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*.

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.
The first step in nitrate assimilation is the reduction of nitrate \((\text{NO}_3^-)\) to nitrite \((\text{NO}_2^-)\). 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 \((\text{NO}_2^-)\) to ammonium \((\text{NH}_4^+)\). 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 AmrR 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 KNO₃, KNO₂ or NH₄Cl as sole source of nitrogen, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 10% ADS, 0.2% glycerol and 0.05% Tween 80 (1 l of the basal medium contained 1 g KH₂PO₄, 2.5 g NaH₂PO₄, 2 g K₂SO₄ and 2 ml of trace elements; 1 l of trace elements contained 40 mg ZnCl₂, 200 mg FeCl₃·6H₂O, 10 mg CuCl₂·4H₂O, 10 mg MnCl₂·4H₂O, 10 mg Na₂B₄O₇·10H₂O and 10 mg (NH₄)₂MoO₄·2H₂O). To test for accumulation of nitrite, as a nitrogen-limiting medium a modified Proskauer Beck was used that contained, per litre, 5 g KNO₃, 0.6 g MgSO₄·7H₂O, 2.5 g CaH₂MgO₂, 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 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 3595 bp *MluI* fragment containing the *nirBD* gene cluster, and a 1582 bp *EcoRV–Smal* 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 OD₆₀₀ between 0.7 and 1.0. The cultures were washed with nitrogen-limiting medium before the OD₆₀₀ 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 OD₆₀₀.

### 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 KNO₃, or on fully supplemented 7H10 plates. Two *M. smegmatis* strains, #5192, which were unable to grow with 10 mM KNO₃, were transformed with an integrating cosmid library of *M. tuberculosis* H37Rv. Cosmid pMW32 was recovered from a complemented *M. smegmatis* clone. From this cosmids, a *NofI* 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 *PvuII*. pBA7 and pBA8 contained only Rv0818 and its own promoter.

### Accumulation of nitrite.

Bacteria were adjusted to an OD₆₅₀ of 0.2 in nitrogen-limiting medium containing 10 mM KNO₃ and incu-
bated at 37 °C with good aeration. At indicated time points, 100 μl sulfuric 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 pPM261 was fused to nirBD. For the initial PCR, 261Fus#1 (3’-TGTGTTGAGAATCTGGC-3’) and 261Fus#2 (5’-CCGGAACTCAGCAGGCAAATGGTGTCC-3’) were used, whereas the second PCR was performed with nirBfs#1 (5’-TCCGAGAATCTGGCATAAGGCACGCCC-3’) and nirBfs#2 (5’-GACTGATCACTGATACTCCG-3’). PCR products were used as a template for a self-primed PCR with the terminal primer pair 261Fus#1 and nirBfs#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. ΔglnR 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 digest (NEB) was performed with the eluate for 45 min. After 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’-CAGCGAAGGACGCCCAGAATCTGGC-3’) and qrt_qriA_mtu4 (as control) (5’-TACCGGCAAGCTGGCACTGCGGCAAATGGTGTCC-3’), qrt_nirB_mtu3 (5’-TACCGGCAAGCTGGCACTGCGGCAAATGGTGTCC-3’) and qrt_nirB_mtu4 (5’-CAGCGGAAATCTGGC-3’). The PCR conditions were 95 °C for 5 min; then 35 cycles of 95 °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’-CATATGTGGAGATATACGCGGACG-3’ and 5’-AAGCTTCT-ATTTTTCGACTTGGGTTGCTAATCGACGTGGGGAAGG-3’ by adding a sequence encoding a C-terminal StrepII-tag. The product was subcloned into pJOE2775, under the control of the Prham 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; 15000 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’-AGCCATGGCCGATAAGGCGGACCGG-3’ and 5’-AGCCATGGCCGATAAGGCGGACCGG-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 Microspin columns (GE Healthcare). Fragment labelling was performed via PCR using the Cy5-labelled primer 5’-AGCCATGGCCGATAAGGCGGACCGG-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 44 003 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 ΔnarG M. tuberculosis and ΔnirB 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 ΔnarG mutant on
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 ΔnirB 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 ΔnirBD 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. 1. Growth of ΔnarG M. tuberculosis and ΔnirB 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 OD 600 . Cultures were kept with good aeration. The following strains were compared: wild-type M. tuberculosis (●), ΔnarG M. tuberculosis (∆) or ΔnirB M. tuberculosis (∗), ΔnarG M. tuberculosis::narGHJI (○ in a) and ΔnirB M. tuberculosis::nirBD (○ in b). Wild-type M. tuberculosis was cultured in nitrogen-limiting medium without nitrate or nitrite as a control (□).

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+N03, 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 Rv0839 (#2009::pBA4), and the nitrate assimilation mutant of M. smegmatis #2009 transformed with ORF Rv0818 only (#2009::pBA7, #2009::pBA8).
Growth of a ΔglnR mutant of *M. tuberculosis* on nitrate

We generated a ΔglnR (Rv0818) mutant in *M. tuberculosis* by introducing an unmarked 323 bp deletion within glnR on the chromosome of *M. tuberculosis*. We compared growth of ΔglnR *M. tuberculosis* with that of the wild-type in nitrogen-limiting medium, with 10 mM nitrate as the sole source of nitrogen. The ΔglnR mutant of *M. tuberculosis* was unable to grow on nitrate. Expression of glnR on a plasmid in the ΔglnR mutant restored the wild-type phenotype (Fig. 3). Likewise, glnR from *S. coelicolor* also complemented ΔglnR *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, ΔnirB *M. tuberculosis* and ΔglnR *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, ΔglnR *M. tuberculosis* was cultured on ammonium. The ΔglnR *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 could not exclude that the accumulation of nitrite might be caused also by other factors such as transport of nitrite, we hypothesized that the ΔglnR 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.
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 ΔglnR 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 ΔglnR 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 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 ΔglnR mutant of M. tuberculosis. It is noteworthy that under the same conditions no difference in expression of narGHIJ (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 ΔglnR mutant.

DISCUSSION

When we started characterizing narGHIJ of M. tuberculosis, we favoured the idea that NarGHIJ is a purely anaerobic nitrate reductase (Weber et al., 2000). Sohaskey & Wayne (2003) thoroughly studied expression of narGHIJ 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...
transcription of the nitrate-assimilatory gene determined by semiquantitative RT-PCR. GlnR activates the effect of GlnR on the transcription of upstream of nirB in E. coli, which is similar to that of NarGHJI 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 ΔglnR mutant was also unable to grow on nitrate as the sole source of nitrogen. The ΔglnR 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 nirBD was determined by semiquantitative RT-PCR. GlnR activates transcription of the nitrate-assimilatory gene nirBD, which may explain the inability of the ΔglnR 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 ΔglnR 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 ΔglnR 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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**REFERENCES**

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