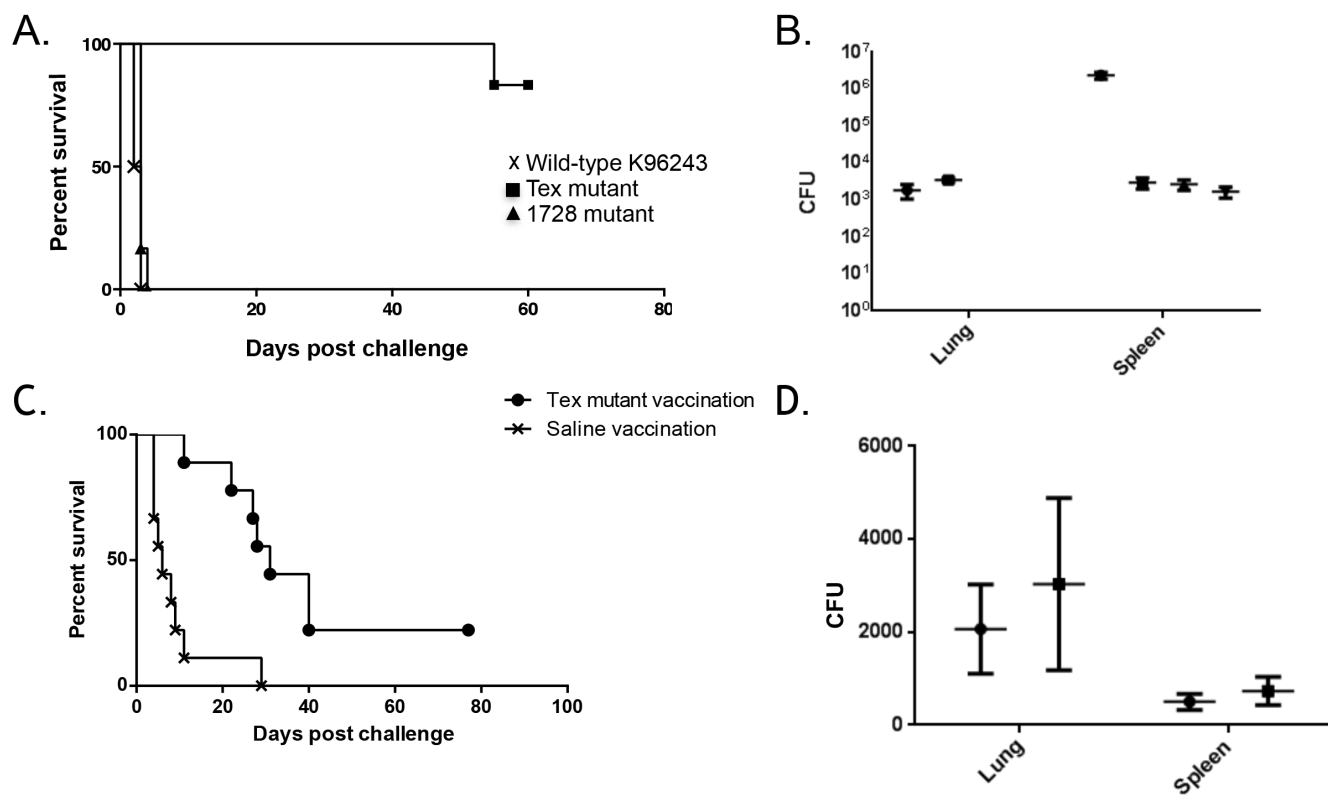
733 **Figure 5. Sensitivity of *B. pseudomallei* TraDIS mutants to human sera**

734 10^6 CFU of *B. pseudomallei* K96243 wild-type, Δ RpiR, Δ BapA, Δ 1728, Δ tex, and Δ 2248 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, Δ RpiR, Δ BapA, and Δ tex were resistant to killing by
737 human sera as has been previously reported for *B. pseudomallei* K96243, Δ 1728 and Δ 2248
738 were both sensitive to complement killing by human sera (p value 0.0029 and 0.0000056
739 respectively).

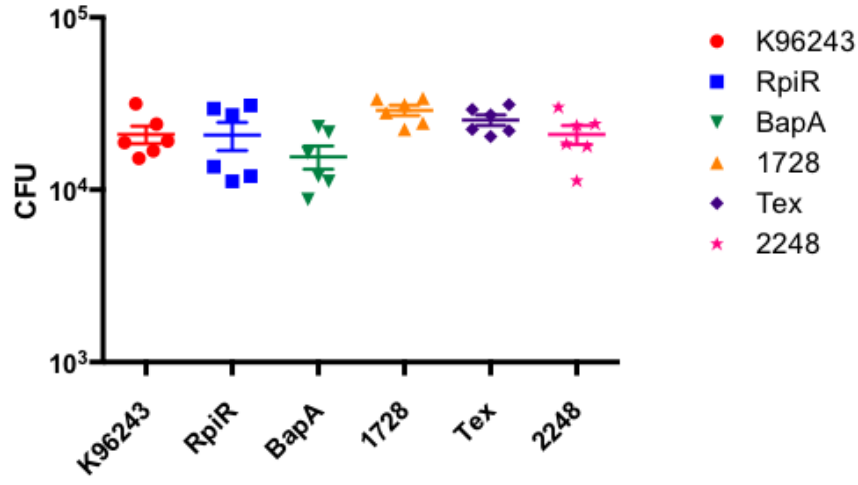
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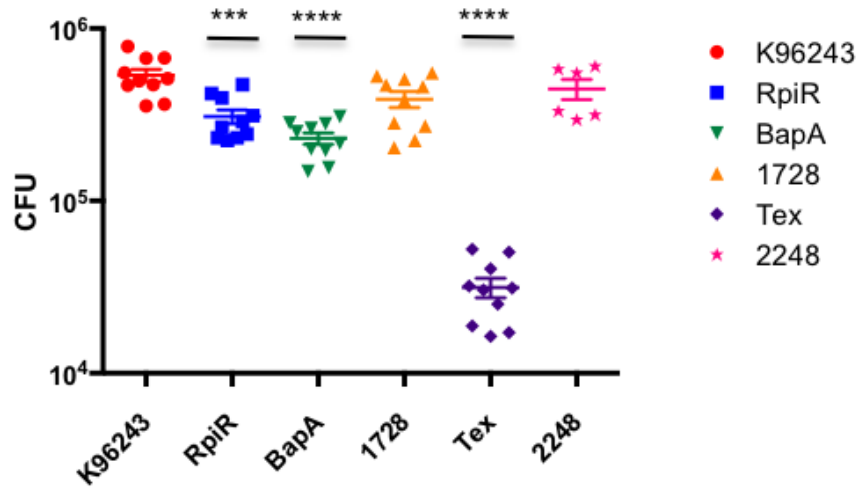




A.



B.



C.

