**Influence of the molybdenum cofactor biosynthesis on anaerobic
respiration, biofilm formation and motility in *Burkholderia
thailandensis***

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Abstract

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

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

1. Introduction

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

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

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

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

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

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

2. Materials and Methods

2.1 Bacterial strains, growth conditions and medium

B. thailandensis (E264) and *Escherichia coli* were routinely grown in Luria-Bertani broth (L-broth) at 37 °C, solidified when required with 1.5 % (w/v) bacteriological agar. When appropriate, antibiotics were added to the medium at the following concentrations; 100 µg/ml gentamicin, 50 µg/ml kanamycin, 100 µg/ml ampicillin or 50 µg/ml tetracycline. Anaerobic growth studies were conducted statically within an anaerobic cabinet (10 % CO₂, 80 % N₂ and 10 % H₂) using primarily in M9 minimal medium supplemented with 20 mM sodium succinate, a non-fermentable carbon source, or L-broth. M9 medium was composed of 2 mM MgSO₄, 0.1 mM CaCl₂, 20 % 5x M9 salts (5 x M9 salts: 85.5 g/l Na₂HPO₄, 15 g/l KH₂PO₄, 2.5 g/l NaCl, 5 g/l NH₄Cl). When appropriate the anaerobic growth medium was supplemented 20 mM sodium nitrate (NaNO₃⁻).

2.2 Transposon mutagenesis

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

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

2.3 Polymerase Chain Reaction

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

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

2.4 Reverse transcriptase PCR

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

2.5 Southern blot

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

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

A mutant complement was created, using the constitutive expression vector pDA-17 (7,360 bp) encoding a tetracycline resistance cassette. BTH_I1704 (1,299 bp) was cloned into pDA-17 in front of the *dhfr* promoter region via *NdeI* and *XbaI* restriction sites and transformed into DH5 α competent cells. Successful DH5 α pDA-17::BTH_I1704 transformants were screened using PCR and sequenced prior to conjugating into CA01 using a *E. coli* helper strain (pKR2013). Any potential complements (CA01_pDA-17::BTH_I1704) were confirmed using PCR and maintained on medium containing tetracycline 50 μ g/ml. All *in vitro* experiments with CA01_pDA-17::BTH_I1704 were performed using media supplemented with tetracycline 50 μ g/ml.

2.7 Nitrate reductase assays

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

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

2.8 Biofilm formation

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

2.9 Motility assay

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

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

2.10 Virulence study using *Galleria mellonella*

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

2.11 Statistical tests

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

3. Results

3.1 *B. thailandensis* grows anaerobically on nitrate

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

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

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

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

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

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

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

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

To further confirm the lack of NAR activity *in vitro* seen in CA01, spectrophotometric assays (nitrate dependent reoxidation of reduced methyl viologen) were performed on cell membrane fractions. Cultures were grown under aerobic conditions to generate biomass, prior to anaerobic incubation (4 hours) with nitrate. CA01 showed a statistically significant reduction in NAR activity (T-test p-value < 0.05) displaying $0.04 \mu\text{mol} [\text{NO}_3^-]/\text{min/g}$ (ww) activity compared to $0.134 \mu\text{mol} [\text{NO}_3^-]/\text{min/g}$ (ww) NAR activity seen with wild-type *B. thailandensis* (Fig. 1 C). The combined results demonstrate the inability of CA01 to respire using nitrate as the sole respiratory substrate is due to a reduced NAR activity.

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

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

3.4 Transposon insertion into BTH_I1704 affects motility and biofilm formation

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

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

The present work has shown *B. thailandensis* to grow anaerobically using nitrate as the sole electron acceptor and, like other well studied systems, nitrate dependent growth requires a molybdopterin biosynthetic pathway for the functional assembly of the respiratory reductase. Molybdenum-dependent enzymes fall into two distinct groups; bacterial nitrogenases and pterin based molybdoenzymes (Magalon, 2011). *B. thailandensis* and *B. pseudomallei* encode a wide range of predicted molybdoenzymes, including formate dehydrogenase, sulfite oxidase, xanthine dehydrogenase and nitrate reductase, sharing 90 to 99 % sequence identity. Many of these are predicted to play roles in anaerobic metabolism and electron transport (Table 1). Random transposon insertion into BTH_I1704 (Fig. 2) identified the molybdopterin biosynthetic pathway as not only being required for anaerobic growth, but also showed an effect on biofilm formation and motility. Because the transposon disrupted the formation of the Mo-*bis* MGD cofactor required for multiple molybdoproteins one cannot be sure that all observed 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 (Bever *et al.*, 2008, Bever *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

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

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

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

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

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

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

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

MOSC^h domain-containing protein	<i>ycbX^d</i>	BPSS0707	BTH_II1722	93	Putative role in N-hydroxylaminopurine (HAP) detoxification ^d
MOSC^h domain-containing protein	<i>yiiM</i>	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	<i>nuoABCD EFGHJK</i>	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 BPSS1241 share 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)

Figure legends

Figure 1. Nitrate-dependent growth and NAR activity of *B. thailandensis* and CA01.

B. thailandensis was grown in M9 minimal medium aerobically or anaerobically in the presence or absence of nitrate. (A) Anaerobic growth of wild-type *B. thailandensis* (WT) using nitrate as the sole electron acceptor (squares). Nitrite production was measured using the Griess reagent system (triangles). (B) Nitrite production, measured using the Griess reaction, during aerobic growth in M9 minimal media supplemented with 20 mM sodium nitrate, for WT (dark grey), CA01 (light grey) and CA01_pDA-17::BTH_I1704 (white with cross-hatched lines). (C) Nitrate reductase activity (in $\mu\text{mol} [\text{NO}_3^-]/\text{min/g}$ (ww)) of membrane fractions from anaerobically acclimatised wild-type (dark grey) and CA01 (grey) cultures. (D) Anaerobic growth of wild-type *B. thailandensis* (squares), CA01 (circles) and CA01_pDA-17::BTH_I1704 (triangles). Results are the average at least three biological replicates. Statistically significant results ($p\text{-values} \leq 0.05$) are shown with asterisk (*). Error bars $\pm$ standard deviation (SD).

Figure 2. CA01 and CA01_pDA-17::BTH_I1704 confirmation and expression of wild-

type *B. thailandensis* BTH_I1704 and BTH_I2200 under anaerobic conditions. (A) Southern blot and agarose gel (lanes 1 - 4) of *Xho*I digested WT or CA01 DNA and using WT probe (lanes 5-8) or mutant probe (lanes 9-10). Agarose gel - lane 1, 1 kb DNA ladder (Thermo Scientific); lane 2, *B. thailandensis* digested DNA; lane 3, CA01 digested DNA; lane 4, WT probes DNA (300 bp); Southern blots - lane 5, 1 kb GeneRuler ladder; lane 6, *B. thailandensis* DNA (2,979 bp band); lane 7 digested CA01 (2,000 bp band); lane 8, 300 bp WT probe DNA; lane 9, digested *B. thailandensis* DNA; lane 10, digested CA01 DNA. (B) CA01 transposon mutant 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 $\pm$ 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 $\pm$ 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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627 Figure 4