

The roles of the nitrate reductase NarGHJI, the nitrite reductase NirBD and the response regulator GlnR in nitrate assimilation of *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis can utilize various nutrients including nitrate as a source of nitrogen. Assimilation of nitrate requires the reduction of nitrate via nitrite to ammonium, which is then incorporated into metabolic pathways. This study was undertaken to define the molecular mechanism of nitrate assimilation in *M. tuberculosis*. Homologues to a *narGHJI*-encoded nitrate reductase and a *nirBD*-encoded nitrite reductase have been found on the chromosome of *M. tuberculosis*. Previous studies have implied a role for NarGHJI in nitrate respiration rather than nitrate assimilation. Here, we show that a *narG* mutant of *M. tuberculosis* failed to grow on nitrate. A *nirB* mutant of *M. tuberculosis* failed to grow on both nitrate and nitrite. Mutant strains of *Mycobacterium smegmatis* mc²155 that are unable to grow on nitrate were isolated. The mutants were rescued by screening a cosmid library from *M. tuberculosis*, and a gene with homology to the response regulator gene *glnR* of *Streptomyces coelicolor* was identified. A Δ *glnR* mutant of *M. tuberculosis* was generated, which also failed to grow on nitrate, but regained its ability to utilize nitrate when *nirBD* was expressed from a plasmid, suggesting a role of GlnR in regulating *nirBD* expression. A specific binding site for GlnR within the *nirB* promoter was identified and confirmed by electrophoretic mobility shift assay using purified recombinant GlnR. Semiquantitative reverse transcription PCR, as well as microarray analysis, demonstrated upregulation of *nirBD* expression in response to GlnR under nitrogen-limiting conditions. In summary, we conclude that NarGHJI and NirBD of *M. tuberculosis* mediate the assimilatory reduction of nitrate and nitrite, respectively, and that GlnR acts as a transcriptional activator of *nirBD*.

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INTRODUCTION

Mycobacterium tuberculosis has only limited access to nutrients in infected tissue (Munoz-Elias & McKinney, 2006). Nitrate, however, is available in infected tissue, as it

is generated spontaneously from nitric oxide (NO), the product of nitric oxide synthase (Bogdan, 2001). Assimilation of nitrogen into mycobacterial metabolism is essential for the survival of *M. tuberculosis* *in vitro* and *in vivo*. Assimilation of nitrate by *M. tuberculosis* was reported more than 40 years ago (DeTurk & Bernheim, 1958; Hedgecock & Costello, 1962; Virtanen, 1960). However, its molecular basis remains unknown.

Abbreviation: EMSA, electrophoretic mobility shift assay.

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The first step in nitrate assimilation is the reduction of nitrate (NO_3^-) to nitrite (NO_2^-). In *Bacillus subtilis*, assimilatory nitrate reduction is mediated by a distinct cytoplasmic enzyme. A different nitrate reductase, encoded by *narGHJI*, is membrane bound and serves respiratory functions. NarJ assembles the subunits NarG, H and I to the functional nitrate reductase. In *B. subtilis*, its expression is typically induced under anaerobic conditions (Gennis & Stewart, 1996; Nakano & Zuber, 1998; Ogawa *et al.*, 1995). Analysis of the genome of *M. tuberculosis* revealed genes with 30% and 50% homology at the amino acid level to those found in the NarGHJI of *B. subtilis*. We previously reported that NarGHJI of *M. tuberculosis* mediates reduction of nitrate, not only under anaerobic, but also under aerobic conditions (Stermann *et al.*, 2004; Weber *et al.*, 2000). This is in accordance with data from Sohaskey & Wayne (2003), who reported that expression of *narGHJI* was not dependent on anaerobiosis. As genes with homology to a distinct assimilatory nitrate reductase were not identified in the genome of *M. tuberculosis*, the initial goal of this study was to examine whether NarGHJI of *M. tuberculosis* has an assimilatory function.

The second step in nitrate assimilation is reduction of nitrite (NO_2^-) to ammonium (NH_4^+). The genome of *M. tuberculosis* revealed genes with 40% and 50% homology, at the amino acid level, to *nirBD* of *Escherichia coli* (Cole *et al.*, 1998). This operon encodes a sirohaem-dependent NADH-nitrite reductase, an enzyme that typically mediates nitrate assimilation in various bacteria and fungi (Lin & Stewart, 1998). Only in *E. coli* and in other enterobacteria is *nirBD*-encoded nitrite reductase enzyme induced under anaerobic conditions, and it does not function as an assimilatory nitrite reductase but detoxifies nitrite that accumulates from nitrate respiration (Gennis & Stewart, 1996). We included analysis of *nirB* of *M. tuberculosis* with respect to its assimilatory function in this study.

Regulation of nitrate assimilation may be subject to a general nitrogen regulation (*ntr*) system, which depends on the presence of the preferred nitrogen source. It might also be pathway specific, and is then controlled by the availability of nitrate. In two actinobacteria, *Corynebacterium* and *Streptomyces*, the transcriptional regulation of nitrogen assimilation has been analysed. In *Corynebacterium glutamicum*, the regulator protein AmtR is responsible for this process (Beckers *et al.*, 2005; Burkovski, 2007). In *Streptomyces coelicolor*, GlnR controls regulation of genes involved in nitrogen metabolism (Fink *et al.*, 2002; Tiffert *et al.*, 2008; Wray *et al.*, 1991). Regulation of nitrate assimilation has not been specifically addressed in either species, or in *M. tuberculosis*.

In the present study, assimilation of nitrate was measured as growth on nitrate as a sole source of nitrogen. We found robust growth of *M. tuberculosis* wild-type, whereas neither a *narG* mutant nor a *nirB* mutant grew under these conditions, suggesting assimilatory functions for both genes. We also identified GlnR as a regulator for expression of the *nirBD*-encoded assimilatory nitrite reductase.

METHODS

Strains and cultures. *Mycobacterium tuberculosis* H37Rv and *Mycobacterium smegmatis* mc²155 (Snapper *et al.*, 1990) were cultured in 7H9 broth or on 7H10 plates (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80 for all liquid media, and 10% ADS (0.5% Bovine Albumin Fraction V, 0.2% glucose, 140 mM NaCl) unless indicated otherwise. Assimilation of nitrate or nitrite was tested under nitrogen-limiting conditions using a basal medium, if necessary solidified with 1.5% agar, supplemented with KNO_3 , KNO_2 or NH_4Cl as sole source of nitrogen, 0.5 mM MgCl_2 , 0.5 mM CaCl_2 , 10% ADS, 0.2% glycerol and 0.05% Tween 80 (1 l of the basal medium contained 1 g KH_2PO_4 , 2.5 g Na_2HPO_4 , 2 g K_2SO_4 and 2 ml of trace elements; 1 l of trace elements contained 40 mg ZnCl_2 , 200 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 10 mg $\text{CuCl}_2 \cdot 4\text{H}_2\text{O}$, 10 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 10 mg $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ and 10 mg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$). To test for accumulation of nitrite, as a nitrogen-limiting medium a modified Proskauer Beck was used that contained, per litre, 5 g KH_2PO_4 , 0.6 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g $\text{C}_6\text{H}_6\text{MgO}_7$, 15 ml glycerol, 2.5 ml 20% Tween 80 and 10 mM nitrate.

Generation of mutants in *M. tuberculosis*. Construction of the *narG* mutation in *M. tuberculosis* has been described previously (Stermann *et al.*, 2003). Cosmids from a genomic library of *M. tuberculosis* (Bange *et al.*, 1999) carrying *nirB* or *glnR* were obtained by colony hybridization. A 1352 bp fragment and a 323 bp fragment were deleted in *nirB* and *glnR*, respectively, and appropriate fragments were cloned into the *PacI* site of the previously described pYUB657 (Pavelka & Jacobs, 1999). *M. tuberculosis* was transformed and screened for clones resistant to hygromycin. Sucrose was used for counter-selection, as described previously, to select clones in which double crossovers had occurred (Pavelka & Jacobs, 1999).

For complementation experiments, an 8652 bp *EcoRV* fragment containing the *narGHJI* gene cluster, a 3959 bp *MluI* fragment containing the *nirBD* gene cluster, and a 1582 bp *EcoRV*–*SmaI* fragment containing *glnR* were chosen. The fragments were subcloned from cosmids into the promoterless pMV306 vector. In addition, the 1087 bp *HindIII*–*BamHI* fragment containing *glnR* of *S. coelicolor* was inserted into the *HpaI* site of pMV261.

Testing for growth on nitrate, nitrite or ammonium. Bacteria were cultured in 7H9 broth to an OD_{600} between 0.7 and 1.0. The cultures were washed with nitrogen-limiting medium before the OD_{600} was adjusted to 0.1. Cultures were incubated at 37 °C with good aeration. Medium was supplemented with 10 mM nitrate, 1 mM nitrite or 10 mM ammonium chloride and growth was tested by measuring the OD_{600} .

Isolation and complementation of *M. smegmatis* mutants, and mapping of the corresponding mutations. Ethyl methane sulphonate-mutagenized *M. smegmatis* mc²155 clones (McKinney *et al.*, 2000) were cultured on nitrogen-limiting agar containing either no nitrate or 10 mM KNO_3 , or on fully supplemented 7H10 plates. Two *M. smegmatis* strains, #2009 and #5192, which were unable to grow with 10 mM KNO_3 , were transformed with an integrating cosmid library of *M. tuberculosis* H37Rv. Cosmid pIW32 was recovered from a complemented *M. smegmatis* clone. From this cosmid, a *NotI* fragment containing Rv0816c to Rv0820 was subcloned into the promoterless integrating mycobacterial shuttle vector pMV306 (Stover *et al.*, 1992), resulting in the construct pBA4. This construct was further subcloned into the construct pBA7 using *EcoRV*, and into pBA8 using *NruI* and *PvuI*. pBA7 and pBA8 contained only Rv0818 and its own promoter.

Accumulation of nitrite. Bacteria were adjusted to an OD_{600} of 0.2 in nitrogen-limiting medium containing 10 mM KNO_3 and incu-

bated at 37 °C with good aeration. At indicated time points, 100 µl sulfanilic acid and 100 µl *N,N*-dimethyl-1-naphthylamine (API system, bioMérieux) was added to 1 ml culture. Absorbance of the supernatant was measured at 440 nm and quantified, relating the values to a standard curve of nitrite.

Expression of *nirBD* under the control of the *hsp60* promoter in the *glnR* *M. tuberculosis* mutant. The constitutive *hsp60* promoter from pMV261 was fused to *nirBD*. For the initial PCR, 261Fus#1 (5'-TGTGGTGGCATCCGTGGC-3') and 261Fus#2 (5'-CGCGAACTCCCAGCCGTAGGCATTGCGAAGTGATTCCTCC-3') were used, whereas the second PCR was performed with *nirBFus*#1 (5'-TCCG-GAGGAATCACTTCGCAATGCCTACGGCTGGGAGTTCG-3') and *nirBFus*#2 (5'-GACTCGATCGACTCGGTACCG-3'). PCR products were used as a template for a self-primed PCR with the terminal primer pair 261Fus#1 and *nirBFus*#2. This fusion molecule was cut with *KpnI* and cloned via the *KpnI* sites into the promoterless, integrating mycobacterial plasmid pMV306 containing *nirBD* on a *MluI* fragment. This construct was transformed into the *glnR* mutant. Growth curves were obtained by culturing the respective strains as described above.

Preparation of RNA from *M. tuberculosis*. Δ *glnR* *M. tuberculosis* and the wild-type strain were cultured in 7H9 to the mid-exponential growth phase. Then, the cells were washed with nitrogen-limiting medium supplemented with 5 mM KNO₃ and incubated for 18 h or 48 h before RNA was extracted. Cultures were incubated with an equal volume of GTC buffer [5 M guanidinium thiocyanate, 0.5% (w/v) *n*-laurylsarcosine, 0.7% (w/v) sodium citrate, 0.7% β -mercaptoethanol], centrifuged and resuspended in 1 ml Trizol reagent (Invitrogen). The cells were disrupted and extracted once with CHCl₃, and re-extracted using the RNeasy Mini-kit (Qiagen). The optional DNase I on-column digest was extended to 1 h and an additional DNase I digest (NEB) was performed with the eluate for 45 min. After that, RNA was purified again with the RNeasy Mini-kit.

RT-PCR of RNA from *M. tuberculosis*. RNA was extracted after incubation of bacteria in nitrogen-limiting medium for 18 h. cDNA, which was generated by random priming of 2 µg RNA, was diluted 1:20 and 1:5. PCRs were performed with 2 µM of each of the following primers: *qrt_sigA_mtu3* (as control) (5'-CACGCAAGGACGCCGAATC-3'), *qrt_sigA_mtu4* (as control) (5'-TACAGGCCAGCCTCGATCCG-3'), *qrt_nirB_mtu3* (5'-GTCCCGTTTCGTTTCC-TTCG-3') and *qrt_nirB_mtu4* (5'-CGCGGGATACCAATGGACAC-3'). The PCR conditions were 95 °C for 5 min; then 35 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 20 s; and finally 5 min at 72 °C.

Expression of *glnR* from *M. tuberculosis* in *E. coli*. *GlnR* from *S. coelicolor* was expressed and purified as described previously (Tiffert *et al.*, 2008). *glnR* of *M. tuberculosis* was amplified with the primers 5'-CATATGTTGGAGTTATTACTGCTGAC-3' and 5'-AAGCTTTC-ATTTTCGAACTGCGGGTGGCTCCACTGACTGCGCAACGGGTC-3' by adding a sequence encoding a C-terminal StrepII-tag. The product was subcloned into pJOE2775, under the control of the P_{rham} promoter. Gene expression was induced with 0.2% rhamnose. Cells were harvested, washed with a solution of 50 mM Tris, 150 mM NaCl, 10 mM MgCl₂, 5% glycerol and 10 mM β -mercaptoethanol, pH 8, and broken by French press (American Instruments). Then, Complete protease inhibitor cocktail (Roche) was added to the mixture. Cell debris and membrane fractions were removed by centrifugation (45 min; 15 000 r.p.m.; 4 °C). Purification of *GlnR*-Strep-tagged proteins from the soluble fraction was performed at 7 °C with StrepTactin Superflow gravity flow columns (IBA).

Electrophoretic mobility shift assay (EMSA). DNA fragments containing *M. tuberculosis* H37Rv upstream regions were PCR-

amplified using genomic DNA of *M. tuberculosis* H37Rv as template. The primers 5'-AGCCAGTGGCGATAAGCCACACCGGACGCGA-CCAC-3' and 5'-AGCCAGTGGCGATAAGGGTCTGAGGGTATGAGGGGC-3' for the upstream region of *nirB* were used. The underlined 5'-extensions have no homology to the template and were used for PCR labelling. The DNA fragments were purified using S-400 Microspins (GE Healthcare). Fragment labelling was performed via PCR using the Cy5-labelled primer 5'-AGCCAGTGGCGATAAG-3'.

Two nanograms of DNA was used in each EMSA reaction. For *GlnR* mixed with the *nirB* upstream region of *M. tuberculosis*, 16 µM purified *GlnR* was used. The DNA and protein were incubated in a reaction buffer (50 mM Tris, 100 mM NaCl, 10 mM β -mercaptoethanol, pH 8) for 10 min at 24 °C. The fragments were separated on 2% TAE agarose gels. DNA bands were visualized by fluorescence imaging using a Typhoon Trio+ Variable Mode Imager (GE Healthcare).

Transcriptome analysis. *M. tuberculosis* wild-type and mutant were cultured in 7H9 to an OD₆₀₀ of between 0.7 and 1.0. Bacteria were harvested by centrifugation and washed with nitrogen-limiting medium containing 5 mM KNO₃. The OD₆₀₀ was adjusted to approximately 0.7 with nitrogen-limiting medium supplemented with 5 mM KNO₃ and cultures were incubated for about 18 h and 48 h at 37 °C with agitation before RNA was extracted as described above.

Equal amounts of RNA (between 3 and 10 µg) were subjected to reverse transcriptase reaction following the Affymetrix protocol. The cDNA was purified using the QIAquick PCR Purification. Custom-made microarrays for *M. tuberculosis* H37Rv were used for transcriptome analysis (Affymetrix). The chip contains 44 033 probe pairs representing 4003 coding sequences, and 7902 probe pairs representing 1413 intergenic regions. Hybridization was done following the Affymetrix protocol. Microarrays were stained in the GeneChip Fluidics Station 450 according to the modified FlexMidi_euk2v3 program for *Pseudomonas aeruginosa*. Analysis of microarray data was performed using the Affymetrix GCOS 1.4 software. For normalization, all array experiments were scaled to a target intensity of 150, otherwise using the default values of GCOS 1.4. Signal intensities obtained from the individual measures for mutant *M. tuberculosis* and the wild-type strain at indicated time points were grouped and compared using *t*-test statistics. The entire dataset was submitted in MIAME-format to the GEO database (<http://www.ncbi.nlm.nih.gov/projects/geo/>), accession number GSE13246.

RESULTS

Growth of Δ *narG* *M. tuberculosis* and Δ *nirB* *M. tuberculosis* on nitrate and nitrite

Previously, we constructed a mutant of *M. tuberculosis* that carried a deletion in the *narG* gene (Stermann *et al.*, 2003). Constitutive expression of *narGHJI* in *M. tuberculosis* has been reported, but the issue of whether the *narGHJI*-encoded nitrate reductase provides assimilatory functions has not been addressed. Thus, we tested growth of the *narG* mutant in nitrogen-limiting medium, with nitrate as the sole nitrogen source. The mutant had lost the ability to assimilate nitrate. Introduction of *narGHJI* from *M. tuberculosis* into the mutant restored the wild-type phenotype (Fig. 1a). Growth of the Δ *narG* mutant on

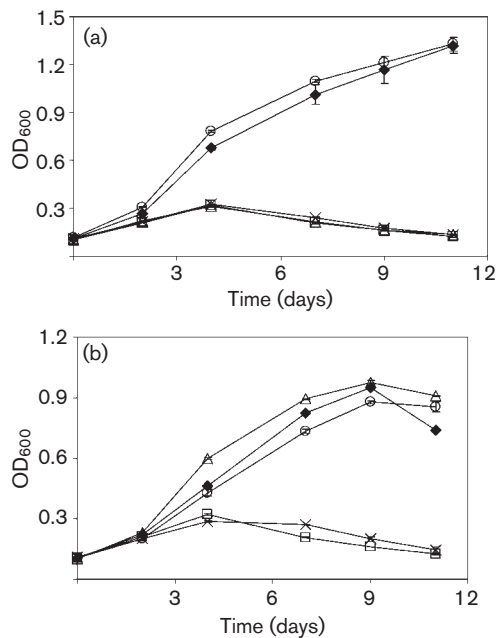


Fig. 1. Growth of $\Delta narG$ *M. tuberculosis* and $\Delta nirB$ *M. tuberculosis* on nitrate and nitrite. Bacteria were cultured in nitrogen-limiting medium containing (a) 10 mM nitrate or (b) 1 mM nitrite as the only nitrogen source. Aliquots were tested for growth by measuring OD₆₀₀. Cultures were kept with good aeration. The following strains were compared: wild-type *M. tuberculosis* (◆), $\Delta narG$ *M. tuberculosis* (△) or $\Delta nirB$ *M. tuberculosis* (×), $\Delta narG$ *M. tuberculosis* :: *narGHJI* (○ in a) and $\Delta nirB$ *M. tuberculosis* :: *nirBD* (○ in b). Wild-type *M. tuberculosis* was cultured in nitrogen-limiting medium without nitrate or nitrite as a control (□).

nitrite was unimpaired (Fig. 1b). These results suggest that *narGHJI* mediates nitrate assimilation in *M. tuberculosis*.

Nitrate assimilation requires the concerted action of a nitrate and nitrite reductase, followed by incorporation of ammonium into cellular metabolism via glutamine synthetase and glutamate synthase. Sequencing of *M. tuberculosis* revealed homology to only one nitrite reductase encoded by *nirB* and *nirD* (Rv0252, Rv0253) (Cole *et al.*, 1998). As no functional studies have been conducted to date, we constructed a deletion mutant of *nirB* in *M. tuberculosis* to elucidate its role in the second step of assimilation of nitrate. A 1352 bp fragment within *nirB* was deleted from the chromosome, and growth of $\Delta nirB$ *M. tuberculosis* (Fig. 1b) was compared to that of the wild-type in nitrogen-limiting medium, containing 1 mM nitrite as the sole source of nitrogen. The mutant had lost its ability to assimilate nitrite. Cloning of *nirBD* from *M. tuberculosis* into the mutant restored the wild-type phenotype. Growth of $\Delta nirB$ *M. tuberculosis* was also tested in nitrogen-limiting medium with nitrate as the sole source of nitrogen. Again, no growth was detected (Fig. 1a). Thus, *nirB* encodes the assimilatory nitrite reductase of *M. tuberculosis*.

Selection and characterization of mutants of *M. smegmatis* defective in growth on nitrate

An ethyl methane sulphonate (EMS) mutagenized *M. smegmatis* mc²155 library was screened for mutants that were unable to grow on nitrogen-limiting agar, containing 10 mM nitrate. We found two mutants, #2009 and #5192, that failed to grow on nitrate and nitrite, but showed robust growth on ammonium. We were able to complement these two mutants with an integrating cosmid library from *M. tuberculosis* for growth on nitrate (data not shown). Cosmids were isolated from complemented mutants, and sequence analysis showed that cosmids from both mutants had overlapping DNA fragments, including Rv0813c to Rv0839, corresponding to position 907 338 to 936 389 of the *M. tuberculosis* genome. Cosmids recovered from the mutant #2009 also complemented the mutant #5192, and vice versa. These findings suggest that the mutations in #2009 and #5192 mapped to adjacent genes or the same gene. The complementing DNA fragment was narrowed down to Rv0818, which was able to rescue the *M. smegmatis* mutant #2009 for growth on nitrate (Fig. 2). Mutant #2009 grew less well on fully supplemented 7H10 medium. At present, the reason for this minimal growth defect remains unclear. It might be due to an unspecific effect as a result of the EMS mutagenesis in *M. smegmatis*, or to pleiotropic effects of the mutation. Rv0818 also complemented *M. smegmatis* mutant #5192 (data not shown). A search of the TubercuList database (<http://genolist.pasteur.fr/TubercuList/>) showed that Rv0818 shares 61.5% identity with the transcriptional regulator GlnR of *S. coelicolor*. GlnR is the global regulator of nitrogen assimilation in *S. coelicolor*.

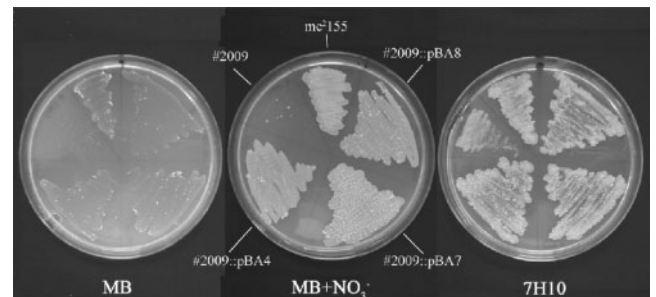


Fig. 2. Complementation of a *M. smegmatis* mutant for growth on nitrate. Strains were cultured on nitrogen-limiting agar (here referred to as MB) containing 10 mM nitrate (MB+NO₃⁻, middle), nitrogen-limiting agar without nitrate (MB, left), and rich 7H10 agar (7H10, right). Going from the top counterclockwise, the following strains were used: wild-type *M. smegmatis* mc²155, the nitrate assimilation mutant of *M. smegmatis* (#2009), the nitrate assimilation mutant of *M. smegmatis* #2009 transformed with a chromosomal fragment from *M. tuberculosis* containing ORFs Rv0816c to Rv0820 (#2009 :: pBA4), and the nitrate assimilation mutant of *M. smegmatis* #2009 transformed with ORF Rv0818 only (#2009 :: pBA7, #2009 :: pBA8).

Growth of a $\Delta glnR$ mutant of *M. tuberculosis* on nitrate

We generated a $\Delta glnR$ (Rv0818) mutant in *M. tuberculosis* by introducing an unmarked 323 bp deletion within *glnR* on the chromosome of *M. tuberculosis*. We compared growth of $\Delta glnR$ *M. tuberculosis* with that of the wild-type in nitrogen-limiting medium, with 10 mM nitrate as the sole source of nitrogen. The $\Delta glnR$ mutant of *M. tuberculosis* was unable to grow on nitrate. Expression of *glnR* on a plasmid in the $\Delta glnR$ mutant restored the wild-type phenotype (Fig. 3). Likewise, *glnR* from *S. coelicolor* also complemented $\Delta glnR$ *M. tuberculosis* for growth on nitrate (Fig. 3).

The inability to reduce nitrite may result in the secretion of nitrite and rising levels in the medium. To elucidate the role of GlnR in nitrate assimilation of *M. tuberculosis*, we compared nitrite levels during culture of *M. tuberculosis* wild-type, $\Delta nirB$ *M. tuberculosis* and $\Delta glnR$ *M. tuberculosis*, as well as the complemented mutants. Strains were cultivated in nitrogen-limiting medium, with 10 mM nitrate as the sole source of nitrogen. *M. tuberculosis* wild-type did not accumulate nitrite, because nitrate is reduced to nitrite by *narGHJI*-encoded nitrate reductase, and further reduced to ammonium by *nirBD*-encoded nitrite reductase. Both mutants, however, accumulated nitrite when cultured on nitrate (Fig. 4). To test whether the *glnR* deletion affected the nitrate/nitrite assimilatory pathway beyond nitrite reduction, $\Delta glnR$ *M. tuberculosis* was cultured on ammonium. The $\Delta glnR$ *M. tuberculosis* was phenotypically indistinguishable from *M. tuberculosis* wild-type, as both strains utilized ammonium as a sole source of nitrogen (Fig. 3). Even though at this point we

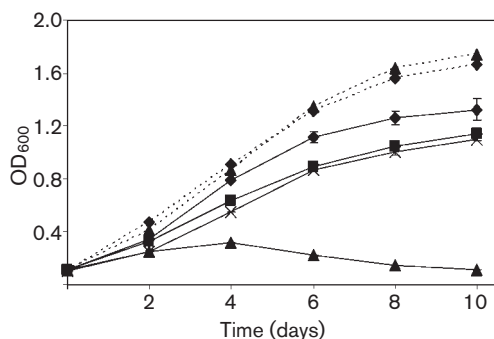


Fig. 3. Growth of $\Delta glnR$ mutant of *M. tuberculosis* on different sources of nitrogen. *M. tuberculosis* $\Delta glnR$ was cultured in nitrogen-limiting medium containing 10 mM nitrate (continuous lines) or 10 mM ammonium (dotted lines) as the only nitrogen source. Aliquots were tested for growth by measuring the OD_{600} . Cultures were kept with good aeration. The following strains were compared: wild-type *M. tuberculosis* (◆), $\Delta glnR$ *M. tuberculosis* (▲), $\Delta glnR$ *M. tuberculosis*::*glnR*_{MTB} (×), $\Delta glnR$ *M. tuberculosis*::*glnR*_{SCO} (■).

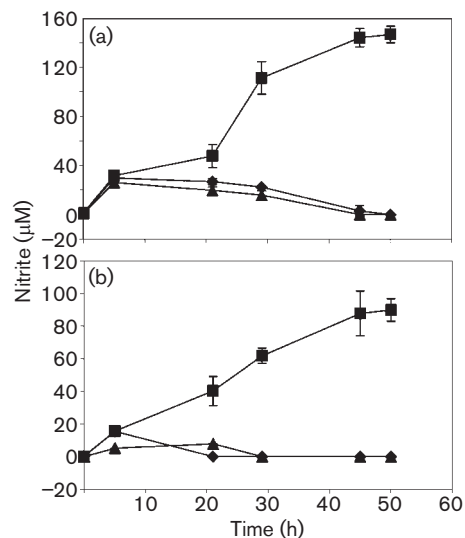


Fig. 4. Nitrite levels during culture of (a) $\Delta nirB$ *M. tuberculosis* and (b) $\Delta glnR$ *M. tuberculosis* in nitrogen-limiting medium containing 10 mM nitrate. At indicated time points aliquots were tested for accumulation of nitrite. The following strains were compared: (a) *M. tuberculosis* wild-type (▲), *M. tuberculosis* carrying a deletion of *nirB* (■), *M. tuberculosis* carrying a deletion of *nirB* transformed with *nirB* from *M. tuberculosis* (◆); (b) *M. tuberculosis* wild-type (▲), *M. tuberculosis* carrying a deletion of *glnR* (■), *M. tuberculosis* carrying a deletion of *glnR* transformed with *glnR* from *M. tuberculosis* (◆).

could not exclude that the accumulation of nitrite might be caused also by other factors such as transport of nitrite, we hypothesized that the $\Delta glnR$ mutant reduces nitrate to nitrite, but is blocked in further reduction of nitrite to ammonium.

Complementation of the *M. tuberculosis glnR* deletion mutant with *nirBD*

In order to confirm that the lack of nitrate assimilation of the *glnR* mutant is due to the lack of upregulation of *nirBD* in this mutant strain, we expressed *nirBD* under the transcriptional control of the constitutive *hsp60* promoter in the *glnR* mutant. We compared growth on nitrate as a sole source of nitrogen of the *glnR* mutant strain and the mutant complemented with *nirBD*; growth of the wild-type in the same medium without nitrate was used as a control. The *glnR* mutant strain was not able to grow on nitrate and behaved like the wild-type control cultured without nitrate (Fig. 5). The *glnR* mutant strain complemented with *nirBD* showed an extended lag and acceleration phase, then replicated stably from day 4 until day 16 (Fig. 5). These results show that expression of *nirBD* allows the *glnR* mutant to use nitrate as a sole source of nitrogen.

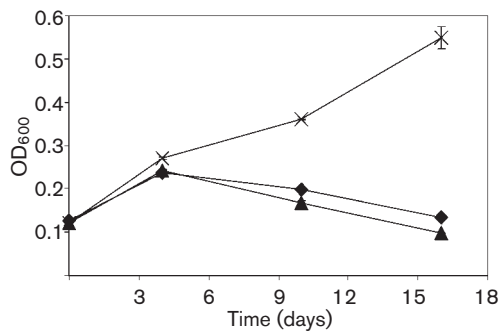


Fig. 5. Growth on nitrate of the *glnR* mutant of *M. tuberculosis* transformed with *nirBD* expressed from a heterologous promoter. Bacteria were cultured in nitrogen-limiting medium containing 10 mM nitrate as the only nitrogen source. Aliquots were tested for growth by measuring the OD₆₀₀. Cultures were kept with good aeration. The following strains were compared: $\Delta glnR$ *M. tuberculosis* (▲), $\Delta glnR$ *M. tuberculosis* :: *nirBD* under control of the heterologous hsp60 promoter (×), and the wild-type cultured without nitrogen as a control (◆).

GlnR directly controls *nirB* expression

Tiffert *et al.* (2008) showed that expression of *nirB* was lost in a *glnR* mutant that had been generated by chemical mutagenesis of *S. coelicolor*. In the present study a putative GlnR-binding site within *nirB* of *M. tuberculosis* was analysed by performing an EMSA with purified GlnR of *M. tuberculosis* as well as purified GlnR of *S. coelicolor*. For purification of GlnR of *M. tuberculosis*, the *glnR* gene was fused to a StrepII-tag. An N-terminal Strep-*glnR* fusion gene could not be expressed in *E. coli* XL1 blue, as assessed by SDS-PAGE and Western blot analysis (data not shown). However, by using a C-terminal *glnR*-Strep construct, *glnR* was successfully overexpressed and purified (data not shown). To investigate the ability of GlnR to bind to the *nirB* upstream region agarose EMSA was performed. Specific binding of GlnR of *M. tuberculosis* and *S. coelicolor* to the *nirB* upstream region of *M. tuberculosis* was observed (Fig. 6a, b).

To analyse the impact of GlnR on *nirB* expression, semiquantitative reverse transcription-PCR (RT-PCR) was performed, using wild-type *M. tuberculosis* H37Rv and the $\Delta glnR$ mutant strain. RNA was isolated from cultures that were grown in complex medium and subsequently transferred to nitrogen-limiting medium containing 5 mM KNO₃ for 18 h. Internal primers for the GlnR-regulated gene *nirB* were utilized (*sigA* was used as control, Fig. 6c). In contrast to the parent strain of *M. tuberculosis*, expression of the *nirB*-encoded nitrite reductase was greatly reduced in the $\Delta glnR$ mutant (Fig. 6c).

To verify these results, we performed whole-genome expression profiling comparing the *glnR* mutant of *M. tuberculosis* and the parent strain cultured in nitrogen-limiting medium with 5 mM nitrate as the sole source of

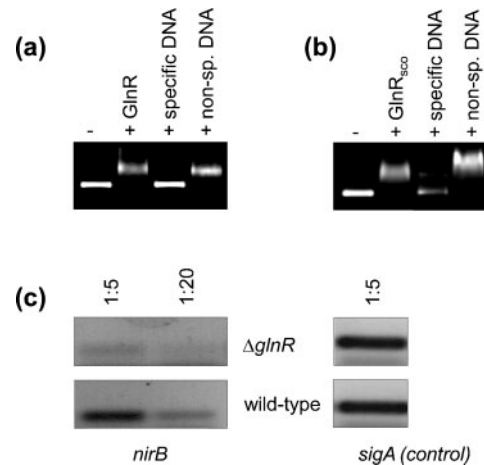


Fig. 6. GlnR regulates *nirB* transcription. (a) Purified GlnR protein of *M. tuberculosis* was incubated with 2 ng of 250 bp, Cy5-labelled PCR fragments containing the upstream regions of *nirB*. The shifts were verified to be specific by adding 500-fold excess of specific and non-specific DNA (non-labelled). (b) Purified GlnR_{SCO} protein of *S. coelicolor* was incubated with 2 ng of 250 bp, Cy5-labelled PCR fragments containing the upstream regions of *nirB*. The shifts were verified to be specific by adding 500-fold excess of specific and non-specific DNA (non-labelled). (c) RT-PCR analysis of GlnR target gene *nirB* in *M. tuberculosis* wild-type and $\Delta glnR$ mutant. Two micrograms of RNA was used for reverse transcription. cDNA was diluted 1 : 5 and 1 : 20 and used for PCR analysis. *sigA* was used as control.

nitrogen as described above. For whole-genome expression profiling, we exposed bacilli to nitrogen-limiting medium providing 5 mM KNO₃ for 18 h or 48 h. Labelled cDNA from two independent experiments for each time point was subjected to array analysis. Two different time points from two independent experiments thus generated four datasets that were pooled and analysed. The results showed that *nirB* was upregulated 9.8-fold ($P=0.003$), and that *nirD* was upregulated 6.6-fold ($P=0.017$) in the wild-type strain compared with the *glnR* mutant of *M. tuberculosis*. It is noteworthy that under the same conditions no difference in expression of *narGHJI* (*narG*, 1.2-fold, $P=0.639$; *narH*, 1.1 fold, $P=0.826$; *narJ*, 1.2-fold, $P=0.437$; *narI*, 1.2-fold, $P=0.599$) was found between *M. tuberculosis* wild-type and the *glnR* mutant.

DISCUSSION

When we started characterizing *narGHJI* of *M. tuberculosis*, we favoured the idea that *NarGHJI* is a purely anaerobic nitrate reductase (Weber *et al.*, 2000). Sohaskey & Wayne (2003) thoroughly studied expression of *narGHJI* of *M. tuberculosis* under various conditions. They found that the nitrate reductase activity of *M. tuberculosis* was sensitive to inhibition by both tungstate and azide, suggesting that the enzyme is a membrane-bound molybdenum-containing

complex, which is typical for a respiratory enzyme. NarGHJI of *M. tuberculosis* also appeared to be functionally similar to that of *E. coli*, and complemented a *narGHJI*-defective strain of *E. coli* to support anaerobic growth (Sohaskey & Wayne, 2003). These findings pointed to a potential role for this protein in anaerobic metabolism. However, the authors also reported that *M. tuberculosis*, unlike *E. coli* and *B. subtilis*, constitutively expressed *narGHJI* independent of the level of oxygen. Like *M. tuberculosis*, *C. glutamicum* and *S. coelicolor* belong to the order of *Actinomycetales*. *C. glutamicum* has one copy of *narGHJI*, whereas *S. coelicolor* has three copies of the gene cluster. In *C. glutamicum*, *narGHJI* serves as a respiratory nitrate reductase (Nishimura *et al.*, 2007). The organism is not able to utilize nitrate as a sole source of nitrogen, suggesting the absence of nitrate assimilation (Nishimura *et al.*, 2007; Takeno *et al.*, 2007). In *S. coelicolor* the role of *narGHJI* is unclear. The organism utilizes nitrate as a sole source of nitrogen (Hodgson, 2000). However, it is not known whether this activity is mediated by *narGHJI*, and whether *narGHJI* encodes a respiratory nitrate reductase. Thus, at present *M. tuberculosis* appears to provide the first example of a *narGHJI*-encoded nitrate reductase that mediates assimilation of nitrate under aerobic conditions.

In *E. coli*, NirBD encodes an NADH-dependent nitrite reductase that is composed of two subunits. The enzyme is only synthesized during anaerobiosis, presumably to regenerate NAD and detoxify nitrite that accumulates as a result of nitrate respiration (Gennis & Stewart, 1996). However, this is the exception to the rule, as most NAD(P)H-dependent nitrite reductases consist of a single polypeptide, and have an assimilatory function (Lin & Stewart, 1998). Here, we demonstrate that in *M. tuberculosis* NirBD functions as an assimilatory nitrate reductase. Homologues to *nirBD* of *M. tuberculosis* have been identified in *S. coelicolor* (SCO2486–SCO2488) but not in *C. glutamicum* (<http://www.ncbi.nlm.nih.gov/sites/entrez>). In the published literature, we found no direct evidence reporting reduction of nitrite in either species. However, as nitrate assimilation has been reported in *S. coelicolor* and requires formation of ammonium from nitrite, the homologues of *nirBD* might encode the assimilatory nitrite reductase in *S. coelicolor* (Tiffert *et al.*, 2008).

In the present study, a *M. tuberculosis* Δ *glnR* mutant was also unable to grow on nitrate as the sole source of nitrogen. The Δ *glnR* mutant of *M. tuberculosis* grew on ammonium as well as the wild-type strain. One explanation for this observation is that GlnR controls the expression of *narGHJI* and/or *nirB*. We identified a GlnR-binding site upstream of *nirB* but not of *narGHJI* of *M. tuberculosis*. The effect of GlnR on the transcription of *nirB* was determined by semiquantitative RT-PCR. GlnR activates transcription of the nitrate-assimilatory gene *nirB*, which may explain the inability of the Δ *glnR* mutant to grow on nitrate as a sole source of nitrogen as well as the accumulation of nitrite. Whole-genome expression profiling of the *glnR* mutant and the wild-type strain confirmed

regulation of *nirBD* expression by GlnR, and showed that the regulator does not control expression of *narGHJI*. In *S. coelicolor*, it has been reported that GlnR also activates the expression of the nitrite reductase gene *nirB* (Tiffert *et al.*, 2008). This and the successful complementation of the phenotype of the *M. tuberculosis* Δ *glnR* mutant with the *glnR* gene of *S. coelicolor* provides further evidence that the role of NarGHJI, NirBD and GlnR in assimilation of nitrate is conserved between *M. tuberculosis* and *S. coelicolor*.

The signalling cascade for GlnR-mediated *nirBD* activation is unknown. Normally, OmpR-like regulators are specifically phosphorylated. OmpR itself is phosphorylated at a conserved aspartate residue (D-55) by the cognate sensor kinase EnvZ, resulting in a modulation of its DNA-binding affinity (Delgado *et al.*, 1993). GlnR represents an orphan regulator with no coupled sensor kinase gene in the up- or downstream region. It is likely that GlnR is modified by an as yet unidentified kinase, as the protein contains the conserved aspartate residue (D-49) in its N terminus. A second gene, Rv2884, with homology to *glnR* of *S. coelicolor*, has been identified on the chromosome of *M. tuberculosis*. In *S. coelicolor* a second *glnR*, *glnRII*, has been described (Fink *et al.*, 2002). However, its inactivation did not produce a phenotype corresponding to that of a *S. coelicolor* Δ *glnR* mutant strain, pointing to GlnR as the principal regulator of nitrogen metabolism in *S. coelicolor*. Thus the role of Rv2884 in *M. tuberculosis* remains unclear at present.

In summary, this study describes the molecular mechanisms required for assimilatory nitrate and nitrite reduction, and its transcriptional control by GlnR in *M. tuberculosis*. Nitrate has been shown to accumulate in chronically infected tissue and might therefore be available to *M. tuberculosis* as a nutrient in the host. It might be utilized as an alternative substrate in cases of nitrogen limitation. In *S. coelicolor* GlnR has been suggested to play a global role in nitrogen metabolism (Tiffert *et al.*, 2008). Further studies are ongoing that include additional genome expression profiling experiments, combined with confirmatory PCR and DNA-binding analysis, to address this issue in *M. tuberculosis*.

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