



20 **ABSTRACT**

21 The melioidosis bacterium, *Burkholderia pseudomallei*, is increasingly being recognized as a  
22 pathogen in patients with cystic fibrosis (CF). We have recently catalogued genome-wide  
23 variation of paired, isogenic *B. pseudomallei* isolates from seven Australasian CF cases, which  
24 were collected between four and 55 months apart. Here, we extend this investigation by  
25 documenting the transcriptomic changes in *B. pseudomallei* in five cases. Following growth in  
26 an artificial CF sputum medium, four of the five paired isolates exhibited significant differential  
27 gene expression (DE) that affected between 32 and 792 genes. The greatest number of DE  
28 events was observed between patient CF9 strains, consistent with the hypermutator status of  
29 the latter strain, which is deficient in the DNA mismatch repair protein MutS. Two patient  
30 isolates harbored duplications that concomitantly increased expression of the  $\beta$ -lactamase gene  
31 *penA*, and a 35kb deletion in another abolished expression of 29 genes. Convergent expression  
32 profiles in the chronically-adapted isolates identified two significantly downregulated and 17  
33 significantly upregulated loci, including the antibiotic resistance-nodulation-division (RND) efflux  
34 pump BpeEF-OprC, the quorum-sensing *hhqABCDE* operon, and a cyanide- and pyocyanin-  
35 insensitive cytochrome *bd* quinol oxidase. These convergent pathoadaptations lead to  
36 increased expression of pathways that may suppress competing bacterial and fungal pathogens  
37 and that enhance survival in oxygen-restricted environments, the latter of which may render  
38 conventional antibiotics less effective *in vivo*. Treating chronically-adapted *B. pseudomallei*  
39 infections with antibiotics designed to target anaerobic infections, such as the nitroimidazole  
40 class of antibiotics, may significantly improve pathogen eradication attempts by exploiting this  
41 Achilles heel.

42

## 43 INTRODUCTION

44 The Gram-negative soil-dwelling bacterium *Burkholderia pseudomallei* causes melioidosis, an  
45 opportunistic tropical infectious disease of humans and animals that has a high fatality rate  
46 (Wiersinga et al. 2012). *B. pseudomallei* is found in many tropical and subtropical regions  
47 globally, and has been unmasked in temperate and even arid environments following unusually  
48 wet weather events (Yip et al. 2015; Chapple et al. 2016; Sarovich et al. 2016). Infection occurs  
49 following percutaneous inoculation from contaminated soil or water, inhalation, or ingestion.  
50 Melioidosis symptoms vary widely due to the ability for *B. pseudomallei* to infect almost any  
51 organ, with pneumonia being the most common presentation (Leelarasamee and Bovornkitti  
52 1989; Currie et al. 2010). Individuals most at risk of contracting melioidosis include diabetics,  
53 those with hazardous alcohol consumption, and the immunosuppressed. There has been  
54 increasing recognition that people with chronic lung diseases such as cystic fibrosis (CF) are  
55 also at a heightened risk (Holland et al. 2002; O'Carroll et al. 2003).

56 CF is a heritable disorder of the *CFTR* gene, and defects in CFTR lead to exaggerated and  
57 ineffective airway inflammation, an imbalance in salt regulation in the lungs and pancreas, and a  
58 chronic overproduction of thick and sticky mucus in the airways and digestive system (Amaral  
59 2015). Impaired immunity and mucus clearance encourage infection and subsequent  
60 persistence and adaptation of opportunistic bacterial pathogens in the CF lung, leading to the  
61 development of bronchiectasis with subsequent progressive pulmonary decline, and ultimately,  
62 loss of pulmonary function and death (Cohen and Prince 2012).

63 The most common pathogens of the CF lung comprise *Pseudomonas aeruginosa*,  
64 *Staphylococcus aureus*, *Haemophilus influenzae*, and less commonly, *Achromobacter*  
65 *xylosoxidans*, non-tuberculosis mycobacteria, *Stenotrophomonas maltophilia* and certain  
66 *Burkholderia* species, including *B. cepacia* complex species and *B. pseudomallei* (Coutinho et

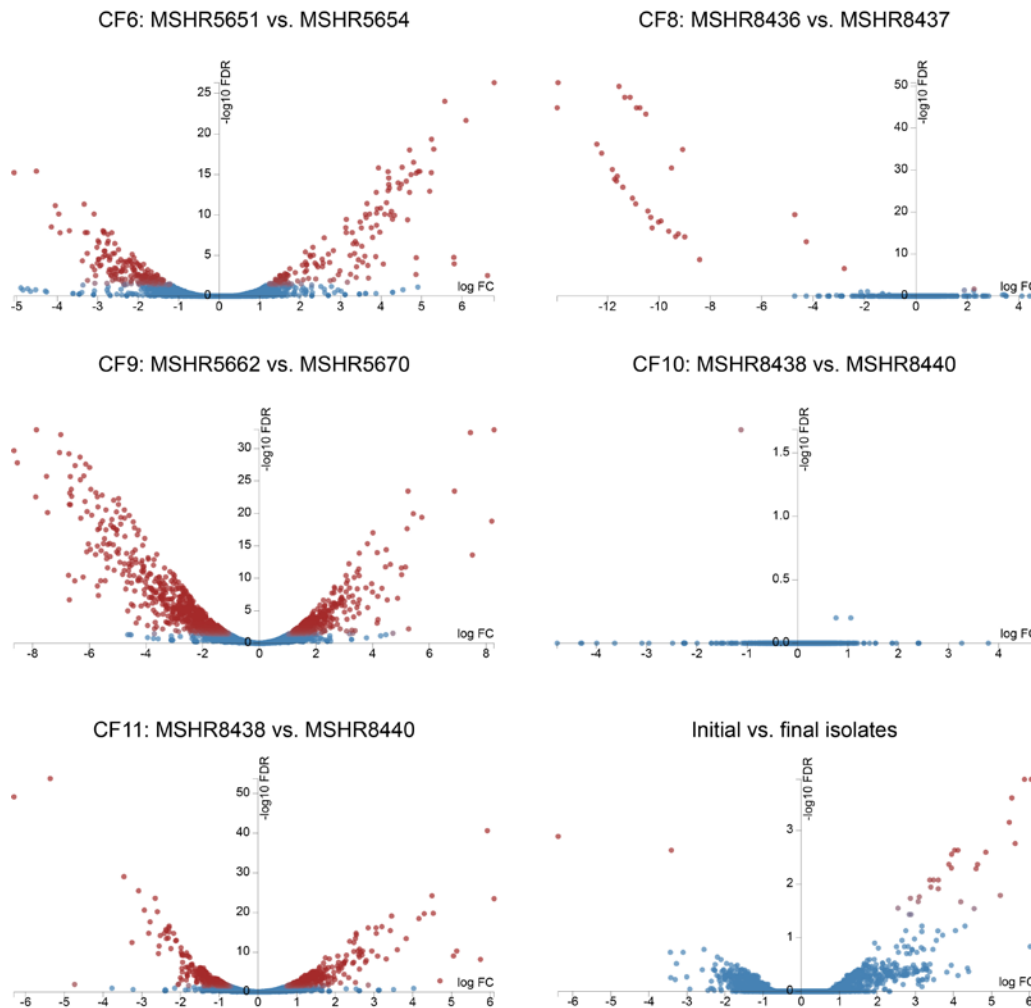
67 al. 2008). The most common and best-studied CF pathogen is *P. aeruginosa*, which can adapt  
68 to the CF lung environment via various mechanisms. Convergent pathoadaptations in *P.*  
69 *aeruginosa* include the downregulation or loss of virulence factors and motility-encoding loci,  
70 emergence of hypermutators, enhanced antibiotic resistance and immune evasion facilitated by  
71 a switch to mucoidy and a biofilm-based lifestyle, and altered expression of other loci enhancing  
72 bacterial metabolism and survival within the nutrient-poor CF lung environment (Cohen and  
73 Prince 2012; Winstanley et al. 2016).

74 Improving life expectancy for those with CF has led to an increased risk of exposure to *B.*  
75 *pseudomallei* following travel to melioidosis-endemic regions. Although uncommon, infection of  
76 the CF lung by *B. pseudomallei* has now been documented in at least 25 cases worldwide  
77 (Geake et al. 2015). Due to low total case numbers, comparatively little is understood about the  
78 pathogenic role of *B. pseudomallei* in CF pulmonary disease. The most common clinical  
79 presentation is chronic carriage (76%), which is associated with accelerated lung function  
80 decline (Geake et al. 2015). This prevalence contrasts with melioidosis in non-CF patients,  
81 where chronic carriage is exceedingly rare, occurring in only 0.2% of cases (Price et al. 2015).  
82 To better understand *B. pseudomallei* pathoadaptation in the CF lung, we recently investigated  
83 the genome-wide evolution of isogenic *B. pseudomallei* strains isolated from seven Australasian  
84 CF patients, which were collected between 4 and 55 months apart (Viberg et al. 2017).  
85 Hallmarks of these infections included *B. pseudomallei* persistence despite multiple eradication  
86 attempts, multidrug resistance, mutations in virulence, metabolism and cell wall components,  
87 and the first-documented case of hypermutation in *B. pseudomallei*. In all except one case,  
88 multiple single-nucleotide polymorphism (SNP) and insertion-deletion (indel) mutations were  
89 identified, with a high rate of nonsynonymous mutations, many of which were predicted to affect  
90 protein function (Viberg et al. 2017).

91 RNA-seq provides a detailed view of the transcriptional landscape in bacterial isolates grown  
92 under different conditions or niches (Sharma et al. 2010), and is now a well-established method  
93 for examining differential gene expression (DE) in bacterial pathogens (Creecy and Conway  
94 2015). Here, we performed bacterial RNA-seq on five of the CF cases that we have recently  
95 described (Viberg et al. 2017) to catalogue both within-host and convergent transcriptional  
96 evolution during long-term *B. pseudomallei* infection in the CF lung. Paired isolates representing  
97 the initial and the most recent cultures available from each patient were compared. *B.*  
98 *pseudomallei* cultures were grown in an artificial sputum medium (Sriramulu et al. 2005; Fung et  
99 al. 2010) to mimic the conditions found in the CF lung environment.

## 100 RESULTS

101 **Differential gene expression among CF isogenic pairs.** DE was observed in four of the five  
102 CF pairs, with only the CF10 pair failing to yield significant transcriptional differences (i.e. no  
103 genes with a  $\geq 1.5$   $\log_2$ -fold change and a false discovery rate (FDR) of  $\leq 0.01$ ; Figure 1). We  
104 have previously shown that no genetic variants separate the CF10 strains, which had the  
105 shortest time between collection of only 10 months (Viberg et al. 2017). This lack of significant  
106 transcriptional differences rules out epigenetic effects on gene expression between this pair, at  
107 least under the tested growth conditions, and illustrates that RNA-seq is a robust methodology  
108 that is not readily prone to false-positive results.



109

110 **Figure 1.** Degust volcano plots showing differentially expressed (DE) genes between paired  
111 *Burkholderia pseudomallei* isolates retrieved from five cystic fibrosis (CF) lungs, and between  
112 initial and latter isolates. Four of the five pairs exhibited DE; CF10, with the shortest time  
113 between isolates, exhibited no genetic or significant transcriptomic changes. CF6, CF8, CF9  
114 and CF11 pairs were separated by 229, 32, 792 and 169 DE loci, respectively. The 32 DE loci in  
115 CF8 were all downregulated; DE genes in CF6, CF9 and CF11 were down- or up-regulated.  
116 Nineteen loci were DE between initial and latter isolates, of which 17 were upregulated. Blue,  
117 genes with no significant DE; red, genes with significant DE.

118

119 Of the four pairs with significant DE, the CF8 pair had the least with 32 loci, followed by CF11,  
120 CF6 and CF9 with 169, 229 and 792 DE loci, respectively (Figure 1; Table 1). These paired  
121 isolates were collected 46, 14, 27 and 55 months apart, respectively. There was good  
122 correlation between the proportion of DE loci and the genome-wide mutations catalogued  
123 between these isolate pairs (Viberg et al. 2017), with 12, 15, 24, and 112 mutational events (i.e.  
124 SNPs, indels, deletions or gene duplications) identified in CF8, CF11, CF6 and CF9,  
125 respectively (Table 1). The elevated number of mutations seen in CF9 is due to a *mutS*  
126 mutation in the latter strain, which confers a hypermutator phenotype, the first time  
127 hypermutation has been described in *B. pseudomallei* (Viberg et al. 2017); this in turn  
128 contributes to a high number of DE genes. However, when comparing the ratio of DE genes to  
129 mutational events, CF11 had the highest proportion of DE genes (11.3), followed by CF6 (9.5),  
130 CF9 (7.1) and CF8 (2.7). There was a significant skew in DE towards genes located on  
131 chromosome II, which contains a lower proportion of housekeeping genes than chromosome I  
132 (Holden et al. 2004). Despite encoding only 44% of the genome by size and 41% of coding  
133 sequences, chromosome II loci were significantly overrepresented in the non-hypermutator CF

134 pairs (Pearson's  $X^2$  test  $p < 0.001$ ), with between 63 and 97% of the DE genes residing on  
 135 chromosome II. In CF9, there was a non-significant trend towards chromosome II loci, with 52%  
 136 of DE loci located on this chromosome, pointing to the more random nature of mutations in the  
 137 CF9 hypermutator compared with the other cases (Table S1).

138 **Table 1.** Summary of the genetic mutations and differentially expressed (DE) genes between  
 139 paired, sequential *Burkholderia pseudomallei* isolates obtained from five cystic fibrosis patients<sup>a</sup>.

Patient	Initial and final isolate IDs	Months between collection	No. genome-wide mutational events (Chr I, Chr II)	No. genes affected by mutations (Chr I, Chr II)	No. DE genes (Chr I, Chr II)	No. DE genes (downregulated, upregulated)
CF6	MSHR5651, MSHR5654	27	24 (14, 10)	29 (14, 15)	229 (69, 160)	229 (124, 105 <sup>b</sup> )
CF8	MSHR8436, MSHR8437	46	12 (7, 5)	39 (5, 34)	32 (1, 31)	32 (32 <sup>c</sup> , 0)
CF9	MSHR5662, MSHR5670	55	112	79 (40, 39)	792 (381, 411)	792 (558, 234)
CF10	MSHR8438, MSHR8440	10	0	0	0	0
CF11	MSHR8441, MSHR8442	14	15	38 (10, 28)	169 (62, 107)	169 (68 <sup>d</sup> , 101)

140 <sup>a</sup>Adapted from (Viberg et al. 2017).



141 <sup>b</sup>Seven of these genes are upregulated due to a 30x duplication affecting these loci in  
142 MSHR5654.

143 <sup>c</sup>Twenty-nine of these genes are downregulated due to a 35kb deletion affecting these loci in  
144 MSHR8437.

145 <sup>d</sup>Twenty-five of these genes appear as downregulated due to a 10x duplication affecting a  
146 36.7kb locus in MSHR8441 compared with only a 2x duplication in MSHR8442 (Viberg et al.  
147 2017).

148

149 **Many DE genes are absent in *Burkholderia mallei* and in chronic-carriage melioidosis**

150 **Patient 314.** *B. mallei*, the causative agent of glanders, is an equine-adapted clone of *B.*  
151 *pseudomallei* that continues to undergo dramatic reductive evolution, having already shed ~1.3-  
152 1.5Mbp of its genome since its divergence from *B. pseudomallei* (Holden et al. 2004; Price et al.  
153 2013). As a consequence, this bacterium does not survive in the environment, although it  
154 remains highly pathogenic (Dvorak and Spickler 2008). *B. mallei* therefore provides a useful  
155 comparison for examining DE in the CF *B. pseudomallei* strains due to similar genome-wide  
156 patterns of locus loss and adaptation to a mammalian host (Viberg et al. 2017). We also  
157 compared the DE loci in the CF strains to those genes mutated in chronic-carriage melioidosis  
158 case P314. P314 has the longest *B. pseudomallei* infection ever documented, and despite  
159 multiple eradication attempts, continues to harbor this bacterium in her lungs since she was first  
160 diagnosed in 2000. The genome of a 139-month isolate from P314, MSHR6686, shows  
161 dramatic adaptation to the lung environment, including the loss of 285kb of chromosome II at  
162 four separate locations that collectively encompass 221 genes; ~50% of these genes are also  
163 absent in *B. mallei* (Price et al. 2013).

164 When compared with genes lost in 17 *B. mallei* strains (092700E 11, 2000031063,  
165 2000031281, 2002721280, 6, A188, A193, ATCC10399 , ATCC23344, BMQ, China5,  
166 China7, FMH23344, GB8horse4, JHU, and PRL-20), there was a 23, 27, 33 and 100% overlap  
167 with DE genes in the latter isolates from CF9, CF6, CF11 and CF8, respectively, and when  
168 compared with genes lost in MSHR6686, there was a 9, 15, 20 and 97% overlap, respectively  
169 (Table S1). The proportion of downregulated genes varied across this dataset, ranging from  
170 13% for CF11 to 100% for CF8, demonstrating that the effect on gene expression at these loci  
171 is not unidirectional, with certain overlap loci in fact being upregulated in CF6, CF9 and CF11.  
172 Of note, all 29 genes (*BPSS1131-BPSS1159*) that were lost due to a large deletion in the latter  
173 CF8 isolate (Viberg et al. 2017) were also absent in *B. mallei* and MSHR6686 (Price et al.  
174 2013), providing further evidence of their dispensability for long-term *B. pseudomallei* survival in  
175 the mammalian host. As expected, DE showed dramatic downregulation of between 8- and 14-  
176 log<sub>2</sub> fold (341 to 15,886x) of these loci (Figure 1).

177 **DE of surface antigens in the CF6, CF9 and CF11 pairs.** *B. pseudomallei* produces four  
178 capsular polysaccharides (CPS-I to -IV) and a lipopolysaccharide (LPS). However, only CPS-I  
179 (encoded by *BPSL2786-BPSL2810*) and LPS (encoded by *BPSL1936* and *BPSL2672-*  
180 *BPSL2688*) are associated with virulence in mammals (Reckseidler-Zenteno et al. 2010; Stone  
181 et al. 2014). The remaining three capsule clusters are found in *B. thailandensis* but are not  
182 present or intact in *B. mallei*. Our previous genomic analysis of the CF pairs identified missense  
183 mutations affecting the LPS loci *wzt* (*BPSL2681*) and *rmIA* (*BPSL2685*) in CF6, a missense  
184 mutation affecting the putative LPS biosynthesis gene *BPSL1119* in CF11, and a CPS-I  
185 frameshift mutation affecting *wcbA* (*BPSL2809*) in CF11 (Viberg et al. 2017). Consistent with  
186 being under heavy selection during chronic infection, we observed DE of several surface  
187 polysaccharide loci in the CF6, CF9 and CF11 pairs, the most dramatic of these being in CF9,  
188 with forty-six downregulated surface polysaccharide genes (Table S1).

189 The LPS loci *wbiE* (*BPSL2676*) and *wbiD* (*BPSL2677*) were downregulated by ~1.8-fold (3x) in  
190 the latter CF9 isolate. In addition, the poorly characterized LPS biosynthesis-related membrane  
191 protein loci *BPSS1683-BPSS1685* were downregulated by ~5.9-fold (60x). This isolate also  
192 exhibited downregulation of all CPS-I loci (except *wcbC*) by between 1.7- and 3.1-fold (3 to 8x).  
193 In contrast, the latter isolate from CF11 showed upregulation of the CPS-I loci *BPSL2793-*  
194 *BPSL2797* (*wcbN-wcbM-gmhA-wcbL-wcbK*) when compared with its initial isolate, with  
195 increases ranging from 2.7 to 6.1-fold (7 to 69x). However, when compared with initial isolates  
196 from CF6, CF8, CF9 and CF10, expression of *BPSL2793-BPSL2797* in the latter CF11 strain  
197 was in fact downregulated (3.4 to 4.5-fold; 11 to 23x). This observation was confirmed as  
198 significant downregulation of these loci in the initial CF11 strain (by between 6.1- and 10.1-fold;  
199 69 to 1,098x) when compared with all other initial strains, rather than significant upregulation of  
200 these CPS-I loci in the latter CF11 strain.

201 Unlike CPS-I, expression of the CPS-II cluster (*BPSS0417-BPSS0429*) is induced when grown  
202 in water, suggesting that this polysaccharide plays a role in environmental survival (Reckseidler-  
203 Zenteno et al. 2010). One locus involved in CPS-II biosynthesis, *BPSS0425*, was  
204 downregulated (1.8-fold; 4x) in CF6, and the entire cluster was downregulated in CF9 (range:  
205 2.5- to 5.2-fold; 6 to 37x). Conversely, *BPSS0417* and *BPSS0418* were upregulated in CF11  
206 (1.6- and 1.7-fold; 3x), respectively. However, as with CPS-I, both CF11 strains exhibited  
207 significant downregulation of *BPSS0417* and *BPSS0418* when compared with initial strains from  
208 CF6, CF8, CF9 and CF10 (2.0- and 3.1-fold; 4 to 9x). The genes encoding CPS-III (*BPSS1825-*  
209 *BPSS1835*) were significantly downregulated in the latter isolates from CF6 (2.4- to 3.1-fold; 5  
210 to 9x) and CF9 (5.6- to 7.5-fold; 50 to 178x). Finally, two genes within the CPS-IV cluster  
211 (*BPSL2769-BPSL2785*) were downregulated in CF9 (*BPSL2782* and *BPSL2785*; 1.8- to 2.8-  
212 fold; 3 to 7x) but 11/17 loci from this cluster were upregulated in CF11 by 1.7- to 5.9-fold  
213 (*BPSL2769*, *BPSL2775-BPSL2784*). However, unlike CPS-I and CPS-II, this pattern of

214 upregulation in both CF11 isolates was maintained for CPS-IV loci *BPSL2769* and *BPSL2775-*  
215 *BPSL2781* even when compared with the initial CF6, CF8, CF9 and CF10 isolates (2.0- to 4.0-  
216 fold; 4 to 16x).

217 **DE of other virulence-associated loci.** In addition to CPS-I and LPS, *B. pseudomallei*  
218 encodes for several other virulence factors that enhance organism survival and replication upon  
219 infection or that subvert or disarm host defenses. These factors include adhesins, flagella,  
220 fimbriae, pili, specialized secretion systems, actin motility proteins, secreted factors and  
221 secondary metabolites (Stone et al. 2014). Although virulence factors are often critically  
222 important during the acute stages of infection, they can become disadvantageous for long-term  
223 survival, presumably due their immunogenicity (Price et al. 2013; Winstanley et al. 2016).  
224 Consistent with loss-of-virulence as a pathoadaptive mechanism in chronic *B. pseudomallei*  
225 infections, we have previously documented missense mutations affecting the Type 3 secretion  
226 system 3 (T3SS-S) gene *bsaW* in CF6, and *Burkholderia* biofilm factor A *bbfA* and fimbrial  
227 protein BPSL1628 in CF9 (Viberg et al. 2017). When examining RNA-seq profiles, other  
228 virulence genes lacking genetic mutations were found to be significantly downregulated in the  
229 latter CF isolates. These loci included three Type IV pilus 7 (TFP7) loci (*pilR*, *pilG* and *pilN*; 1.7-  
230 fold; 3x), the lysozyme inhibitor *BPSL1057* (3.4-fold; 11x), *Burkholderia* lethal factor 1 (3.2-fold;  
231 10x), four T3SS-3 loci (*bsaS*, *bsaP*, *bsaO* and *bsaN*), and 16 flagellum loci in CF9 (average 2.5-  
232 fold; 6x), and a trimeric autotransporter adhesin (*bpaC*; *BPSL1631*) in CF11 (1.6-fold; 3x). Of  
233 these, the four T3SS-3 loci are also missing in chronic P314 isolates.

234 **Several regulators with decreased transcription in the CF isolates are absent in *B. mallei*.**  
235 We hypothesized that downregulation of transcriptional regulators, particularly those absent in  
236 *B. mallei*, would be identified in the latter CF isolates due to niche adaptation. As predicted, two  
237 of the four pairs exhibited significant downregulation of transcriptional regulators. The first of

238 these, the Fis family regulatory protein YfhA (encoded by *BPSL0350*), was downregulated by  
239 ~2.7-fold (~7x) in both CF6 and CF9. In *E. coli*, Fis is a global regulator that is induced under  
240 nutrient-rich conditions and plays a role in the regulation of myriad processes including the  
241 initiation of DNA replication, ribosomal RNA transcription activation and capsule expression  
242 (Beach and Osuna 1998). Thus, the downregulation of Fis in CF6 and CF9 may be responsible  
243 for concomitant downregulation of CPS-II and CPS-III loci in these two patients, among other  
244 loci. Additional regulators downregulated in CF9 that are absent in *B. mallei* include the  
245 transmembrane regulator PrtR (*BPSL0069*; 2.7-fold), two LysR-family transcriptional regulators  
246 (*BPSS0438* and *BPSS2207*; both 1.7-fold) and the metal-related two-component system  
247 response regulator IrlR2 (*BPSS1994*; 2.7-fold).

248 **High-level TMP/SMX resistance in *B. pseudomallei* involves *bpeEF-oprC* upregulation.**

249 The combination antibiotic trimethoprim/sulfamethoxazole (TMP/SMX), the antibiotic of choice in  
250 the eradication phase of melioidosis treatment (Lipsitz et al. 2012), was administered to CF6,  
251 CF9 and CF11 during their *B. pseudomallei* eradication attempts. Acquired resistance towards  
252 this antibiotic emerged in the latter isolates from CF6 and CF11, and in midpoint isolates from  
253 CF9 (Viberg et al. 2017). The resistance-nodulation-cell division (RND) efflux pump, BpeEF-  
254 OprC, is responsible for widespread TMP resistance in *B. pseudomallei* and has been  
255 implicated in TMP/SMX resistance (Podnecky et al. 2017). BpeEF-OprC (*BPSS0292*-  
256 *BPSS0294*) expression is under the control of two LysR-type regulators, BpeT (*BPSS0290*) and  
257 BpeS (*BPSL0731*). We therefore expected to observe upregulation of *bpeEF-oprC* in CF strains  
258 with elevated TMP/SMX MICs, consistent with defective *bpeT* or *bpeS* loci.

259 The latter isolate from CF6, which encodes a T314fs mutation in *bpeT* and is highly resistant  
260 towards TMP/SMX (MIC  $\geq$ 32  $\mu$ g/mL), showed 5.2 to 6.8-fold upregulation of *bpeEF-oprC* (38 to  
261 111x; Table S1). This isolate also harbours an R20fs mutation in *ptr1* (*BPSS0039*; *foIM*), which

262 encodes a pteridine reductase that is involved in TMP/SMX resistance (Podnecky et al. 2017);  
263 this frameshift truncates Ptr1 from 267 to 91 residues. Both strains isolated from CF11 are also  
264 highly resistant to TMP/SMX (MIC  $\geq$ 32  $\mu$ g/mL) and encode the BpeS missense variants V40I  
265 and R247L (K96243 annotation) when compared with wild-type *B. pseudomallei* strains (Viberg  
266 et al. 2017). They also encode a three-residue in-frame insertion of R20-A22 in Ptr1 (Podnecky  
267 et al. 2017; Viberg et al. 2017). Because both CF11 strains are TMP/SMX-resistant, DE was  
268 determined by comparison with TMP/SMX-sensitive isolates in our dataset. Using this  
269 approach, significant upregulation was observed for BpeEF-OprC (5.3 to 6.9-fold; 40 to 121x).  
270 DE was not observed for *ptr1* or other genes involved in the folate biosynthesis pathway in  
271 either the CF6 or CF11 isolates.

272 **Ceftazidime resistance can occur by upregulation of *penA*.** Ceftazidime (CAZ) is a third-  
273 generation cephalosporin antibiotic that is the most commonly recommended therapy for the  
274 primary phase of melioidosis treatment (Currie 2015). In addition to TMP/SMX, the latter isolate  
275 from CF6 is highly resistant to CAZ (MIC  $\geq$ 256  $\mu$ g/mL) (Viberg et al. 2017). High-level CAZ  
276 resistance is often conferred by a C94Y substitution (C69Y using Ambler's (Ambler et al. 1991)  
277 numbering scheme) in PenA  $\beta$ -lactamase (Sam et al. 2009; Rholi et al. 2011; Sarovich et al.  
278 2012). We have recently shown that the latter CF6 strain also harbors a  $\sim$ 30x duplication of a  
279 7.5kb region that encompasses *penA*; all 30 copies encode the C69Y variant of this enzyme  
280 (Viberg et al. 2017). Consistent with this duplication event, *penA* (*BPSS0946*) expression  
281 increased by 4.5-fold (22x) in the latter CF6 strain. Six proximal genes (*BPSS0945*; *BPSS0948*-  
282 *BPSS0952*) were also upregulated by 3.1- to 4.7-fold (9 to 26x; Table S1). One of these,  
283 *BPSS0945*, is a peptidase and a putative virulence factor that may play a role in multinucleated  
284 giant cell formation (Singh et al. 2013).

285 A gene duplication event encompassing *penA* has also been documented in the CF11 isolates.  
286 The initial strain showed an elevated MIC towards CAZ (12 µg/mL), corresponding with a ~10x  
287 duplication of a 36.7kb region that includes *penA*, whereas the latter strain had a 2x duplication  
288 of this region and a wild-type CAZ MIC (2 µg/mL) (Viberg et al. 2017). As expected, *penA* was  
289 downregulated by 2.1-fold (4x) in the latter isolate due to five times fewer copies of this gene.  
290 Downregulation of other genes within the 36.7kb locus ranged from 1.4 to 3.3-fold (3 to 10x;  
291 Table S1).

292 **Increased doxycycline MICs in CF11 are due to *amrAB-oprA* upregulation and *BPSL3085***  
293 **mutation.** The RND efflux pump, AmrAB-OprA (*BPSL1802-BPSL1804*), efficiently effluxes  
294 aminoglycoside- and macrolide-class antibiotics (Moore et al. 1999). We have recently shown  
295 that synergistic mutations affecting both its regulator AmrR (*BPSL1805*) and an S-adenosyl-L-  
296 methionine (SAM)-dependent methyltransferase (*BPSL3085*) led to doxycycline resistance in an  
297 Australian melioidosis case (Webb et al. 2017). Doxycycline was administered to CF11 in  
298 combination with TMP/SMX as part of a final attempt to eradicate *B. pseudomallei* (Currie 2015;  
299 Geake et al. 2015). This lengthy administration led to a doxycycline MIC of 4-8 µg/mL in the  
300 CF11 isolates, both of which were retrieved post-treatment (Viberg et al. 2017).

301 Both CF11 strains encode a large deletion in *amrR* (*amrR*<sup>ΔV62-H223</sup>). This mutation results in a  
302 2.6- to 3.2-fold (6 to 9x) upregulation of *amrAB-oprA* in these isolates. In addition, a previously  
303 undocumented S130L mutation in *BPSL3085* likely contributes to the decreased susceptibility  
304 observed towards doxycycline.

305 **Evidence of convergent DE between early and latter CF isolates.** Finally, we performed a  
306 comparison of expression profiles from all CF cases to identify a signal of convergent gene  
307 expression (pathoadaptation) across early vs latter isolates. To yield the most robust and  
308 relevant analysis, we excluded the latter isolate from CF10 due to a lack of DE in this strain, and

309 the initial isolate from CF11, which was retrieved >3 years after infection and had already  
310 undergone substantial genetic and transcriptional modifications. Exclusion of both strains was  
311 supported by a lack of convergent signal when they were included in the analysis (data not  
312 shown). Using these parameters, 17 genes were found to be significantly upregulated, and two  
313 were significantly downregulated (Table S2). Five (26%) loci encode for hypothetical proteins  
314 with no known function, of which four were upregulated. Among the convergently upregulated  
315 genes with known function was the RND efflux pump BpeEF-OprC (4.8- to 6.1-fold; 28 to 69x),  
316 the CydAB cytochrome *bd* quinol oxidase (5.5- to 5.9-fold; 45 to 60x), and the quorum sensing  
317 *hhqABCDEFG* (*BPSS0481-BPSS0487*) operon (3.4- to 4.1-fold; 11 to 17x). The downregulated  
318 locus, *BPSS0351*, encodes the MerR family transcriptional regulator CueR (3.4-fold; 11x).

319



## 320 DISCUSSION

321 The causative agent of the tropical disease melioidosis, *B. pseudomallei*, is an uncommon  
322 pathogen in CF, with fewer than 30 cases documented worldwide to date (Geake et al. 2015).  
323 We have recently performed comparative genomic analysis of isogenic strains collected  
324 between 4 and 55 months apart from the airways of seven of these cases (Viberg et al. 2017).  
325 Here, we sought to further characterize these chronic cases by examining the transcriptomes of  
326 five paired *B. pseudomallei* isolates retrieved between 10 and 55 months apart. Isolates were  
327 cultured in an artificial CF sputum medium (Fung et al. 2010) to mimic their original *in vivo*  
328 environment.

329 Under these conditions, DE was detected in four of the five cases and ranged from 32 to 792  
330 genes, with the hypermutator strain from CF9 contributing the greatest number of DE loci (Table  
331 S1). Interestingly, when compared with the number of genetic changes occurring in each isolate  
332 pair, the latter isolate from CF11 had a higher proportion of DE loci to mutations (11.3) than CF9  
333 (7.1), demonstrating that hypermutation does not necessarily lead to a similarly high number of  
334 transcriptional differences. The one case with no DE, CF10, exhibited no genetic changes (i.e.  
335 SNPs, small indels, copy-number variants, or large deletions) and had the shortest time  
336 between isolate collection at 10 months (Viberg et al. 2017). All other cases encoded genetic  
337 differences between pairs. The DE genes fell into several functional categories (Table S1),  
338 reflecting the diversity and versatility of pathoadaptive pathways in *B. pseudomallei*. Our RNA-  
339 seq analysis revealed that many of the DE genes were absent in the chronic P314 strain (range:  
340 9-97%) or in *B. mallei* (range: 23-100%), providing further evidence that these loci are not  
341 required for long-term survival in the human airways. Perhaps most striking was the observation  
342 that nearly one-third (32%) of DE genes lack a known function, highlighting the relative paucity  
343 of functional studies into this important yet under-recognized pathogen.

344 *B. pseudomallei* has a ~7.3Mbp genome that is encoded on two replicons; a ~4.1Mbp  
345 'housekeeping' chromosome I, and a ~3.2Mbp 'accessory' chromosome II. The genome of  
346 archetypal strain K96243 consists of 3,460 and 2,395 coding sequences on chromosomes I and  
347 II, respectively (Holden et al. 2004). There was a greater proportion of DE genes (between 52  
348 and 97%) on chromosome II in all cases, despite its smaller size and fewer coding sequences.  
349 This bias towards DE on the accessory chromosome contrasts with a 2009 study of a CF-  
350 derived *B. cenocepacia* isolate, which displayed a greater proportion of DE genes on  
351 chromosome I compared with chromosomes II and III when the isolate was grown in an artificial  
352 sputum medium (Yoder-Himes et al. 2009). However, the study by Yoder-Himes and colleagues  
353 compared DE of a single isolate grown in sputum versus a soil medium, rather than between  
354 longitudinal clinical isolates, which may explain the discordance between studies. We have  
355 previously shown that a greater proportion of mutational events affect chromosome II of *B.*  
356 *pseudomallei* in a long-term chronic-carriage isolate from P314 (Price et al. 2013), and in the  
357 mammalian-adapted *B. pseudomallei* clone *B. mallei*, a greater proportion of genes have been  
358 lost from chromosome II than chromosome I, with chromosome II representing 44% of the *B.*  
359 *pseudomallei* K96243 genome (Holden et al. 2004) but only 40% of the ATCC 23344 *B. mallei*  
360 genome (Nierman et al. 2004). The skew towards DE loci on chromosome II in chronic *B.*  
361 *pseudomallei* isolates points towards a lesser role for chromosome II loci in bacterial survival  
362 and persistence within the human host, which is reflected by the greater degree of reductive  
363 evolution affecting this replicon (Price et al. 2013; Viberg et al. 2017). In their study of the  
364 transcriptional landscape of *B. pseudomallei*, Ooi and co-workers found that only ~28% of  
365 chromosome II genes were expressed under a single condition, compared with ~72% of  
366 chromosome I genes (Ooi et al. 2013). Taken together, these results confirm the 'accessory'  
367 role of the chromosome II replicon in *B. pseudomallei*.

368 Attenuation of immunogenic surface antigens and other virulence factors are hallmarks of  
369 chronically persistent infections across many pathogenic bacterial species, including *B.*  
370 *pseudomallei* (Price et al. 2013; Viberg et al. 2017). Encoded by the 34.5kb *wcb* operon  
371 *BPSL2786-BPSL2810* (Reckseidler et al. 2001; Reckseidler-Zenteno et al. 2005), the *B.*  
372 *pseudomallei* CPS-I is a potent virulence determinant that imparts high-level serum resistance  
373 and facilitates phagocytic evasion (Reckseidler-Zenteno et al. 2005). This capsule is also intact  
374 in *B. mallei* and has been shown to be essential for its virulence (DeShazer et al. 2001; Atkins et  
375 al. 2002). Our prior genomic analysis identified only a single CF pair with mutated CPS-I in  
376 CF11. This frameshift mutation in *wcbA* results in a truncated protein (Viberg et al. 2017) that  
377 would likely cause reduced, although not abolished, CPS-I production (Cuccui et al. 2012). The  
378 DE analysis provides further evidence of CPS-I inactivation in the CF pairs, with downregulation  
379 of all but one of the CPS-I loci in the latter CF9 isolate, and downregulation of *wcbN-wcbM-*  
380 *gmhA-wcbL-wcbK* in both CF11 isolates. In both cases, it is likely that CPS-I production was  
381 either substantially reduced or abolished. Although CPS-III is not required for virulence, it is  
382 noteworthy that this locus was also downregulated in CF9 and CF11, as it has been previously  
383 shown that CPS-III expression is tied to that of CPS-I genes (Ooi et al. 2013).

384 Like CPS-I, the *B. pseudomallei* LPS is required for capsule biosynthesis, virulence and serum  
385 resistance (DeShazer et al. 1998). Its immunogenic outer membrane component is readily  
386 recognized by the host innate immune system (Tuanyok et al. 2012), which makes LPS a target  
387 for inactivation in chronic bacterial infections. We have previously uncovered missense  
388 mutations in the LPS *wzt* and *rmlA* loci of CF6, and a missense mutation affecting *BPSL1119* in  
389 CF11 (Viberg et al. 2017). DE analysis identified additional evidence for reduced or abolished  
390 LPS production in a third CF case, CF9, due to the significant downregulation of *wbiD*, *wbiE*,  
391 and *BPSS1683-BPSS1685*. The convergent evolution of the chronic strains to attenuate CPS-I  
392 and LPS loci demonstrates the dispensable, and probably highly unfavourable, nature of these

393 surface antigens for long-term survival of this pathogen in the CF airways. Prior work has  
394 suggested that CPS-I and LPS may be disadvantageous for *B. pseudomallei* persistence due to  
395 their virulence potential and immunogenicity (Price et al. 2013). By examining both genomic and  
396 transcriptomic modifications over time, it is now clear that these capsule clusters, in their wild-  
397 type form, pose a major issue for successful long-term *B. pseudomallei* persistence in the CF  
398 lung, with the bacterium either mutating or downregulating key genes in CPS-I and LPS  
399 pathways. We also observed genetic mutation or transcriptional downregulation of other  
400 virulence genes in latter CF strains including TFP7 loci, Burkholderia lethal factor 1, T3SS-3 loci  
401 and flagellum loci, suggesting that these loci are similarly detrimental to long-term *B.*  
402 *pseudomallei* survival in the human host. Importantly, the RNA-seq data identified additional  
403 cases of surface antigen and virulence factor abrogation that were not observable with only  
404 genomic data. This finding underscores the importance of using both genomic and  
405 transcriptomic approaches to identify the functional consequences of within-host evolution of  
406 chronic bacterial infections.

407 TMP/SMX is used during the eradication phase of melioidosis treatment and is recommended  
408 for post-exposure prophylaxis (Peacock et al. 2008). We have previously shown that the latter  
409 isolate from CF6, and both isolates from CF11, had developed high-level ( $\geq 32$   $\mu\text{g/mL}$ )  
410 TMP/SMX resistance over the course of treatment. These elevated MICs were proposed to be  
411 due to mutations within BpeEF-OprC efflux pump regulators (BpeT T314fs in CF6, and BpeS  
412 V40I and R247L in CF11) alongside mutations affecting the R20 residue of Ptr1/FoIM (R20fs in  
413 CF6; R20-A22 duplication in CF11) (Viberg et al. 2017). Here, we have demonstrated that the  
414 efflux pump regulatory mutations cause a dramatic upregulation of *bpeEF-oprC* in these strains  
415 of between 5.2- and 6.9-fold (38 to 121x), mirroring expression levels in *bpeS* and *bpeT* lab-  
416 generated mutants with high-level TMP/SMX resistance (Podnecky et al. 2017). Our results  
417 confirm those of Podnecky and colleagues (Podnecky et al. 2017) showing that upregulation of

418 *bpeEF-oprC* via BpeS or BpeT dysregulation, together with Ptr1/FolM alteration, leads to a  
419 significant increase in TMP/SMX MICs that would render this antibiotic ineffective *in vivo*. RNA-  
420 seq is thus a useful tool for confirming the functional consequences of regulatory mutations that  
421 control RND efflux pump expression.

422 In addition to TMP/SMX resistance, the initial isolate from CF11 is resistant to CAZ (12 µg/mL)  
423 and the latter isolate from CF6 is highly resistant to CAZ (≥256 µg/mL). Our prior genomic study  
424 showed that CAZ resistance in the initial CF11 strain was due to a 10x duplication of a 36.7kb  
425 region encompassing the β-lactamase gene, *penA*, the first time that gene duplication has been  
426 shown to confer CAZ resistance in *B. pseudomallei* (Viberg et al. 2017). In contrast, a 2x  
427 duplication of this region in the latter strain did not raise the CAZ MIC above wild-type levels  
428 (Viberg et al. 2017). Similarly, the latter strain from CF6 exhibited a 30x duplication of a 7.5kb  
429 region encompassing *penA*; however, all 30 copies encoded a C69Y missense mutation, which  
430 by itself causes high-level (≥256 µg/mL) CAZ MICs (Sam et al. 2009). As anticipated, RNA-seq  
431 provided confirmation of the effects of gene duplications affecting *penA*, with the 30x duplication  
432 event in the latter CF6 strain resulting in a 4.5-fold (22x) corresponding increase in DE of this  
433 gene. Similarly, a 2.1-fold (4x) downregulation of *penA* in the latter strain from CF11 was linked  
434 to a 5x greater copy number of this gene in the early strain (Viberg et al. 2017). Thus, RNA-seq  
435 provided excellent correlation with gene copy number variation determined from whole-genome  
436 sequence coverage data. Taken together, the combined genetic and transcriptional changes  
437 affecting antibiotic resistance genes in the CF airways-adapted *B. pseudomallei* strains  
438 illustrates both the intractability of eradicating chronic bacterial infections and the unintended  
439 consequences of prolonged antibiotic use in CF treatment.

440 Although determining within-host transcriptional differences in longitudinal isolates yields  
441 valuable insights into the infection dynamics within individual patients, identifying convergent

442 transcriptional changes provides a potential means to predict pathogen behavior and evolution  
443 across multiple CF cases in a relatively straightforward manner. Such predictability could  
444 conceivably be exploited to improve the diagnosis or treatment of intractable CF infections, or  
445 ideally, to prevent them from progressing in the first place. Therefore, a major objective of this  
446 study was to identify evidence of convergence in *B. pseudomallei* gene expression during its  
447 transition to a chronic infection. Despite the small number of CF melioidosis patients available  
448 for this study, a signal of convergent pathoadaptation was identified between the initial and latter  
449 isolates, with 19 significantly DE loci identified, 17 of which were upregulated (Table S2). This  
450 convergence is noteworthy given the large size of the *B. pseudomallei* genome and the many  
451 redundant pathways that could lead to similar adaptive phenotypes, a phenomenon that is well-  
452 recognized in *P. aeruginosa* (Marvig et al. 2015). One advantage of identifying convergence  
453 using transcriptomics rather than genomic data is that it can reveal the transcriptional  
454 consequence of multiple genetic mutations; for example, we have observed that multiple  
455 missense mutations in the RND efflux pump regulator AmrR lead to the same transcriptional  
456 outcome of *amrAB-oprA* upregulation (Sarovich et al. 2017). As such, RNA-seq data can  
457 simplify the identification of convergently expressed loci that are under the influence of several  
458 genetic variants.

459 The development of antibiotic resistance is a recurring theme in *P. aeruginosa* isolated from the  
460 CF airways (Winstanley et al. 2016), and we have recently shown that the same adaptive  
461 phenomenon can be observed in the genome of *B. pseudomallei* in response to prolonged,  
462 high-dose antibiotic therapy (Viberg et al. 2017). It was therefore not surprising to identify the  
463 convergent upregulation of *bpeEF-oprC* (4.8- to 6.1-fold; 28 to 69x), which was significantly  
464 upregulated in two of the four patients with DE (CF6 and CF11), and which led to TMP/SMX  
465 resistance as discussed above. The second convergently upregulated locus was the *cydAB*  
466 operon (*BPSL0501* and *BPSL0502*), which encodes for cytochrome *bd* quinol oxidase (5.5- to

467 5.9-fold; 45 to 60x); this locus was significantly DE in CF6 and CF9. *CydAB* is an aerobic  
468 terminal oxidase that oxidizes ubiquinol-8 and reduces oxygen to water under oxygen-limiting  
469 conditions. This enzyme is better able to scavenge oxygen under microaerobic conditions  
470 compared with cytochrome *o* oxidase, which otherwise predominates as the terminal respiratory  
471 enzyme in electron transport-associated energy production (Cotter et al. 1997). Voggu and  
472 colleagues demonstrated that the *cydAB* loci encoded by non-pathogenic *Staphylococcus*  
473 species were better able to resist *P. aeruginosa* antagonism in the CF lung compared with the  
474 *cydAB* loci encoded by *S. aureus*. This resistance was imparted by an insensitivity of the non-  
475 pathogenic staphylococci cytochrome *bd* quinol oxidases to the presence of the small  
476 respiratory inhibitors hydrogen cyanide and pyocyanin, which are commonly secreted by *P.*  
477 *aeruginosa* in the CF lung. In contrast, Voggu *et al.* showed that *S. aureus* was exquisitely  
478 sensitive to the co-presence of *P. aeruginosa* due to their less resistant *cydB* locus, which is  
479 inhibited by these small respiratory inhibitors (Voggu et al. 2006). Thus, it is feasible that the  
480 convergent upregulation of *cydAB* loci represents a defense mechanism employed by *B.*  
481 *pseudomallei* to counteract the toxic effects of small respiratory inhibitors produced by *P.*  
482 *aeruginosa* in the CF lung. In support of this hypothesis, *P. aeruginosa* was co-isolated in all five  
483 CF cases examined in this study (Viberg et al. 2017). Alternatively, *cydAB* upregulation may  
484 simply represent a physiological response to the oxygen-limited environment of the CF airways,  
485 as its expression is known to be induced in *B. pseudomallei* in hypoxic conditions (Hamad et al.  
486 2011). Under such conditions, many pathogens including *B. pseudomallei* become less  
487 susceptible to conventional antibiotics, which are typically more effective under aerobic  
488 conditions, but more susceptible to antibiotics that target anaerobic infections, such as the  
489 nitroimidazole class of antibiotics (Hamad et al. 2011). This phenomenon may explain the  
490 difficulty of chronic *B. pseudomallei* eradication using conventional antibiotics like CAZ and  
491 TMP/SMX, and raises the exciting but not yet tested possibility that nitroimidazoles may be a

492 highly effective therapeutic option for chronic, hypoxia-adapted *B. pseudomallei* infections such  
493 as those adapted to the CF airways.

494 A third convergently upregulated locus, the quorum sensing operon *hhqABCDEFG* (3.4- to 4.1-  
495 fold; 11x to 17x), is homologous to the *B. cepacia* complex *hmqABCDEFG* operon (Chapalain et  
496 al. 2017). This operon synthesizes a class of compounds known as 4-hydroxy-3-methyl-2-  
497 alkylquinolines (HMAQs), the methylated counterparts of 2-alkyl-4(1H)-quinolones (AHQs; also  
498 known as HAQs). AHQs were first recognized in *P. aeruginosa* and are produced by the  
499 signaling system *pqsABCDE* (Diggle et al. 2006). This cluster produces over 50 different AHQs,  
500 and these compounds exhibit diverse biological activities that enable cell-to-cell communication  
501 within and between bacterial species and the regulation of various functions including  
502 secondary metabolism, virulence, antibacterial activity and biofilm formation (Diggle et al. 2006).  
503 In contrast, little is currently known about the role of HMAQs and AHQs in *Burkholderia* spp.  
504 (Chapalain et al. 2017). The AHQ precursor molecule 2-heptyl-4(1H)-quinolone (HHQ) that is  
505 produced by *P. aeruginosa* actively suppresses the host innate immune response (Kim et al.  
506 2010), a role that could be shared by *B. pseudomallei* HHQ. A second possibility is that these  
507 compounds impart a competitive advantage in the CF lung environment as HMAQs produced by  
508 *B. cepacia* exhibit antifungal activity (Kilani-Feki et al. 2011), so it is feasible that the  
509 *hmqABCDEFG* operon of *B. pseudomallei* produces similarly potent compounds that can inhibit  
510 fungal species from establishing residence in the CF lung. The convergent upregulation of  
511 *hhqA-G* in the *B. pseudomallei* CF isolates points to a putative role for AHQ-based compounds  
512 in *B. pseudomallei* signaling, immune evasion or competition in the CF lung. More work is  
513 needed to elucidate the myriad functions of AHQ compounds in *B. pseudomallei*, and  
514 particularly their role in promoting bacterial persistence in the CF airways.



515 Of the two convergently downregulated loci, only one, *BPSS0351*, has an assigned function,  
516 although little is known about the role of this gene and its product in *B. pseudomallei*. This gene  
517 encodes CueR (3.4-fold; 11x), a MerR family copper response regulator that is highly sensitive  
518 to the presence of copper (Cu) and which regulates the transcription of genes that protect  
519 against toxic metal ion concentrations (Brown et al. 2003; Singh et al. 2004). Cu has a long  
520 history as an effective antimicrobial agent due its ability to generate reactive oxygen species,  
521 with Cu accumulation in the mammalian host purported to act as an innate immune defense  
522 mechanism to restrict pathogen growth (Samanovic et al. 2012). Thus, downregulation of *cueR*  
523 in the latter CF isolates may represent a mechanism for mitigating Cu toxicity in the host,  
524 similarly to *E. coli* (Singh et al. 2004). However, there are contradictory reports as to whether Cu  
525 levels are elevated in CF sputa (Gray et al. 2010; Smith et al. 2014), and the artificial sputum  
526 growth medium does not appear to contain elevated Cu levels (Fung et al. 2010). CueR  
527 regulates the Cu/silver ATPase CopA and the multicopper oxidase CueO enzymes in *E. coli*,  
528 which correspond to *BPSS0224* and *BPSL0897* in *B. pseudomallei* K96243, respectively;  
529 however, neither of these genes were DE in any of the patient pairs. The absence of  
530 concomitant increased expression of *cueO*, which converts periplasmic Cu<sup>+</sup> to less toxic Cu<sup>2+</sup>  
531 *in vivo* (Singh et al. 2013), suggests that other enigmatic pressures are responsible for  
532 decreasing *cueR* expression in latter CF isolates. The biological role of these other factors  
533 requires further exploration.

534 We recognize that there are limitations to our study. Growth conditions are known to be an  
535 important consideration for mRNA-based investigations due to the alteration of the  
536 transcriptome when isolates are grown under different environments or media components  
537 (Yoder-Himes et al. 2009). Although our *in vitro* conditions do not completely mimic the  
538 conditions seen in the CF lung, the artificial sputum medium is designed to reflect the nutrient  
539 conditions of this environment (Fung et al. 2010), and our shaking parameters provided a robust

540 way of measuring cellular growth over time while avoiding non-uniform cellular growth, which  
541 ensured harvest of *B. pseudomallei* cultures at the same growth phase (Figure S1). Additionally,  
542 the use of isogenic strain pairs with genomic data (Viberg et al. 2017) enabled us to  
543 comprehensively assess the effects of transcriptional adaptation to the CF lung compared with  
544 their underlying genetic variants. Our conditions provided transcriptomic data that was  
545 consistent with expected expression differences based on genome-wide alterations. Other  
546 studies have used artificial sputum media and additional mechanical methods to mimic the CF  
547 lung conditions. A rotating wall vessel has been developed to simulate the low fluid shear  
548 conditions encountered in CF mucus due to pathological effects of *CFTR* dysfunction on  
549 mucociliary clearance (Crabbé et al. 2008), with CF-derived *P. aeruginosa* isolates  
550 demonstrating transcriptional differences depending on shear conditions (Dingemans et al.  
551 2016). The culturing methods for bacterial RNA-seq are a critical consideration in experimental  
552 design as they can affect transcriptomic profiles, and the impact of conditions should be  
553 considered when comparing transcriptional differences between studies. Another shortcoming is  
554 that we only examined five patient pairs due to the relative paucity of melioidosis CF cases  
555 worldwide, and only two isolates from each patient due to limited bacterial colony selection and  
556 storage at the time of sputum collection and processing. Deeper sampling efforts across a  
557 greater number of melioidosis CF patients would be needed to provide greater confidence in our  
558 convergent adaptation findings and would allow a more advanced and detailed understanding of  
559 *B. pseudomallei* population dynamics and diversity to be attained. Nevertheless, the findings  
560 from our study provide important new insights into *B. pseudomallei* evolution in the CF airways,  
561 with many, although not complete, parallels with the common CF pathogens, *P. aeruginosa* and  
562 *B. cepacia* complex species.

563

564 **METHODS**

565 **Ethics statement.** Ethics approval for this study was obtained as previously described (Currie  
566 et al. 2010; Geake et al. 2015).

567 **CF isolates.** The *B. pseudomallei* strains used in this study are summarized in Table 1. The  
568 history and genomic analysis of these cases and strains are detailed elsewhere (Viberg et al.  
569 2017).

570 **Artificial sputum medium.** This medium was made as previously described (Sriramulu et al.  
571 2005; Fung et al. 2010), with modifications detailed here. Antibiotics were not used to maintain  
572 media sterility due to concerns that their addition would alter expression profiles. Due to  
573 impracticality in its filtration (Dingemans et al. 2016), 1 g porcine stomach mucin (Sigma-Aldrich,  
574 Castle Hill, NSW, Australia) dissolved in 40 mL ultrapure water was autoclaved prior to use. All  
575 other solutions were sterilized using a 0.22 µm vacuum filter, apart from the UV-irradiated egg  
576 yolk emulsion (Oxoid, Thebarton, SA, Australia), which was treated aseptically. A stock solution  
577 of diethylenetriaminepentaacetic acid (Sigma-Aldrich) was made by dissolving 59.5 mg into 5  
578 mL of very basic water (pH=14). CaCl<sub>2</sub> was added at a final concentration of 0.22 g/L (J. Manos,  
579 pers. comm.). Final concentrations of the components were: 10 g/L mucin, 1.39 g/L salmon  
580 sperm DNA (Sigma-Aldrich), 5 g/L NaCl, 2.2 g/L KCl, 0.22 g/L CaCl<sub>2</sub>, 5 g/L casein acid  
581 hydrosylate (Sigma-Aldrich), 10 g/L bovine serum albumin (Roche Diagnostics, Castle Hill,  
582 NSW, Australia), 0.005% diethylenetriaminepentaacetic acid, and 0.5% of egg yolk emulsion.  
583 Each batch was tested for sterility prior to use by plating 100 µL onto Luria Bertani (LB) agar  
584 (Oxoid) and incubating aerobically for 24 h. pH was tested using an aliquot of the medium to  
585 ensure it was within the desired range (pH ~6.5-7). The medium was stored at 4 °C for no  
586 longer than four weeks prior to use.

587 **Viability counts.** Two sets of viability counts were performed for this study. The first was  
588 conducted to determine the number of colony-forming units (cfu) at OD<sub>590</sub>, which enabled us to  
589 standardize the starting number of cells inoculated into the artificial sputum medium. The  
590 second was conducted to verify the final concentration of cells across all CF isolates, which  
591 enabled us to determine the number of cells for nucleic acid extraction to ensure that  
592 approximately equal cell amounts were processed for each pair. The CF isolates were  
593 subcultured from glycerol stocks onto LB agar at 37 °C for 24 h. Cells were suspended into  
594 phosphate-buffered saline (PBS) followed by spectrophotometric measurement at OD<sub>590</sub>=1.0 in  
595 a WPA CO 8000 cell density meter (Biochrom Ltd, Blackburn, VIC, Australia). Tenfold dilutions  
596 and plating of cultures onto LB agar was carried out, followed by enumeration at 24 h. Viable  
597 counts demonstrated that all CF isolates exhibited similar cell density when normalized to an  
598 OD<sub>590</sub>=1.0 (range 1.3x10<sup>8</sup> to 4.9x10<sup>8</sup>). Based on these counts, the starting amount of culture for  
599 the CF isolates was standardized to 10<sup>5</sup> cfu for all subsequent experiments.

600 **Growth curves in the artificial sputum medium.** To minimize laboratory passage, each  
601 culture was again subcultured from the original glycerol stocks onto LB agar at 37 °C for 24 h,  
602 followed by a replication of OD<sub>590</sub> measurements as determined previously. Based on the  
603 viability count data, samples were then diluted to 10<sup>6</sup> cfu/mL in PBS. One hundred µL of this  
604 suspension (~10<sup>5</sup> cfu) was used to inoculate 1.9 mL of the sputum medium, which was  
605 aliquoted into 14 mL Nunc round-bottom culture tubes (Thermo Fisher Scientific, Scoresby, VIC,  
606 Australia). Due to biosafety concerns, cells were grown in closed-capped tubes, and were  
607 incubated at 37 °C by shaking in an orbital incubator shaker (model BL8500; Bioline, Eveleigh,  
608 NSW, Australia) at 50, 200 or 230 rpm for 44 h. Growth curves were obtained by measuring  
609 OD<sub>590</sub> at regular intervals over this period using un-inoculated sputum medium as the control  
610 blank. Shaking at 50 rpm was initially performed to mimic the low-oxygen conditions of the CF  
611 lung; however, this speed caused heavy sedimentation of cells and media components, and

612 biofilm formation at the aerobic interface, both of which led to a decrease in OD values over  
613 time and unpredictable, non-uniform growth. Similarly, shaking at 230 rpm was too vigorous for  
614 the cells, as observed by inconsistent, non-reproducible viable counts. When shaking at 200  
615 rpm, highly reproducible OD values that correlated with viability counts were obtained (Figure  
616 S1), and the medium did not readily sediment. We therefore used this speed for subsequent  
617 experiments, including for RNA harvest. Viability counts were performed at the time of harvest  
618 to ensure uniformity of cell concentrations across all isolates.

619 **RNA extraction from isolates grown in the artificial sputum medium.** The CF strains were  
620 grown in duplicate at 200 rpm as detailed above. Based on the growth curve analysis, nucleic  
621 acids for all cultures were extracted at late log phase (17 h). At the point of harvest, the OD<sub>590</sub> of  
622 each replicate was measured to ensure that consistent cell density had been obtained prior to  
623 combining replicates; final viability counts were also performed. Due to the highly labile nature  
624 of bacterial mRNA, two 100 µL aliquots for each strain were immediately placed into 200 µL of  
625 RNeasy Protect (Qiagen, Doncaster, VIC, Australia) and incubated for 5 min to preserve their  
626 transcription profiles. Cells were pelleted by centrifugation at 5000 x g for 10 min, and the  
627 supernatant discarded. Total RNA was extracted using the RNeasy Protect Bacteria Mini Kit  
628 (Qiagen). *B. pseudomallei* cells were lysed following the protocol for genomic DNA extraction  
629 (Currie et al. 2007), with an extended incubation time in Proteinase K to 1.5 h. Lysates were  
630 loaded onto the RNeasy mini columns and extractions were carried out according to the  
631 manufacturer's instructions, including the recommended on-column DNase I digestion. In our  
632 hands, we found this DNase I treatment to be insufficient for removing all contaminating DNA.  
633 Extractions for RNA-seq were therefore further treated with TURBO DNA-free kit (Ambion,  
634 Scoresby, VIC, Australia). For each sample, 35 µL of extracted RNA was incubated with 6 U  
635 TURBO DNase at 37 °C for 32 min. The remaining RNA was not treated with this second round  
636 of DNase; instead, this sample was used as template for PCR contamination screening, as

637 described below. All samples were transferred to clean RNase/DNase-free tubes for  
638 downstream processing.

639 **RNA quality control.** To verify the removal of DNA from the total RNA extractions, two  
640 contamination screens were performed. The first was used to determine the removal of salmon  
641 sperm DNA, and the second was to determine the removal of *B. pseudomallei* DNA. Both the  
642 pre- and post-treated RNA samples were used to test for contamination, in duplicate, with the  
643 former acting as the positive control. The RNA samples were diluted 1/10 into molecular-grade  
644 H<sub>2</sub>O (Fisher Biotech) prior to PCR. Identification of residual salmon sperm DNA was  
645 investigated by targeting the mitochondrial 12S rDNA region of vertebrates (Humair et al. 2007).  
646 Primers 12S-6F (5'- CAAACTGGGATTAGATACC) and B-12S-9R (5'-  
647 AGAACAGGCTCCTCTAG) were used at a final concentration of 1 µM in a mix containing 1X  
648 PCR buffer (Qiagen), 1 U HotStarTaq, 0.2 mM dNTPs, 1 µL template and molecular-grade H<sub>2</sub>O  
649 in a 15 µL total reaction volume. Thermocycling conditions comprised 94 °C for 5 min, followed  
650 by 35 cycles of 94 °C for 30 sec, 52 °C for 30 sec, and 72 °C for 30 sec, and a final extension at  
651 72 °C for 2 min. Amplicons were detected by agarose gel electrophoresis.

652 Real-time PCR was used to detect *B. pseudomallei* DNA contamination. The *mmsA*  
653 (methylmalonate-semialdehyde dehydrogenase) housekeeping gene was targeted using the  
654 primers Bp\_266152\_3012-F1-flap (5'-AATAAATCATAAACGTGAGGCCGGAGATGT) and  
655 Bp\_266152\_3012-R1-flap (5'-AATAAATCATAAGACCGACATCACGCACAGC) in combination  
656 with a *B. pseudomallei*-specific TaqMan MGB probe, 266152-T\_Bp (5'-VIC-  
657 CGGTCTACACGCATGA) (Price et al. 2012), as previously described, with the following  
658 modifications: 0.2 µM probe and 0.4 µM each primer was used, reactions were carried out in a 5  
659 µL total reaction volume, and cycling was performed to 50 cycles.

660 **RNA storage, shipment and RNA-seq.** For each sample, 20  $\mu$ L of total RNA was added to an  
661 RNAsable tube (Biomatrica, San Diego, CA, USA), gently mixed with the preservation agent  
662 and left to air-dry in a biosafety cabinet for 48 h. Samples were shipped at ambient temperature  
663 to Macrogen Inc. (Geumcheon-gu, Seoul, Rep. of Korea) for RNA-seq. Ribosomal RNA was  
664 removed by treatment with the Ribo-Zero rRNA Removal Kit for Bacteria (Epicentre, Madison,  
665 WI, USA), followed by 100 bp paired-end, stranded library construction using the TruSeq rapid  
666 SBS Kit (Illumina Inc., San Diego, CA, USA). Libraries were sequenced on either the  
667 HiSeq2000 or HiSeq2500 platform (Illumina Inc.). All samples were extracted from two separate  
668 experiments to account for biological variation, except for MSHR8442, which was extracted  
669 thrice. Between 36 and 80 million reads were generated for each sequence, corresponding to  
670 between 3.6 and 8.1 billion base pairs each.

671 **RNA-seq analysis.** Illumina read filtering was first performed with Trimmomatic v0.33 using the  
672 following parameters: TruSeq2-PE adapter removal, leading=3, trailing=3, sliding window=4:15,  
673 and minimum length=36. Reads were mapped to the prototypic *B. pseudomallei* K96243  
674 reference genome (RefSeq IDs NC\_006350 and NC\_006351 for chromosomes 1 and 2,  
675 respectively (Holden et al. 2004)) using Bowtie 2 v2.2.1 (Langmead and Salzberg 2012).  
676 Transcript quantification was performed with HTSeq (v0.6.1p1) (Anders et al. 2015) using the  
677 intersection non-empty mode and --stranded=reverse parameters. DE analysis was carried out  
678 using the glmFit function of edgeR v3.18.1 (Robinson et al. 2010), implemented in the online  
679 Degust 3.1.0 tool (<http://www.vicbioinformatics.com/dequst/index.html>). DE loci were visualized  
680 using the volcano plot function within Degust. Several different groups were compared to  
681 determine DE. The first analyses compared initial and latter isolates within CF patients (Table 1)  
682 without summing technical replicates (i.e. the RNA-seq data from each independent experiment  
683 of a single strain) to identify DE within each patient. To determine convergent DE loci, we  
684 summed the reads for each technical replicate prior to analysis and then compared all initial CF

685 isolates vs. all latter CF isolates, with the latter CF10 isolate excluded due to a lack of DE in this  
686 strain and the initial CF11 isolate excluded due to >3 years of infection prior to its isolation  
687 (Viberg et al. 2017). For all analyses, DE was defined as a  $\log_2$  fold change of  $\geq 1.5$  and a false  
688 discovery rate of  $\leq 0.01$ . To improve visualization of DE loci in the volcano plot of the initial and  
689 latter comparison (Figure 1), highly expressed DE loci in only a single strain were omitted.

## 690 **DATA ACCESS**

691 The RNA-seq data generated in this study are available on the Sequence Read Archive  
692 database under BioProject number PRJNA398168 and submission numbers SRR6031143 to  
693 SRR6031152.



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705 **AUTHOR CONTRIBUTIONS**

706 EPP, BJC and DSS conceived of and obtained funding for the study. EPP, LTV and DSS  
707 designed the laboratory experiments LTV carried out experiments. EPP, LTV and DSS  
708 performed bioinformatic analyses. TJK, SCB and BJC supplied the *B. pseudomallei* isolates and  
709 clinical information. EPP and DSS wrote the manuscript. All authors read and approved the final  
710 manuscript.

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712 **DISCLOSURE DECLARATION**

713 The authors declare no conflicts of interest.

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