The contribution of the NarB and NarGHI enzymes to nitrate reduction in *Mycobacterium smegmatis*

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Abstract

Background: Nitrate reduction in bacteria is an essential step in the nitrogen cycle. For this, the reduction of nitrate to nitrite is catalyzed by a variety of nitrate reductase enzymes. In the pathogen *Mycobacterium tuberculosis*, nitrate reduction is driven by the NarGHI respiratory and assimilatory nitrate reductase. In addition to this enzyme, *Mycobacterium smegmatis* carries a second putative narB-encoded nitrate reductase and the contribution of this enzyme to nitrate reduction remains unknown. Herein, we set out to investigate this. **Results:** To assess the relative contribution of NarGHI and NarB, the corresponding gene loci we deleted using two-step allelic replacement, individually and in combination, followed by investigation of nitrate reduction using the Griess assay. However, previous reports demonstrated that this assay was unable to report on nitrate reduction in *M. smegmatis*, as it yielded no detectable levels of the nitrite product. To address this, we modified the assay through the addition of zinc, which reduces nitrate remaining in the reaction to nitrite thus allowing for assessment of nitrate depletion. This then serves as a surrogate for nitrate reductase activity. The mutant strains lacking narB and/or narGHI retained the ability to reduce nitrate at levels comparable to the wild type. We further investigated nitrate assimilation and all strains defective for these enzymes were able to grow on nitrate as the sole nitrogen source. **Conclusions:** Collectively, these data confirm that NarB and NarGHI are individually and collectively dispensable for both respiratory and assimilatory nitrate reduction in *M. smegmatis*. Furthermore, we identified MSMEG_4206 as a putative, previously unannotated, nitrate reductase in this organism.

Background

Nitrogen is an essential element for all life, being a vital component of proteins and nucleic acids in both eukaryotes and prokaryotes. The nitrogen cycle, shown in Figure 1A, is well characterized and involves the cycling of nitrogen from the atmosphere in various forms through the earth and back. The entire cycle involves different processes including nitrogen fixation, ammonification, nitrification and denitrification [1]. Bacteria are critical to this process as they are the only organisms capable of carrying out a number of the processes [1-3]. Although nitrogen is abundant in the atmosphere as a gas in a diatomic state (N₂), the triple bond between the two nitrogen atoms requires a large amount of energy for hydrolysis and subsequent incorporation of nitrogen into other compounds. In addition to N₂, other available sources of nitrogen include inorganic nitrate-containing compounds, urea, ammonia and proteins/amino acids. Nitrate reduction is considered the most important stage of nitrogen turnover in the nitrogen cycle and this reaction is catalyzed by nitrate reductase (NR) enzymes [4].

Prokaryotic NR's are mononuclear molybdenum-containing enzymes and are assigned to one of three classes based on the ultimate purpose of the nitrate being reduced as well as their sub-cellular localization [2]. Assimilatory NR's found in the cytoplasm are commonly referred to as Nas enzymes and catalyze the incorporation of nitrogen into cellular material thus directly contributing to growth [2, 5, 6]. Dissimilatory or periplasmic NR's, known as Nap enzymes are generally involved in respiration through redox balancing via electron transfer. Nap proteins can also serve as respiratory enzymes in bacteria that

lack the respiratory NR, Nar [2, 7, 8]. Nar enzymes are similar to their dissimilatory counterparts in that they are involved in electron transfer; however the electron flow through respiratory NR's is coupled with proton translocation and thus the generation of a proton motive force (PMF) across the cytoplasmic membrane and energy production [5, 7]. Common to all three NRs is a catalytic site made up of the molybdenum cofactor (MoCo), specifically *bis*-molybdopterin guanine dinucleotide (*bis*-MGD) and iron-sulfur clusters, usually of the type [4Fe-4S] however, a distinct series of steps is followed for nitrate reduction by each enzyme [5].

The respiratory NR is a trimeric enzyme made up of the subunits NarG, NarH and NarI, and is encoded by the *narGHJI* operon. NarJ serves as a chaperone to facilitate the insertion MoCo [9]. In *Escherichia coli* during the catalytic reaction, electrons are donated to NarI from the electron carrier menaquinol (MQH₂) and this is coupled with the transfer of two protons into the periplasm [5]. The two electrons are then transferred to *bis*-MGD for the reduction of nitrate to nitrite, in the process consuming two cytoplasmic protons and thus generating a PMF [5]. The causative agent of tuberculosis, *Mycobacterium tuberculosis*, has a similar respiratory NR, which has been shown to support both assimilatory and respiratory nitrate reduction [10]. Although a second "fused" respiratory NR, NarX, is annotated, no function has been assigned to this enzyme [11]. Nitrate reduction is important for *M. tuberculosis* with several lines of evidence implicating the activity of NarGHI in virulence and pathogenesis, reviewed in [12].

The model organism, *Mycobacterium smegmatis*, also carries a *narGHJI*-encoded NR and it has been demonstrated that strains lacking a functional *bis*-MGD biosynthetic pathway were unable to assimilate nitrate, presumably due to the lack of the catalytic MoCo cofactor required by the NarGHI [13, 14]. However, in addition to this NR, *M. smegmatis* encodes for a second putative NR, *narB*, proposed to contribute to assimilatory nitrate reduction [15]. However, this has not been demonstrated. Furthermore, both NarB and NarGHI are annotated as MoCo-dependent enzymes, Figure 1B. Abrogation of *bis*-MGD biosynthesis would therefore abolish the function of both enzymes, thus making it difficult to determine relative contribution to nitrate reduction. Herein, we aimed to dissect the relative contribution of NarGHI and NarB to respiratory and assimilatory nitrate reduction in *M. smegmatis* using a gene-knockout approach and subsequent characterization of mutant strains.

Results

Deletion of the *narB* and *narGHJI* loci individually and collectively does not affect nitrate utilization. We set out to investigate the role of NarB and NarGHI in nitrate reduction by generating deletion mutant strains lacking each locus as well as a strain lacking both loci. The strains $\Delta narB$, $\Delta narGHJI$ and $\Delta narB \Delta narGHJI$ were generated by homologous recombination and genotypically confirmed by Southern blot analyses (Additional File 1: Figures S1 and S2). Following this, nitrate utilization assays were carried out. *M. smegmatis* has been reported as NR negative in the Griess assay, due to the inability to detect nitrite under anaerobic conditions when nitrate was provided as a substrate [16]. Hence, to investigate NR activity in *M. smegmatis*, we modified the Griess assay to measure nitrate utilization as described in Figure 2A. As a negative control, a $\Delta moaD2 \Delta moaE2$ double mutant, which was previously shown to be

defective for *bis*-MGD production and as a result, nitrate reduction [13, 14], was included as it is unable to assimilate nitrate. When the Griess reagents were added to the culture samples taken at Day 0, no colour change was observed (Figure 2B) which was in contrast to the immediate change to pink observed for the nitrite standards. Once zinc was added to the samples, a dark pink colour was produced, confirming the presence of nitrate at the beginning of the experiment. This colour started fading after 1 day's incubation at 37 °C (Figure 2B). At the end of the experiment, the nitrate was completely consumed for all strains, with the exception of the $\Delta moaD2 \Delta moaE2$ negative control. These effects were quantified (Figure 2C) and further confirmed that loss of both *narB* and *narGHJI* does not affect nitrate utilization.

To confirm that the depletion of nitrate from the medium was because of utilization and not merely transport of nitrate into the cell, intracellular nitrate levels were also quantified. The intracellular levels of nitrate were approximately 10-fold lower than the extracellular levels on Day 1 and were further depleted by Day 6, confirming that the nitrate was being assimilated and not accumulating intracellularly (Figure 2C). No statistically significant differences in the amount of intracellular nitrate were detected between the wild type, $\Delta narB$, $\Delta narGHJI$ and $\Delta narB \Delta narGHJI$ strains. Increased intracellular levels of nitrate were detected in $\Delta moaD2 \Delta moaE2$, again confirming the inability of this strain to reduce nitrate.

NarB and NarGHI are both dispensable for nitrate assimilation in minimal media. After confirming that no change in nitrate utilization occurred upon deletion of both *narB* and *narGHJI*, we next assessed the ability of mutant strains to assimilate nitrate as previously described [14]. The growth curves for both the single mutants and the double deletion mutant in MPLN, were indistinguishable from the wild type (Figure 3A). The $\Delta moaD2 \Delta moaE2$ strain was included as a negative control and as expected was unable to assimilate nitrate in order to grow under these conditions. In addition, no differences in growth were observed between the strains when grown in 7H9 medium (Additional file 1: Figure S3), providing evidence that NarB and NarGHI are dispensable for the growth of *M. smegmatis* under carbon and nitrogen replete conditions.

***M. smegmatis* NarGHI is dispensable under anaerobic conditions.** In *M. tuberculosis*, NarGHI is a respiratory enzyme which serves as the terminal electron acceptor under anaerobic conditions when nitrate is available, thus facilitating growth and/or survival [11]. It has been shown that *M. smegmatis* is able to survive under anaerobic conditions in the presence of nitrate [17]. We sought to assess if this effect is mediated by NarGHI. For this, we scored the proportionate increase in CFUs under anaerobic conditions after 7 days' incubation and normalized this with the initial inoculum. We observed between 30-45 fold increases in bacterial biomass under aerobic conditions (Figure 3B). Under anaerobic conditions, less growth was observed with no significant difference in bacterial yield between the wild type and mutant strains (Figure 3B). The ability of $\Delta narB$, $\Delta narGHJI$ and $\Delta narB \Delta narGHJI$ to grow in MPLN under aerobic and anaerobic conditions suggests that loss of NarB and NarGHI does not abrogate NR activity in *M. smegmatis*. To investigate this further, the expression pattern of *narB* and *narGHJI* was assessed for the different *M. smegmatis* strains grown in 7H9 and MPLN. When grown to late log phase in 7H9, no *narB* or *narG* transcript was detected in any of the strains, suggesting that these genes are not expressed in *M. smegmatis* under the conditions tested (data not shown). In these cases, robust levels of

sigA were detected (Additional file 1: Figure S4). Next, we assessed the expression of *narB* and *narG* in MPLN to determine if growth in nitrate leads to an induction of these genes. We could not detect any *narB* transcript under these conditions (data not shown). For *narG*, transcript was detected in wild type and $\Delta narB$ in equal amounts when grown in MPLN (Additional file 1: Figure S4). Despite the low amount of *narG* transcript in samples grown in MPLN, no transcript was detected when grown in 7H9 (data not shown). These data thus provide evidence that *narGHJI* expression in *M. smegmatis* is induced in the presence of nitrate, albeit to a low level.

Bioinformatics analyses of *M. smegmatis* NR proteins. *M. smegmatis* and *M. tuberculosis* both retain the necessary repertoire of genes encoding proteins required for each step of the nitrate assimilation pathway [18], shown in Figure 4A. The discrepancies observed between the two organisms include the increased number of nitrate/nitrite transporters present in *M. tuberculosis* and the presence of different additional putative NR-encoding genes in *M. tuberculosis* and *M. smegmatis*, namely the *narX* and *narB* respectively. A large degree of sequence homology is observed between the *M. tuberculosis* and *M. smegmatis* *narG* genes (76.8 %). In addition, the single nucleotide polymorphism (SNP) identified in the promoter region of *narG* in *Mycobacterium bovis* (-215 T → C), strongly associated with differences in NR activity [19], is not present in *M. smegmatis* (Additional file 1: Figure S5), suggesting that the expression profile of *narG* in *M. smegmatis* and *M. tuberculosis* could be similar. Furthermore, pairwise protein sequence alignments of NarG performed using the EMBOSS Needle tool [20], revealed high sequence homology (~80%) between different mycobacterial organisms and when compared to the *E. coli* protein, all the catalytic residues are conserved (Additional file 1: Figure S6). These bioinformatics analyses suggest that *M. smegmatis* encodes a functional *narGHJI*-encoded NR.

BLAST [21] analysis revealed that several mycobacterial species harbour a copy of *narB*, including members of the *Mycobacterium avium* complex (MAC), *Mycobacterium fortuitum*, *Mycobacterium yongonense*, *Mycobacterium goodii* and *Mycobacterium kansasii* - all of which are capable of causing disease. However, a *narB* homologue was not identified in any of the organisms belonging to the *M. tuberculosis* complex. Analysis of the NarB protein sequence (A0QW69) revealed four domains, Figure 4B. This domain architecture is most commonly found in the catalytic subunit of periplasmic nitrate reductases and interestingly, a prediction of the 3D structure of *M. smegmatis* NarB models best to the *Desulfovibrio desulfuricans* periplasmic nitrate reductase (Pdb 2V45) [22, 23], Figure 4C. However, this is likely attributable to the lack of a resolved NarB crystal structure. A signal sequence was not identified using SignalP [24], for *M. smegmatis* NarB, suggesting that it is most likely located in the cytoplasm. The protein shares homology (31.1%) with the *Synechococcus* sp. PCC 7942 NarB and according to STRING [25] analysis, it could possibly interact with MSMEG_1847, a ferredoxin which is the predicted physiological electron donor to NarB in *Cyanobacteria* [26].

Considering that nitrate reduction continued to occur after deletion of both *narB* and *narGHJI*, it is possible that an alternate MoCo-dependent NR reductase is present in *M. smegmatis* which is solely responsible for nitrate assimilation or works in combination with other MoCo-dependent NR's for the reduction of nitrate to nitrite. Using the domains shown in Figure 4B, the genome of *M. smegmatis* was

examined in KEGG [27] to identify enzymes with the same architecture which could possibly catalyze the reaction. Seven genes encoding enzymes with the same architecture were identified and are listed in Table 1, two of which were *narB* and *narG*. Three of the seven genes encoded the MoCo-dependent oxidoreductases formate dehydrogenase and NADH dehydrogenase. The two remaining hits were MSMEG_2237 and MSMEG_6816 which are annotated as an anaerobic dehydrogenase and molybdopterin oxidoreductase respectively [28] and could possibly reduce nitrate to nitrite. In addition to the seven genes identified in the *M. smegmatis* genome with the MSMEG annotations, one gene was identified in the *M. smegmatis* genome with the newer MSMEI gene annotations curated by EcoGene-RefSeq [29], MSMEI_4108. MSMEI_4108 is annotated as a putative assimilatory nitrate/sulfite reductase. In order to identify this gene in SmegmaList, a BLAST search was performed using the nucleotide sequence and 100 % homology was detected for an un-annotated region [28]. The region was between coordinates 4288072-4292127 and the surrounding gene organisation for both databases was exactly the same (Additional File 1: Figure S7). No signal sequence or transmembrane domains were detected for MSMEI_4108, suggesting that it is cytoplasmic. In addition, it possesses binding domains for different cytoplasmic electron donors, Figure S7. We thus propose to annotate MSMEG_4206 as a putative assimilatory nitrate/sulfite reductase. Further investigation into this gene and its role in nitrate assimilation is currently being conducted.

Table 1: *M. smegmatis* genes encoding proteins with the same domain architecture as NarB.

Gene	Identifier	EC classification	Gene length	Protein length	NarB % homology*
MSMEG_0161	Formate dehydrogenase	1.2.1.2	2820 bp	939 aa	21.6
MSMEG_2057 (<i>nuoG</i>)	NADH dehydrogenase subunit G	1.6.5.3	2385 bp	794 aa	17.6
MSMEG_3521	Formate dehydrogenase	1.2.1.2	2250 bp	749 aa	21.6
MSMEG_2837 (<i>narB</i>)	Assimilatory nitrate reductase	1.7.7.2	2388 bp	795 aa	-
MSMEG_5140 (<i>narG</i>)	Respiratory nitrate reductase	1.7.5.1/1.7.99.4	3675 bp	1224 aa	16.8
MSMEG_2237	Anaerobic dehydrogenase	Not assigned	2283 bp	760 aa	21.2
MSMEG_6816	Molybdopterin oxidoreductase	Not assigned	2163 bp	720 aa	24.4
MSMEI_4108	Putative assimilatory nitrate reductase/sulfite reductase	Not assigned	4056 bp	1351 aa	18.7

*Alignments were performed using EMBOSS Needle and protein sequences were obtained from KEGG.

Discussion

It has long been accepted that *M. smegmatis* is NR negative, based solely on the results of the Griess assay [16]. When performed following the conventional protocol, this assay cannot be used to assess NR activity in *M. smegmatis* unless the strain carries a constitutively expressed copy of the *M. tuberculosis narGHJI* operon [13, 16], suggesting that *M. smegmatis* is unable to reduce nitrate. However, several studies have shown that *M. smegmatis* is able to use nitrate as the sole nitrogen source [13-15, 17, 30]. Furthermore, it has been demonstrated that NR activity in a strain ($\Delta moaD2 \Delta moaE2$) unable to synthesise the *bis*-MGD cofactor was completely abrogated [13], providing further evidence that a functional NR is present in *M. smegmatis* which like the *M. tuberculosis* NR, is *bis*-MGD dependent. Using a modified Griess assay we confirmed that *M. smegmatis* does indeed reduce nitrate. We show that over time in culture, *M. smegmatis* is able to utilize all available nitrate in the medium for assimilation. The ability of *M. smegmatis* to reduce nitrate is not surprising as two putative NR-encoding genes are annotated in the genome *i.e.* *narB* and *narGHJI*. However, the contribution of each to NR activity was unknown. We sought to address this question.

NR activity is a phenotypic characteristic widely accepted to classify and differentiate between mycobacterial species, with *M. tuberculosis* displaying high NR activity while *M. bovis* and *M. bovis* BCG show no distinct NR activity [30]. The molecular basis for the difference in NR activity between *M. tuberculosis* and *M. bovis* BCG has been attributed to the lack of a functional *narGHJI*-encoded NR as a result of loss of *narH* expression in the latter [31]. The reduced NR activity in the pathogenic *M. bovis* has been attributed to a SNP in the promoter region of *narG*, although the authors couldn't rule out that other SNPs in the coding region of the *narGHJI* operon also contributed to the differential activity between the two strains [19]. *M. smegmatis* retains a fully intact *narGHJI* operon and although there are sequence differences in *narG* between *M. tuberculosis* and *M. smegmatis*, the promoter mutation (-215 T>C) is not present in the latter, suggesting that the enzymes could have similar activities. It has recently been reported that *M. smegmatis* does harbour a T>C mutation in *narG*, however the authors refer to position -125 instead of -215 [32]. The different Griess assay results observed in *M. tuberculosis* and *M. smegmatis* could be due to a lack of extracellular nitrite as a result of either (i) more efficient nitrite reductase activity in *M. smegmatis* which leads to the complete reduction of nitrite to ammonia, (ii) reduced nitrite transporter activity in *M. smegmatis* or (iii) a combination of both. No intracellular nitrite was detected in *M. smegmatis* during this study, suggesting that it is able to completely assimilate nitrite.

NarGHI, encoded by the *narGHJI* operon has been well characterized in *E. coli* and is a respiratory enzyme which serves as the terminal electron acceptor during anaerobic respiration when nitrate is available. The second NR-encoding gene in *M. smegmatis* – *narB*, is a putative assimilatory NR-encoding gene [15]. NarB is a cytoplasmic enzyme that has been well characterized in *Cyanobacteria* where it facilitates growth on nitrate [26]. Based on the genome organisation, sequence similarities and predicted 3D structure, the *M. smegmatis narB* was expected to encode a functional NR. Although this cannot be ruled out, the inability to detect *narB* transcript in our study and the ability of strains lacking *narB* to assimilate nitrate suggests otherwise. Although *narG* transcript was detected in *M. smegmatis*, the strains lacking

narGHJI were also able to assimilate nitrate, suggesting that NarGHI is not the only enzyme involved in nitrate assimilation in contrast to the *M. tuberculosis* counterpart [10]. Furthermore, NarB and NarGHI were dispensable for anaerobic survival in the presence of nitrate, suggesting that either these enzymes are not required for nitrate reduction or that in the absence of each, an alternate enzyme can compensate for the loss.

Conclusions

Herein, we demonstrate that *narB* and *narGHJI* are individually and collectively dispensable for nitrate reduction in *M. smegmatis* under the conditions tested. Our analysis identified MSMEG_4206 as another putative assimilatory NR in *M. smegmatis*. Further analysis of this locus may provide novel insight into nitrogen metabolism in mycobacteria.

Methods

Construction and complementation of mutant strains. The upstream and downstream regions flanking the *narB* gene and *narGHJI* operon were amplified from wild type genomic DNA using the primer sets listed in Additional file 1: Table S1. The amplicons were digested with the appropriate restriction enzymes and a three-way cloning strategy was employed to generate intermediate constructs carrying the upstream and downstream regions of the *narB* gene and the *narGHJI* operon. The selectable – counter-selectable marker cassette was released from the pGOAL19 plasmid with *PadI*, and was subsequently ligated to the *PadI*-linearized p2nil intermediate constructs to generate the suicide vectors pΔ*narB* and pΔ*narGHJI*. *M. smegmatis* mutants were constructed by two-step allelic exchange mutagenesis as previously described [33]. The strains used and generated during this study are listed in Additional file 1: Table S2.

Bacterial growth. *E. coli* strains were grown in Luria Bertani liquid medium (LB) or solid medium (LA) supplemented with the appropriate antibiotics at concentrations of 100 µg/ml Ampicillin (Amp), 200 µg/ml Hygromycin (Hyg) or 50 µg/ml Kanamycin (Kan). *E. coli* strains used for the propagation of suicide vectors were grown at 30 °C to prevent DNA re-arrangements, while all other strains were grown at 37 °C. *M. smegmatis* strains were grown in Middlebrook 7H9 liquid medium (Difco) supplemented with Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco) or 0.085 % NaCl, 0.2 % glucose, 0.2 % glycerol and 0.05 % Tween80 and on Middlebrook 7H10 solid medium (Difco) supplemented with 0.085 % NaCl, 0.2 % glucose and 0.5 % glycerol. For nitrate assimilation and utilization assays, strains were grown in modified *M. phlei* minimal media (MPLN) made up of 5 g KH₂PO₄, 2 g sodium citrate, 0.6 g MgSO₄, 0.85 g NaNO₃, 20 ml glycerol and 5 ml 10 % tyloxapol in 1 L as previously described [14]. Media for *M. smegmatis* growth was supplemented with antibiotics at concentrations of 50 µg/ml Hyg and/or 25 µg/ml Kan where appropriate. Nitrate assimilation assays were carried out as previously described [14]. All liquid *E. coli* and *M. smegmatis* cultures were grown with shaking at 115 rpm.

Gene expression. RNA extractions and quantitative reverse transcriptase PCR (qRT PCR) were carried out as previously described [14]. Primers used for qRT PCR are listed in Additional file 1: Table S3. Expression of each gene was normalized against the *sigA* expression level.

Nitrate utilization. Nitrate utilization was measured using the Griess assay, which is based on the production of a red diazonium dye from the reaction of nitrite with naphthylamide under acidic conditions. This assay therefore relied on the availability of nitrite in the sample being tested. Nitrate reduction leads to the export of nitrite outside the cell, in which case a positive Griess result is observed. Alternatively, nitrite is further assimilated into ammonia or reduced to nitric oxide which leads to a negative Griess result. Therefore, a negative Griess result does not necessarily represent a lack of NR activity. Zinc rapidly reduces nitrate to nitrite [34]. A colour change upon the addition of zinc to a sample containing the Griess reagents measures the remaining nitrate, which allows for a measurement of nitrate depletion, thus confirming NR activity in an indirect way. This method can be exploited to quantify the amount of nitrite present in a sample by generating a nitrite standard curve with absorbance values recorded at $\lambda_{\text{max}} 530\text{nm}$. In addition, the rate of nitrate utilization can be determined. To investigate nitrate utilization *M. smegmatis* cultures were inoculated into MPLN and incubated until stationary phase. The modified Griess assay was performed at the time of inoculation and once cultures had reached the stationary phase, as described by Weber, Fritz [16] with minor modifications, Figure 2A. Briefly, 100 μl of 1 % sulfinilic acid and 100 μl of 1 % *N*-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) were added to 1 ml of culture and mixed thoroughly. In those samples wherein no colour change was observed, a few grains of zinc powder was added, mixed thoroughly and incubated for 5 min at room temperature. The samples were then clarified by centrifugation at $12\,470 \times g$ and the absorbance of the supernatants were measured at 530 nm and compared to known standards of nitrite (0.1 nM- 1000 nM).

Anaerobic growth. Cultures were grown in MPLN overnight to early-log phase. These cultures were used to inoculate, in duplicate, 2 ml MPLN in 10 ml culture tubes to OD 0.05. An aliquot was taken from each tube to determine the starting colony forming units (CFU/ml) and one set of tubes was incubated at 37 °C with no shaking. The other set of tubes were placed in the Oxoid AnaeroGen chamber which creates an anaerobic environment. Once the indicator showed that the environment was anaerobic, the chamber was incubated at 37 °C with no shaking. After 7 days an aliquot from each tube was plated to determine CFU.

Declarations

Ethics approval and consent to participate

This study did not involve human subjects and consequently, an ethics waiver was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (Reference number: W-CJ-160208-2)

Consent for publication

Not applicable

Abbreviations

Amp: Ampicillin, *bis*-MGD: *bis*-molybdopterin guanine dinucleotide, CFU: colony forming units, Hyg: Hygromycin, Kan: Kanamycin, MAC: *Mycobacterium avium* complex, MoCo: molybdenum cofactor, MPLN: modified *M. phlei* minimal medium, MQH2: menaquinol, NEDD: *N*-(1-naphthyl) ethylenediamine dihydrochloride, NR: Nitrate reductase, PMF: proton motive force, qRT PCR: quantitative reverse transcriptase polymerase chain reaction, SNP: single nucleotide polymorphism.

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Availability of data and materials

Data supporting the conclusions in this article are included within the article and additional supporting information. All datasets analyzed in this article are available from the corresponding author.

Authors' contributions

NCC and BDK conceived and designed experiments in this study. NCC performed all experimental procedures. NCC and BDK wrote and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Information Regarding Supplemental File

The supplemental file (downloadable below) contains the following items:

Supplementary tables.

Table S1: Primers used for the construction of suicide vectors

Table S2: Plasmids and strains used and generated during this study

Table S3: Primers used for qRT PCR

Figure S1: Genotypic confirmation of $\Delta narB$. (A) Schematic representation of genomic maps of wild type and mutant *narB* regions. Restriction enzymes, probes and expected fragment sizes for southern blot confirmation are depicted. (B) Southern blot with upstream probe (US). Lane 1: Marker λ IV, Lane 2: Empty, Lane 3: *NotI* digested wild type DNA, Lane 4: *NotI* digested $\Delta narB$ DNA, Lane 5: Empty, Lane 6: *SacI* digested wild type DNA, Lane 7: *SacI* digested $\Delta narB$ DNA. (C) Southern blot with downstream probe (DS). Lane 1: Marker λ IV, Lane 2: Empty, Lane 3: *NotI* digested wild type DNA, Lane 4: *NotI* digested $\Delta narB$ DNA, Lane 5: Empty, Lane 6: *NcoI* digested wild type DNA, Lane 7: *NcoI* digested $\Delta narB$ DNA.

Figure S2: Genotypic confirmation of $\Delta narGHJI$ and $\Delta narB \Delta narGHJI$. (A) Schematic representation of genomic maps of wild type and mutant *narGHJI* regions. Restriction enzymes, probes and expected fragment sizes for southern blot confirmation are depicted. (B) Southern blot with upstream probe (US). Lane 1: Marker λ IV, Lane 2: *BamHI* digested wild type DNA, Lane 3: *BamHI* digested $\Delta narGHJI$ DNA, Lane 4: *BamHI* digested $\Delta narB \Delta narGHJI$ DNA. (C) Southern blot with downstream probe (DS). Lane 1: Marker λ IV, Lane 2: *PstI* digested wild type DNA, Lane 3: *PstI* digested $\Delta narGHJI$ DNA, Lane 4: *PstI* digested $\Delta narB \Delta narGHJI$ DNA, Lane 5: Empty, Lane 6: *MluI* digested wild type DNA, Lane 7: *MluI* digested $\Delta narGHJI$ DNA, Lane 8: *MluI* digested $\Delta narB \Delta narGHJI$ DNA.

Figure S3: Growth kinetics of *M. smegmatis* strains grown in rich 7H9 medium. Cultures were grown in 7H9 supplemented with OADC and tyloxapol with absorbance readings recorded daily for five days. A representative of a single biological replicate is shown.

Figure S4: The expression analysis of strains grown in 7H9 or MPLN. (A) Transcript abundance of *sigA* in 7H9. (B) Transcript abundance of *sigA* in MPLN. (C) The normalized expression of *narG* in MPLN. Strains

were grown to OD600 0,8-1 in MPLN liquid media. Expression was normalized against the house-keeping *sigA* and data from three independent experiments is depicted with standard error bars.

Figure S5: Sequence alignments of *narG* promoter regions. Grey text shows the *narG* upstream promoter region. Black text depicts nucleotides of the *narG* gene sequence. Green text represents the start codon of each homologue while the red uppercase text depicts the promoter mutation site with which differing NR activity is associated. Ecoli: *Escherichia coli*, Mleprae: *Mycobacterium leprae*, Msmeg: *Mycobacterium smegmatis*, *M. tuberculosis*: *Mycobacterium tuberculosis*, BCG: *Mycobacterium bovis* BCG.

Figure S6: Sequence alignments of NarG from different organisms. The amino acids shaded in different colours represent different secondary structures and ligand binding sites. Ecoli: *Escherichia coli*, MSMEG: *Mycobacterium smegmatis*, M.tuberculosis: *Mycobacterium tuberculosis*, M. africanum: *Mycobacterium africanum*, MTBC: *Mycobacterium tuberculosis complex*, M. canetti: *Mycobacterium canettii*, M. bovis: *Mycobacterium bovis* BCG.

Figure S7: The genome organization of MSMEI_4108, an additional putative assimilatory NR in *M. smegmatis*. (A) Shown is the organization of a region of the *M. smegmatis* chromosome as annotated on two different databases, namely Mycobrowser and EcoGene-RefSeq. (B) Shown is a table comparing the gene coordinates and annotations from the two databases. (C) The protein domain architecture of MSMEI_4108 according to KEGG is shown.

Figures

Figure 1

Nitrate reductase forms part of the Nitrogen cycle and is MoCo-dependent. (A) A schematic representation of an example of the Nitrogen cycle in prokaryotes is shown. Different prokaryotic organisms are able to catalyze each of the reactions depicted. Figure adapted from [35]. (B) *M. smegmatis* NarB (Accession: A0QW69) and NarG (Accession: A0R2J8) are MoCo-dependent. The diagram shows the amino acid coordinates of the MoCo-dependent defining domains in both enzymes, as shown on InterPro (<https://www.ebi.ac.uk/interpro/protein/>).

Figure 2

A modified Griess assay used to measure nitrate utilization of *M. smegmatis* in MPLN. (A) The conventional Griess assay involves the visualization of samples following the addition of the Griess reagents. A pink colour represents positivity for nitrate reduction, while no colour change is generally considered negative. In the modified Griess assay, a second step is included for samples that had no colour change during the conventional assay. Upon the addition of zinc, a colour change to pink is

indicative of no NR activity and the sample is then classified as NR negative. No colour change after the addition of zinc represents complete nitrate utilization and thus NR positivity. (B) Cultures were grown at 37° C with shaking at 115 rpm. The modified Griess assay was performed to determine nitrate utilization on Day 0, Day 1 and Day 6. (C) Quantification of extracellular and intracellular nitrate over time, as a function of nitrite detection using the modified Griess assay. The Students t-test was used to compare between strains. *P=0.01

Figure 3

Analysis of nitrate assimilation in minimal media for mutant strains. (A) Growth curve analysis of $\Delta narB$, $\Delta narGHJI$ and $\Delta narB \Delta narGHJI$ in nitrate minimal media Both genes are dispensable for growth under these conditions. Averages of at least three independent experiments were plotted for each strain with standard errors included. (B) Survival of *M. smegmatis* under anaerobic conditions in the presence of nitrate. Shown is the comparison of the aerobic versus anaerobic biomass fold change after 7 days. The fold change was determined by dividing the CFU obtained on Day 7 by the CFU obtained on Day 0 for each strain. The averages of three independent experiments are plotted with standard errors depicted.

Figure 4

Nitrate assimilation in mycobacteria. (A) Genes encoding proteins for each step of the pathway are shown for *M. tuberculosis* and *M. smegmatis*. Gene annotations were obtained from Mycobrowser and reference [26]. (B) NarB domain architecture obtained from InterPro. (C) Predicted crystal structure of NarB (Green) overlaid with the best fit template, the periplasmic NR from *Desulfovibrio desulfuricans*

(Red). The crystal structure prediction was done using i-TASSER and the NarB amino acid sequence obtained from SmegmaList.

Supplementary Files

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