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1 **Influence of the molybdenum cofactor biosynthesis on anaerobic**

2 **respiration, biofilm formation and motility in *Burkholderia***

3 ***thailandensis***

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20 **Abstract**

21 *Burkholderia thailandensis* is closely related to *B. pseudomallei,* a bacterial pathogen
22 and the causative agent of melioidosis. *B. pseudomallei* can survive and persist
23 within a hypoxic environment for up to one year and has been shown to grow
24 anaerobically in the presence of nitrate. Currently little is known about the role of
25 anaerobic respiration in pathogenesis of melioidosis. Using *B. thailandensis* as a
26 model, a library of 1,344 transposon mutants was created to identify genes required
27 for anaerobic nitrate respiration. One transposon mutant (CA01) was identified with
28 an insertion in BTH_I1704 (*moeA*), a gene required for the molybdopterin
29 biosynthetic pathway. This pathway is involved in the synthesis a molybdopterin
30 cofactor required for a variety of molybdoenzymes, including nitrate reductase.
31 Disruption of molybdopterin biosynthesis prevented growth under anaerobic
32 conditions, when using nitrate as the sole terminal electron acceptor. Defects in
33 anaerobic respiration, nitrate reduction, motility and biofilm formation were observed
34 for CA01. Mutant complementation with pDA-17::BTH_I1704 was able to restore
35 anaerobic growth on nitrate, nitrate reductase activity and biofilm formation, but did
36 not restore motility. This study highlights the potential importance of molybdoenzyme
37 dependent anaerobic respiration in the survival and virulence of *B. thailandensis.*

38 Key words – *B. thailandensis;* molybdopterin biosynthesis; anaerobic respiration;
39 nitrate; motility

40 **1. Introduction**

41 The ability of bacteria to utilise a wide range of electron acceptors, provides a
42 survival advantage, allowing colonisation of a wide range of niches. Under oxygen
43 limiting conditions nitrate can be utilised to generate a proton motive force to power
44 growth via the denitrification pathway. The denitrification pathway utilises series of
45 reductase enzymes; nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide
46 reductase (NOR) and nitrous oxide reductase (NOS), to sequentially reduce nitrate
47 to dinitrogen gas (Richardson, 2000, Tavares *et al.*, 2006).

48 NAR requires an active molybdenum cofactor (Moco) in the Mo-*bis* guanine
49 dinucleotide (Mo-*bis* MGD) form, for the reduction of nitrate to nitrite (Jormakka *et*
50 *al.*, 2004, Gonzalez *et al.*, 2006). Moco is found in a number of different molybdo-
51 enzymes, a number of which function under oxygen limiting conditions (Magalon,
52 2011). The formation of an active molybdenum cofactor is critical for activity of all
53 molybdoenzymes. The molybdopterin (MPT) biosynthetic pathway, required for the
54 generation of the molybdenum cofactor (Moco), is a four step enzymatic pathway
55 requiring a variety of molybdate dependent biosynthetic and transport proteins. The
56 first step involves the conversion of guanosine triphosphate (GTP) to the pterin
57 intermediate cyclic pyranopterin monophosphate (cPMP) using MoaA and MoaC.
58 After this cPMP is converted into MPT dithiolate, using a heterotetrameric complex
59 composed of two MoaD and MoaE subunits which together form MPT synthase
60 (Schwarz *et al.*, 2009). The third step requires MoeA and MogA to ligate
61 molybdenum to MPT, generating Mo-MPT (Moco). Finally, for the DMSO reductase
62 family molybdoenzymes, Mo-MPT is converted into molybdenum guanine
63 dinucleotide form (Mo-*bis*-MGD) using MobA (Schwarz *et al.*, 2009, Vergnes *et al.*,
64 2004). Different modifications of Moco have been reported and various derivatives

65 are found in different classes of molybdoenzymes such as the xanthine oxidase and
66 sulfite oxidase family proteins (Magalon, 2011).

67 Anaerobic respiration has been implicated in pathogenesis of a number of
68 bacterial infections. Disruption of NAR, and the lack of synthesis of the Moco, affects
69 anaerobic respiration, and causes defects intracellular growth, virulence,
70 persistence, motility, biofilm formation, invasion and proliferation within Hep-2
71 epithelial cells in a number of different bacterial species (Filiatrault *et al.*, 2006,
72 Williams *et al.*, 2011, Contreras *et al.*, 1997, MacGurn and Cox, 2007, Fritz *et al.*,
73 2002, Van Alst *et al.*, 2007, Weber *et al.*, 2000, Kohler *et al.*, 2002, Sohaskey, 2008).

74 *Burkholderia thailandensis,* a Gram-negative soil dwelling saprophyte (Brett*,*
75 *et al.*, 1998) is closely related to the human pathogen *Burkholderia pseudomallei,* the
76 causative agent of melioidosis (Wiersinga *et al.*, 2012). *B. thailandensis*, although
77 displaying a very high degree of genetic similarity to *B. pseudomallei*, is avirulent
78 rarely causing disease in humans (Glass *et al.*, 2006). Due to the high degree of
79 genetic similarity, ability to survive and replicate intracellularily and lower risk
80 associated with handling, *B. thailandensis* is often used as a surrogate for *B.*
81 *pseudomallei* (Chandler *et al.*, 2009, French *et al.*, 2011, Haraga *et al.*, 2008, Horton
82 *et al.*, 2012, West *et al.*, 2008).

83 Currently little is known about the role anaerobic respiration plays in
84 *Burkholderia spp.* virulence*.* Using transposon mutagenesis this study aimed to
85 identify genes required for anaerobic nitrate respiration in *B. thailandensis,* and
86 determine their roles in biofilm formation, virulence and motility.

87

88 **2. Materials and Methods**

89 *2.1 Bacterial strains, growth conditions and medium*

90 *B. thailandensis* (E264) and *Escherichia coli* were routinely grown in Luria-
91 Bertani broth (L-broth) at 37 $^{o}$C, solidified when required with 1.5 % (w/v)
92 bacteriological agar. When appropriate, antibiotics were added to the medium at the
93 following concentrations; 100 µg/ml gentamicin, 50 µg/ml kanamycin, 100 µg/ml
94 ampicillin or 50 µg/ml tetracycline. Anaerobic growth studies were conducted
95 statically within an anaerobic cabinet (10 % $CO_2$, 80 % $N_2$ and 10 % $H_2$) using
96 primarily in M9 minimal medium supplemented with 20 mM sodium succinate, a non-
97 fermentable carbon source, or L-broth. M9 medium was composed of 2 mM $MgSO_4$,
98 0.1 mM $CaCl_2$, 20 % 5x M9 salts (5 x M9 salts: 85.5 g/l $Na_2HPO_4$, 15 g/l $KH_2PO_4$, 2.5
99 g/l NaCl, 5 g/l $NH_4Cl$). When appropriate the anaerobic growth medium was
100 supplemented 20 mM sodium nitrate ($NaNO_3^-$).

101 *2.2 Transposon mutagenesis*

102 A transposon mutant library was created by conjugation of *B. thailandensis*
103 with *E. coli* strain 19851 *pir*$^+$ containing pUTminiTn5Km2 (Cuccui *et al.*, 2007, de
104 Lorenzo *et al.*, 1990). Briefly *E. coli* 19851 (pUTminiTn5Km2) was grown to
105 exponential phase and mixed at a 1:3 ratio with *B. thailandensis* (E264). After 6
106 hours incubation the mating mix was resuspended, plated out onto antibiotic
107 selective media (250 µg/ml kanamycin and 100 µg/ml gentamicin) and incubated for
108 48 hours. Transconjugates were picked into 96 well plates containing L-broth
109 supplemented with the appropriate antibiotics. The resultant transposon mutant
110 libraries of 1,344 transposon mutants were then screened on M9 minimal medium

111 plates containing 10 mM sodium succinate and 5 mM $NaNO_3^-$ under aerobic and
112 anaerobic conditions, to select for those unable to respire anaerobically on nitrate.

113 *2.3 Polymerase Chain Reaction*

114 Nested PCR using arbitrary primers (Arb3 5' -
115 GGCCACGCGTCGACTAGTACNNNNNNNNNNTGACG-3' or Arb4 5' -
116 GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC-3') and primers specific for
117 the transposon specific primer P7M1 (5'-GTCATTAAACGCGTATTCAGGCTGAC-3'
118 (Tn5Km2) were used to amplify the DNA flanking the transposon (Cuccui *et al.*,
119 2007). The resultant PCR product was used as the template in a second PCR using
120 Arb2 (5'-GGCCACGCGTCGACTAGTAC-3') and P7U (5'-
121 CTGCAGGCATGCAAGCTTCG-3'). The resultant PCR product was cloned into the
122 pJET1.2/blunt cloning vector (Thermo Scientific) and sequenced using primer P7M
123 to identify site of transposon insertion.

124 Wild-type (300 bp internal region of BTH_I1704) and mutant (300 bp region of
125 the kanamycin resistance cassette) DNA probes, used for the southern blot, were
126 generated using PCR using the Phusion High-fidelity PCR master mix (Thermo
127 Scientific).

128 *2.4 Reverse transcriptase PCR*

129 Reverse transcriptase PCR (RT-PCR) was performed, using Invivogen
130 SuperScript One-step RT-PCR mix with Platinum *Taq* polymerase, on RNA samples
131 extracted during anaerobic growth. Primers binding to internal regions of 16S rRNA,
132 BTH_I1704 and BTH_I2200 were used in separate reactions. Two biological
133 replicates were used.

134 *2.5 Southern blot*

135 In order to confirm the site transposon insertion in the mutant a southern blot
136 was performed on *XhoI* digested *B. thailandensis* E264 and CA01 (BTH_I1704-
137 Tn5Km2) genomic DNA using wild-type and mutant DNA probes (100 ng/ml),
138 following the protocol described in Amersham ECL Direct Nucleic acid labelling and
139 Detection system kit (GE Healthcare).

140 *2.6 Construction of a mutant complement using pDA-17 vector with a dhfr promoter*

141 A mutant complement was created, using the constitutive expression vector
142 pDA-17 (7,360 bp) encoding a tetracycline resistance cassette. BTH_I1704 (1,299
143 bp) was cloned into pDA-17 in front of the *dhfr* promoter region via *Nde*I and *Xba*I
144 restriction sites and transformed into DH5α competent cells. Successful DH5α pDA-
145 17::BTH_I1704 transformants were screened using PCR and sequenced prior to
146 conjugating into CA01 using a *E. coli* helper strain (pKR2013). Any potential
147 complements (CA01_pDA-17::BTH_I1704) were confirmed using PCR and
148 maintained on medium containing tetracycline 50 µg/ml. All *in vitro* experiments with
149 CA01_pDA-17::BTH_I1704 were performed using media supplemented with
150 tetracycline 50 µg/ml.

151 *2.7 Nitrate reductase assays*

152 The concentration of nitrite, and relative NAR activity, was measured using
153 the Griess reagent system (Promega) (Green *et al.*, 1982), using samples taken
154 throughout the growth cycle. Three biological replicates were used each with three
155 technical replicates.

156 To further confirm the lack of NAR activity in CA01, wild-type *B. thailandensis*
157 and CA01 were grown aerobically overnight in L-broth to obtain biomass. The
158 cultures were washed and resuspended in M9 minimal media supplemented with
159 $NO_3^-$ and acclimatised to an anaerobic environment for 4 hours. An anaerobic
160 quartz-cuvette viologen assay, using methyl-viologen as the artificial electron donor
161 and $NO_3^-$ as the electron acceptor, was then performed in triplicate on cell
162 membrane fractions (Craske and Ferguson, 1986, Jones and Garland, 1977).

163 *2.8 Biofilm formation*

164 The ability for *B. thailandensis*, CA01 and CA01_pDA-17::BTH_I1704 to form
165 biofilms was assessed under aerobic or anaerobic conditions in L-broth using a
166 modified protocol (O'Toole and Kolter, 1998b). Bacterial cultures were standardised,
167 inoculated into a 96 well plates supplemented with or without nitrate (final volume of
168 200 µl) and incubated 3 days aerobically or anaerobically. After 3 days incubation all
169 the planktonic cells were carefully removed and the biofilm was washed twice with
170 sterile PBS and the biofilm was heat fixed at 80 $^{o}$C for one hour. The biofilm was
171 then stained with 0.1% crystal violet for 15 minutes and the excess crystal violet was
172 washed off prior to solubilisation of the crystal violet stain using 200 µl 70 % ethanol.
173 The degree of biofilm formation was then quantified by absorbance measurement at
174 570 nm. Four biological replicate were used each with five technical repeats.

175 *2.9 Motility assay*

176 To assess whether the transposon mutant exhibited a difference in motility
177 compared to wild-type *B. thailandensis,* 2 µl of a optical density 0.5 (600 nm)
178 standardised culture was inoculated into middle of a semi-solid nutrient broth agar
179 plate containing 0.5 % glucose, solidified with 0.3 % w/v bacteriological agar (NBA).

180 Motility plates were incubated at 37°C for 24 hours. Three biological replicates were
181 used, each with three technical repeats.

182 *2.10 Virulence study using Galleria mellonella*

183 Ten *G. mellonella* larvae were challenged with PBS, or 450 to 500 CFU of
184 wild-type *B. thailandensis* or CA01, via injection into the upper right proleg.
185 Challenged larvae were incubated at 37 °C. The larvae were considered dead when
186 no movement was displayed after gentle prodding. Four independent challenges
187 were performed.

188 *2.11 Statistical tests*

189 Statistical test were performed using a student two tailed T-test or a two-way
190 ANOVA.

191

192 **3. Results**

193 *3.1 B. thailandensis grows anaerobically on nitrate*

194 Bioinformatic analysis identified the presence of a potential denitrification
195 pathway in *B. thailandensis* and in *B. pseudomallei,* with both genomes predicted to
196 encode two membrane-bound NAR enzymes. *B. thailandensis* showed no anaerobic
197 growth when grown in the absence of an alternate electron acceptor ($NO_3^-$ or $NO_2^-$)
198 (data not shown).

199 During the lag phase of *B. thailandensis* anaerobic growth, the levels of nitrite
200 accumulated to approximately 120-140 µM (Fig. 1 A), suggesting an increase in NAR

201 activity and/or expression. At the start of exponential growth the levels of nitrite start
202 to decline and remain low, indicating the likely induction of NIR, NOR and NOS.

203 *3.2 Molybdopterin biosynthesis is required for B. thailandensis anaerobic nitrate*
204 *respiration.*

205 A random transposon mutant library, of 1,344 mutants, was screened to
206 identify gene(s) required for anaerobic growth on nitrate. The site of transposon
207 insertion in one transposon mutant (CA01) was identified as *moeA* (BTH_I1704), a
208 gene required for the molybdopterin biosynthetic pathway. This was confirmed using
209 a southern blot (Fig. 2 A). The southern blot was performed using labelled DNA
210 probes binding to either a 300 bp fragment at the start of BTH_I1704 (wild-type
211 probe), undisrupted by the transposon, or a 300 bp fragment within the kanamycin
212 resistance cassette. Both wild type *B. thailandensis* and CA01 genomic DNA were
213 digested with the restriction enzyme *XhoI* and the fragments were run on a 1 % TAE
214 agarose gel. *XhoI* restriction sites found outside of BTH_I1704 and within the
215 transposon generated a 2,927 bp band for the wild-type and a smaller band
216 (approximately 2000 bp) for the mutant when probed with the wild-type labelled DNA
217 probe. This and the absence of a band in wild-type digest DNA, when probed with
218 DNA recognising the kanamycin resistance gene cassette, confirmed the site of
219 transposon insertion in CA01 (Fig. 2 A).

220 In comparison to the wild-type, CA01 could not grow under anaerobic
221 conditions in the presence of nitrate (Fig. 1 D). Complementation of CA01 using
222 pDA-17::BTH_I1704, confirmed with PCR, successfully restored anaerobic growth
223 on nitrate and NAR activity (Fig. 1 B and D), and did not affect aerobic growth (data

224 not shown). Both the wild-type and mutant could grow aerobically and anaerobically
225 in the presence of nitrite (data not shown).

226 The disruption nitrate reductase was assessed by measuring the
227 accumulation of nitrite in the growth medium using the Griess reaction (Green *et al.*,
228 1982). Since CA01 could not grow under anaerobic conditions using nitrate as a
229 sole electron acceptor, cultures were grown aerobically in M9 minimal medium
230 supplemented with nitrate. Nitrite was detected in the culture medium of wild-type *B.*
231 *thailandensis* and CA01_pDA-17::BTH_I1704, but not CA01 (Fig. 1 B). During
232 aerobic growth the wild-type accumulated nitrite at an earlier time point to the mutant
233 complement, which only showed significant nitrite production after 16 hours growth.
234 After 24 hours CA01_pDA-17::BTH_I1704 nitrite levels were significantly (T-test p-
235 value <0.01) lower (15.5 ± 4 µM) to those seen in the wild-type (21 ± 3 µM). This
236 suggests that the complement may not be able to fully restore nitrate reductase
237 activity to the same extent as that seen in wild-type *B. thailandensis*. In comparison
238 to both the wild-type and CA01_pDA-17::BTH_I107, the *moeA* mutant (CA01) nitrite
239 levels only reached 2 ± 0.9 µM after 24 hours growth.

240 To further confirm the lack of NAR activity *in vitro* seen in CA01,
241 spectrophotometric assays (nitrate dependent reoxidation of reduced methyl
242 viologen) were performed on cell membrane fractions. Cultures were grown under
243 aerobic conditions to generate biomass, prior to anaerobic incubation (4 hours) with
244 nitrate. CA01 showed a statistically significant reduction in NAR activity (T-test p-
245 value < 0.05) displaying 0.04 µmol [$NO_3^-$]/min/g (ww) activity compared to 0.134
246 µmol [$NO_3^-$]/min/g (ww) NAR activity seen with wild-type *B. thailandensis* (Fig. 1 C).
247 The combined results demonstrate the inability of CA01 to respire using nitrate as
248 the sole respiratory substrate is due to a reduced NAR activity.

249 *3.3 B. thailandensis encodes two moeA genes but only BTH_I1704 is expressed*
250 *under both aerobic and anaerobic conditions.*

251 The *moeA* gene was located in a gene cluster encoding *moaA* (BTH_I1706)
252 and *mobA* (BTH_I1705), required for initial and final steps of Mo-*bis* MGD cofactor
253 synthesis. We identified a second *moeA* gene (BTH_I2200) in a gene cluster which
254 included *moaD* and *moaE,* encoding molybdopterin converting subunits 1 and 2 (Fig.
255 2 C). RT-PCR performed on mRNA extracted from bacteria cultures grown
256 anaerobically in M9 minimal media revealed BTH_I1704 to be constitutively
257 expressed in wild-type *B. thailandensis* under anaerobic conditions. No expression
258 was seen for BTH_I2200 under any condition tested (Fig. 2 D). BTH_I1704, but not
259 BTH_I2200, was expressed to a similar degree aerobically in L-broth and L-broth
260 supplemented with nitrate. The significance of the second *moeA* gene (BTH_I2200)
261 is currently unknown.

262 *3.4 Transposon insertion into BTH_I1704 affects motility and biofilm formation*

263 The ability to form biofilms under aerobic and anaerobic conditions was
264 assessed for wild-type *B. thailandensis,* CA01 and CA01_pDA-17::BTH_I1704 in L-
265 broth supplemented with nitrate. In comparison to the wild-type, CA01 displayed a
266 reduction in biofilm formation under all conditions tested (T-test; p-value $\leq 0.01$) (Fig.
267 3 B). No significant growth under anaerobic conditions in the absence of nitrate was
268 seen for any strain tested, resulting in very low biofilm production. Complementation
269 of CA01 with pDA-17::BTH_I1704 was able to successfully restore biofilm formation
270 to the same extent as the wild-type aerobically in the presence or absence or nitrate,
271 but could not restore biofilm formation to the same extent as the wild-type
272 anaerobically in the presence of nitrate (Fig. 3 B).The lack of a complete restoration

of biofilm forming capabilities in the complement to wild-type levels, under anaerobic conditions in the presence of nitrate, could potentially be linked the lower levels of nitrite production, and therefore relative NAR activity, seen after 24 h growth (Fig. 1 B). The ability to form biofilms is often dependent on motility, with flagella being required for initial surface attachment (O'Toole and Kolter, 1998a, Pratt and Kolter, 1998). CA01 displayed a significant reduction in swimming motility when compared to the wild-type (T-test; p-value $\leq 0.01$) (Fig. 3 A). Complementation of CA01 with pDA-17::BTH_I1704 could not restore the motility defect seen in CA01.

*3.5 Disruption of molybdopterin biosynthesis does not affect virulence in G. mellonella*

*G. mellonella* was used as an infection model (Wand *et al.*, 2010) to determine the potential role for *moeA* in virulence of *B. thailandensis.* No significant difference was seen in virulence between the wild-type and mutant CA01 (Two way ANOVA; $df = 1$, $f = 5.2$, $p > 0.05$) (Fig. 4).

## 4. Discussion

*B. pseudomallei* and *B. thailandensis* are environmental saprophytes, commonly found in rice paddy fields in Southeast Asia (Inglis and Sagripanti, 2006). Paddy soil becomes hypoxic at a 3 mm depth where nitrate predominates as the major anion, allowing colonisation by anaerobic microorganisms. In the human body nitrate is obtained as a dietary source or is produced through the oxidation of nitric oxide (Kelm, 1999, Lundberg *et al.*, 2004). Despite the obvious availability of nitrogen-oxyanions, the utilization of nitrate by *Burkholderia* spp. as a respiratory substrate has remained poorly studied.

296 *B. pseudomallei*, the etiological agent of melioidosis, causes acute, chronic
297 and latent infections, persisting within the human body for up to 62 years (Currie *et*
298 *al.*, 2000, Ngauy *et al.*, 2005). Melioidosis is often misdiagnosed as tuberculosis, as
299 both display similar clinical features such as granulomas, which often display a low
300 oxygen tension (Conejero *et al.*, 2011, Vidyalakshmi *et al.*, 2008). Although currently
301 little is known about the mechanisms of persistence of *B. pseudomallei,* it is likely
302 that the ability to survive under anaerobic conditions will play some role. The genes
303 associated with anaerobic respiration in *B. pseudomallei* and *B. thailandensis* display
304 around 95 to 100 % genetic identity, allowing for the use of *B. thailandensis* as a
305 model to determine their role in pathogenesis.

306 The present work has shown *B. thailandensis* to grow anaerobically using
307 nitrate as the sole electron acceptor and, like other well studied systems, nitrate
308 dependent growth requires a molybdopterin biosynthetic pathway for the functional
309 assembly of the respiratory reductase. Molybdenum-dependent enzymes fall into
310 two distinct groups; bacterial nitrogenases and pterin based molybdoenzymes
311 (Magalon, 2011). *B. thailandensis* and *B. pseudomallei* encode a wide range of
312 predicted molybdoenzymes, including formate dehydrogenase, sulfite oxidase,
313 xanthine dehydrogenase and nitrate reductase, sharing 90 to 99 % sequence
314 identity. Many of these are predicted to play roles in anaerobic metabolism and
315 electron transport (Table 1). Random transposon insertion into BTH_I1704 (Fig. 2)
316 identified the molybdopterin biosynthetic pathway as not only being required for
317 anaerobic growth, but also showed an effect on biofilm formation and motility.
318 Because the transposon disrupted the formation of the Mo-*bis* MGD cofactor
319 required for multiple molybdoproteins one cannot be sure that all observed
320 phenotypes is simply due to the disruption of nitrate reductase as other

321 molybdoproteins, such as formate dehydrogenase and sulfite:cytochrome c
322 oxidoreductase, have been implicated in motility and biofilm formation in
323 *Campylobacter jejuni* (Kassem *et al.*, 2012, Tareen *et al.*, 2011).

324 MoeA forms a dimeric protein which is required for molybdenum activation via
325 the ligation of Mo to MPT with MogA, generating Mo-*bis* MGD required for the
326 formation of active molybdoenzymes such as NAR (Hasona *et al.*, 1998, Nichols *et*
327 *al.*, 2007, Sandu and Brandsch, 2002, Schrag *et al.*, 2001). *B. thailandensis* genome
328 encodes two putative *moeA* genes, sharing 40 % identity (BTH_I1704 and
329 BTH_I2200), found within gene clusters encoding other components of the same
330 pathway. In other bacterial species the presence of two *moeA* gene has been
331 suggested to reflect the different requirement for either molybdenum or tungsten
332 metal ions (Bevers *et al.*, 2008, Bevers *et al.*, 2009). To determine whether both
333 BTH_I1704 and BTH_I2200 are expressed under anaerobic conditions RT-PCR
334 performed on mRNA extracted from wild-type *B. thailandensis.* BTH_I1704 was
335 constitutively expressed under anaerobic conditions (Fig. 2 D), indicating it to be the
336 main MoeA encoded in *B. thailandensis*. Although expression of BTH_I2200 was not
337 detected, it is possible that the protein is produced under conditions not tested in this
338 study.

339 BTH_I2200 is encoded on a putative operon encoding *moaD* and *moaE*
340 encoding molybdopterin synthase, essential for addition of dithiolene to cPMP to
341 form MPT (Wuebbens and Rajagopalan, 2003). Considering *moaD* and *moaE* are
342 essential molybdopterin biosynthesis, and no other *moaD* and *moaE* are found within
343 *B. thailandensis* it is plausible that BTH_I2201 and BTH_I2202 are under the control
344 of an alternative promoter that does not control the expression of BTH_I2200 (Fig. 2
345 D). Preliminary Softberry promoter analysis isolated a putative promoter region

346 within BTH_I2200 (data not shown), which could potentially control the expression of
347 BTH_I2201 and BTH_I2202, however further experiments are required to verify this
348 prediction.

349 Disruption of molybdoenzyme function, either directly or indirectly by
350 disrupting molybdopterin biosynthesis has been implicated in motility and virulence in
351 multiple pathogenic bacteria (Van Alst *et al.*, 2007, Baltes *et al.*, 2003, Tareen *et al.*,
352 2011, Filiatrault *et al.*, 2006). *B. pseudomallei* flagella play a role in virulence (Chua
353 *et al.*, 2003) and are required formation of biofilms, which play a role in antimicrobial
354 resistance (Sawasdidoln *et al.*, 2010, Taweechaisupapong *et al.*, 2005). CA01
355 displayed a reduction in both biofilm formation and motility when compared with wild-
356 type *B. thailandensis* (Fig. 3). Complementation of CA01 with pDA-17::BTH_I1704
357 successfully restored biofilm formation to a similar extent to that seen in the wild-
358 type. Interestingly complementation with pDA-17::BTH_I1704 could not restore the
359 motility defect in CA01. This could be due to downstream affects of the over-
360 expression, differential regulation of BTH_I1704, or potentially the loss of pDA-
361 17::BTH_I1704 plasmid. Complementation of CA01 with pDA-17::BTH_I1704 did not
362 fully restore wild-type NAR activity (Fig. 1 C). It is possible that the lack of NAR
363 activity under aerobic conditions may have affected the restoration of motility in
364 CA01. Considering the motility assay was performed under aerobic conditions it is
365 unlikely that the reduced swimming motility is due to bioenergetic constraints.
366 Transposon insertion into BTH_I1704 is not likely to have directly affected genes
367 required for motility as there are no flagella genes within the gene cluster. The
368 reduction in *P. aeruginosa narGH* mutant swarming motility was due to the reduced
369 formation of NO, a signalling molecule for rhamnolipid production (Van Alst *et al.*,
370 2007). It is possible that the reduction in biofilm formation and motility seen in CA01

371 is also due to the lack of NO as a result of limiting the supply of nitrite by disabling
372 the nitrate reductase. However since other molybdo-proteins have been shown to
373 play a role in motility (Kassem *et al.*, 2012, Tareen *et al.*, 2011) the defect in motility
374 and biofilm formation seen in CA01 may not be due to a reduction in NAR activity
375 alone. Further mutagenesis studies on NAR are required to determine its role in
376 motility and biofilm formation in *B. thailandensis.*

377 *P. aeruginosa* and *Mycobacterium* NAR and the molybdopterin biosynthetic
378 pathway have been implicated in virulence in *C. elegans* and murine models of
379 infection (Van Alst *et al.*, 2007, Fritz *et al.*, 2002, Filiatrault *et al.*, 2013, Filiatrault *et*
380 *al.*, 2006). No difference was seen between wild-type *B. thailandensis* and CA01 in
381 virulence when using *G. mellonella* (Fig. 4). It is possible, due to the acute nature of
382 the infection seen when this model organism, that *G. mellonella* may not be the
383 appropriate system for studying the role of anaerobic respiration in virulence of
384 *Burkholderia.* Use of a chronic infection model, allowing for the generation of
385 abscesses or granulomas that may have hypoxic environments, may yet reveal a
386 role for anaerobic respiration and molybdopterin biosynthesis in *B. pseudomallei*
387 pathogenesis.

388 This study has demonstrated the importance of the molybdopterin
389 biosynthetic pathway in nitrate reduction, biofilm formation and motility in *B.*
390 *thailandensis.* Future studies into the role of NAR in *B. pseudomallei* virulence and
391 anaerobic respiration are currently underway.

392

393

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399

400 **6. References**

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564 Table 1 – Putative molybdoproteins in *B. pseudomallei* and *B. thailandensis*

| Name | Gene name(s)[a] | *B. pseudomallei*[a] | *B. thailandensis*[a] | Similarity[b] (%) | Predicted function[c] |
|---|---|---|---|---|---|
| **Membrane bound nitrate reductase(s)** | *narGHJI*<br>*narZYWV* | BPSL2309-2312<br>BPSS1156-1159 | BTH_I1851-1854<br>BTH_II1249-1252 | 90-99 | Dissimilatory nitrate reduction |
| **Nitrate reductase** | *nasA* | BPSL0510 | BTH_I0462 | 95.5 | Assimilatory nitrate reduction |
| **Sulfite oxidase** | *yedZY* | BPSL3177-3178 | BTH_I3032-3033 | 89.7-94.1 | Oxidation of S- and N-oxides |
| **$NAD^+$ formate dehydrogenase** | *fdsGBAD* | BPSL2528-2531 | BTH_I1621-1624 | 93-96 | Formate oxidation |
| **Formate dehydrogenase-N** | *fdoGHI* | BPSS1665-1667 | BTH_II0707-0710 | 96-98 | Formate oxidation |
| **Xanthine dehydrogenase** | *xdhAB* | BPSL2727-2728 | BTH_I1408-1409 | 94-99 | Purine metabolism |
| **Putative DMSO reductase** | *dmsABC* | BPSS2299-2301 | Absent | - | Putative role in anaerobic reduction of DMSO and/or TMAO |

| | | | | | |
|---|---|---|---|---|---|
| **MOSC[h] domain-containing protein** | *ycbX*[d] | BPSS0707 | BTH_II1722 | 93 | Putative role in N-hydroxylaminopurine (HAP) detoxification[d] |
| **MOSC[h] domain-containing protein** | *yiiM* | BPSL0935 | BTH_I0802 | 92.5 | Putative role in N-hydroxylaminopurine (HAP) detoxification[d] |
| **Bifunctional reductase** | - | BPSS1241 | BTH_II1172 | 92.1 | Putative nitrate/sulfite reductase[e] |
| **NADH dehydrogenase** | *nuoABCD EFGHJK* | BPSL1211-1221 | BTH_I1061-1074[f] | 96-100 | Respiratory function |
| **Molybdopterin oxidoreductase** | - | BPSL2207 | BTH_I1975 | 93 | Unknown function |
| | - | BPSL3038 | BTH_I1105 | | |
| | - | BPSS0969 | BTH_II1422 | | |
| **Hypothetical proteins**[g] | - | BPSL0733 | BTH_I0634 | 91-93.4 | Unknown function |
| | - | BPSL1294 | BTH_I2840 | | |
| **Sulfite: cytochrome c oxidoreductase** | - | Absent | BTH_II1622 | - | Unknown function |

[a] Gene name and locus ID determined using GenBank database (NCBI - http://www.ncbi.nlm.nih.gov/)

[b] Determined using KEGG (Kyoto Encyclopaedia of Genes and Genomes - http://www.kegg.jp/)

[c] Predicted based on known molybdo-protein function in other prokaryotic species

[d] BTH_II1722/BPSS0707 and BTH_I0802/BPSL0935 are predicted orthologs of *E. coli* YcbX and YiiM MOSC domain-containing molybdoenzymes (Kozmin *et al.*, 2008)

[e] BTH_II1172 and BPSS1241share orthology with sulfite reductase (NADH) flavoprotein (according to KEGG)

[f]BTH_I1067 and BPSL1217 contain a molybdopterin binding signatures

[g]Hypothetical proteins containing SO-family motifs

[h]MOSC - molybdenum cofactor sulfurase C-terminal domain (Anantharaman and Aravind, 2002)

565 ## Figure legends

566 **Figure 1.** Nitrate-dependent growth and NAR activity of *B. thailandensis* and CA01.
567 *B. thailandensis* was grown in M9 minimal medium aerobically or anaerobically in the
568 presence or absence of nitrate. (A) Anaerobic growth of wild-type *B. thailandensis*
569 (WT) using nitrate as the sole electron acceptor (squares). Nitrite production was
570 measured using the Griess reagent system (triangles). (B) Nitrite production,
571 measured using the Griess reaction, during aerobic growth in M9 minimal media
572 supplemented with 20 mM sodium nitrate, for WT (dark grey), CA01 (light grey) and
573 CA01_pDA-17::BTH_I1704 (white with cross-hatched lines). (C) Nitrate reductase
574 activity (in μmol [$NO_3^-$]/min/g (ww)) of membrane fractions from anaerobically
575 acclimatised wild-type (dark grey) and CA01 (grey) cultures. (D) Anaerobic growth
576 of wild-type *B. thailandensis* (squares), CA01 (circles) and CA01_pDA-
577 17::BTH_I1704 (triangles). Results are the average at least three biological
578 replicates. Statistically significant results (p-values ≤ 0.05) are shown with asterisk
579 (*). Error bars ± standard deviation (SD).

580 **Figure 2.** CA01 and CA01_pDA-17::BTH_I1704 confirmation and expression of wild-
581 type *B. thailandensis* BTH_I1704 and BTH_I2200 under anaerobic conditions. (A)
582 Southern blot and agarose gel (lanes 1 - 4) of *Xho*I digested WT or CA01 DNA and
583 using WT probe (lanes 5-8) or mutant probe (lanes 9-10). Agarose gel - lane 1, 1 kb
584 DNA ladder (Thermo Scientific); lane 2, *B. thailandensis* digested DNA; lane 3, CA01
585 digested DNA; lane 4, WT probes DNA (300 bp); Southern blots - lane 5, 1 kb
586 GeneRuler ladder; lane 6, *B. thailandensis* DNA (2,979 bp band); lane 7 digested
587 CA01 (2,000 bp band); lane 8, 300 bp WT probe DNA; lane 9, digested *B.*
588 *thailandensis* DNA; lane 10, digested CA01 DNA. (B) CA01 transposon mutant
589 complement confirmation, using primers to amplify BTH_I1704 (1,299 bp); lane 1, 1

590 kb gene ruler ladder; lane 2, WT E264; lane 3, CA01; lane 4, CA01_pDA-
591 17::BTH_I1704. (C) Schematic representation of *B. thailandensis moeA* gene
592 clusters involved in molybdopterin biosynthesis identified using NCBI (genes not
593 drawn to scale). (D) RT-PCR of putative *moeA* genes during anaerobic growth using
594 nitrate as a sole electron acceptor. Primers amplifying regions within 16s rRNA,
595 BTH_I1704 and BTH_I2200 were used in separate reactions. mRNA samples were
596 extracted from wild-type *B. thailandensis* cultures. Lane 1 – aerobic LB overnight
597 culture; lane 2 – aerobic LB overnight culture supplemented with nitrate; lane 3 - 2 h
598 (lag phase); lane 4 - 24 h (early exponential); lane 5 - 47 h (mid-exponential); lane 6
599 - 54 h (late exponential); lane 7 - 72 h (stationary phase). mRNA samples used in
600 lanes 3 – 8 are from anaerobic M9 minimal media cultures containing 20 mM sodium
601 nitrate. *B. thailandensis* gDNA (lane 8) was used as a positive control. Images are
602 the representative of two biological replicates.

603 **Figure 3.** A role for BTH_I1704 in motility and biofilm formation. (A) *B. thailandensis*
604 motility on 0.3% nutrient broth agar after 24 hour incubation. Wild-type (dark grey),
605 CA01 (light grey), CA01_pDA-17::BTH_I1704 (white with cross hatched lines). (B)
606 Biofilm assay was performed in the presence or absence of nitrate ($NO_3^-$) for WT *B.*
607 *thailandensis* (dark grey), CA01 (light grey) and CA01_pDA-17::BTH_I1704 (white)
608 in L- broth. A 96 well plate was incubated for three days aerobically (+ $O_2$; dashed
609 columns) or anaerobically (– $O_2$; cross hatched columns), and the degree of biofilm
610 formation was measured using a crystal violet stain. Three or four independent
611 biological replicates were used each with three or five technical replicates. Error bars
612 ± SD. Statistically significant results (p-values ≤ 0.01), comparing WT and CA01 or
613 WT and CA01_pDA-17::BTH_I1704 are shown with asterisks (*). Error bars ± SD.

614 **Figure 4.** *B. thailandensis Galleria mellonella* challenge. Ten wax *G. mellonella* were
615 challenged with either PBS (squares), wild-type *B. thailandensis* (WT - circles) or
616 CA01 (triangles). Data shown is the average of four biological replicates with an
617 average infectious dose 450 - 500 CFU/galleria.

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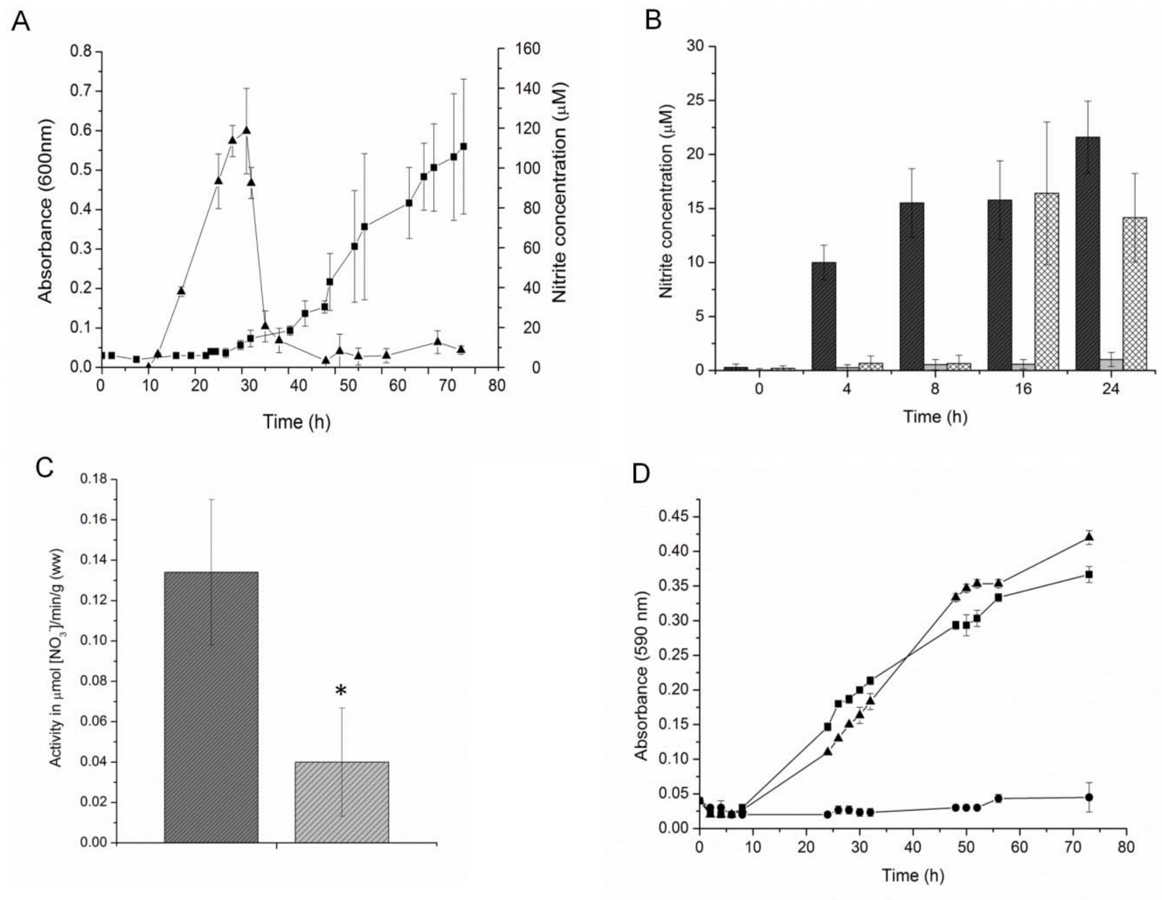
619 Figure 1

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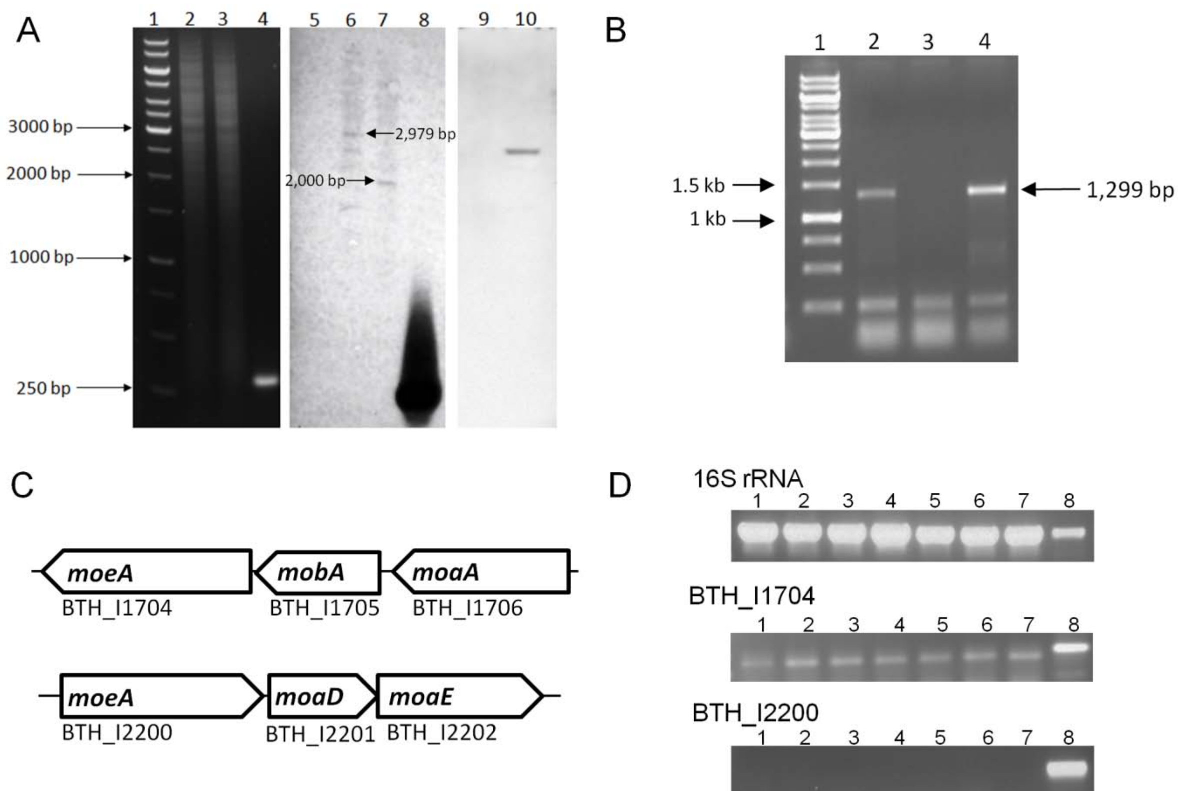
622 Figure 2

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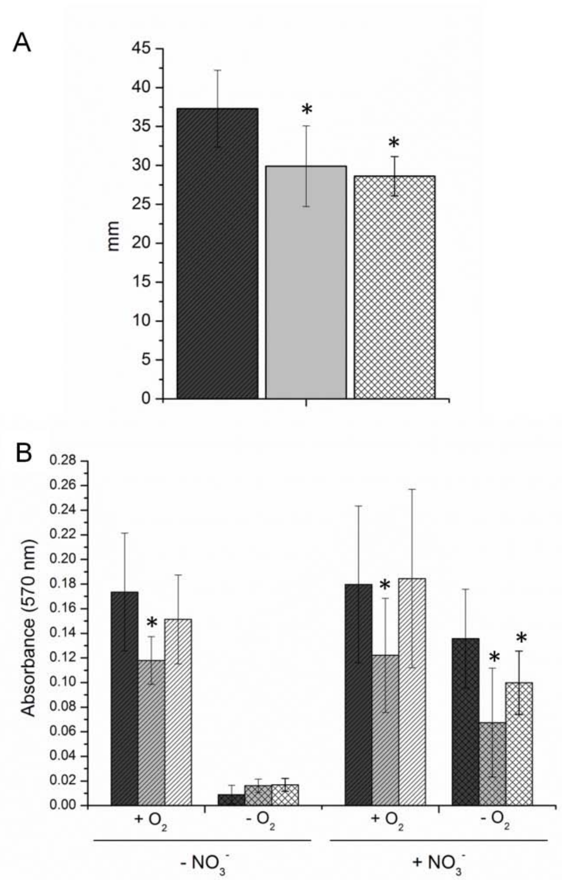
625 Figure 3

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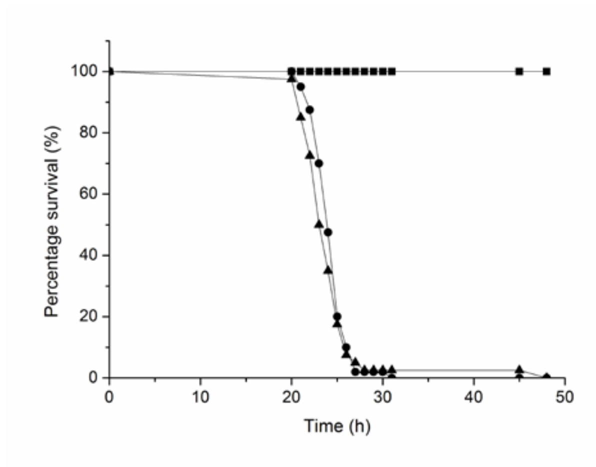
627 Figure 4

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