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Nitrite Reductase NirBD Is Induced and Plays an Important Role during *In Vitro* Dormancy of *Mycobacterium tuberculosis*

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***Mycobacterium tuberculosis* is one of the strongest reducers of nitrate among all mycobacteria. Reduction of nitrate to nitrite, mediated by nitrate reductase (NarGHJI) of *M. tuberculosis*, is induced during the dormant stage, and the enzyme has a respiratory function in the absence of oxygen. Nitrite reductase (NirBD) is also functional during aerobic growth when nitrite is the sole nitrogen source. However, the role of NirBD-mediated nitrite reduction during the dormancy is not yet characterized. Here, we analyzed nitrite reduction during aerobic growth as well as in a hypoxic dormancy model of *M. tuberculosis in vitro*. When nitrite was used as the sole nitrogen source in the medium, the organism grew and the reduction of nitrite was evident in both hypoxic and aerobic cultures of *M. tuberculosis*. Remarkably, the hypoxic culture of *M. tuberculosis*, compared to the aerobic culture, showed 32- and 4-fold-increased expression of nitrite reductase (NirBD) at the transcription and protein levels, respectively. More importantly, a *nirBD* mutant of *M. tuberculosis* was unable to reduce nitrite and compared to the wild-type (WT) strain had a >2-log reduction in viability after 240 h in the Wayne model of hypoxic dormancy. Dependence of *M. tuberculosis* on nitrite reductase (NirBD) was also seen in a human macrophage-based dormancy model where the *nirBD* mutant was impaired for survival compared to the WT strain. Overall, the increased expression and essentiality of nitrite reductase in the *in vitro* dormancy models suggested that NirBD-mediated nitrite reduction could be critical during the persistent stage of *M. tuberculosis*.**

Tuberculosis (TB) accounts for 1.4 million deaths annually and remains a serious health problem worldwide (1). The ability of the causative agent, *Mycobacterium tuberculosis*, to adapt to changing hostile environments within the host and shift into a dormant state has been recognized as one of the major reasons for its successful survival in humans (2). The nonreplicating persistent form not only helps the pathogen escape from the host defense mechanisms but also gives the bacilli the advantage of remaining unaffected by standard antitubercular drugs (3). Long-term persistence of *M. tuberculosis* in the latent stage, during antitubercular therapy, could also assist the pathogen to develop not just tolerance but resistance to currently used drugs (4). Therapeutic intervention which can target and kill the dormant tubercle bacilli therefore would not only provide a novel approach to combat TB but could also pave the way for complete eradication of the disease. A comprehensive knowledge of the metabolic state and physiology of *M. tuberculosis* during latent disease is required in order to discover such therapeutic options. The limited methods available for studying dormancy in *M. tuberculosis*, though not well characterized, have shown some degree of resemblance to actual *in vivo* latency (5–7). Based on the observation that human lung granulomas, where the TB bacilli reside, are hypoxic, an *in vitro* model in which oxygen depletion is induced gradually has been commonly used to study dormancy of *M. tuberculosis* (5, 8). In this model, replication ceases as the oxygen level decreases, and certain changes in energy metabolism are observed, including increased nitrate reductase activity (9). While the expression of nitrate reductase (NarGHJI) remained constant, the increase in the nitrate reductase activity during hypoxia was due to the increased expression of a nitrate transporter, NarK2 (10). *Mycobacterium smegmatis* was also reported to express a respiratory nitrate reductase and to have the ability to undergo hypoxia-induced *in vitro* dormancy, similar to *M. tuberculosis* (11, 12). Assimilatory reduction of nitrite produced by NarGHJI was also evident in this saprophytic strain, and a complete pathway of nitrate assimilation was discovered with the use of a minimal medium having nitrate, nitrite, or ammonium as the sole nitrogen source (13). By employing the defined nitrogen source for growth, the presence of functional nitrite reductase (NirBD) was confirmed in *M. tuberculosis* as well (14). However, the reduction of nitrite during the dormant stage of *M. tuberculosis* was not evaluated yet.

In this study, we characterized the reduction of nitrite and the expression of *nirBD* in the nonreplicating hypoxic stage, to evaluate the role of nitrite reductase (NirBD) during dormancy of *M. tuberculosis*. Survival of a *nirBD* mutant of *M. tuberculosis* in the Wayne hypoxic model of tuberculosis, as well as in a human macrophage-based dormancy model, was also compared with that of the wild-type (WT) strain to further determine the importance of nitrite reductase during persistent infection.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *M. tuberculosis* H37Ra (ATCC 25177) was obtained from IMTECH, Chandigarh, India, and *M. tuberculosis* H37Rv (ATCC 27294) was obtained from the ATCC, Manassas, VA. The stock cultures of both the strains were first subcultured in 7H9 broth to an optical density at 620 nm (OD_{620}) of ~1.0 (14). The

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TABLE 1 Primer sequences, annealing temperatures, and amplification sizes

Gene	Direction	Primer sequence	Annealing temp (°C)	Amplification size (bp)
narG	F	5′-ACTACGCCGACAACACCAAGTTCGCCGACG-3′	68	158
	R	5′-AGCGGCGCACATAGTCGACAAAGAACGGAA-3′		
nirB	F	5′-GTCCCGGTTCGTTTCCTTCG-3′	68	155
	R	5′- CGCGGGATACCAATGGACAC-3′		
glnA	F	5′-CAACTTCTTTGTGCACGACCCGTT-3′	64	423
	R	5′-AACTGGTAGTTGATCTCGGCCTGT-3′		
narK2	F	5′-TGCTTCGTGATGCACCCTACTTTCGGCCCA-3′	68	120
	R	5′-CCGCCGAACACGATCGCGTACAGAAACGAC-3′		
16S[a]	F	5′-ATGCATGTCTTGTGGTGGAAAGCG-3′	58	350
	R	5′-TTCACGAACAACGCGACAAACCAC-3′		

[a] 16S gene PCR was done for 25 cycles, while PCRs of other genes were done for 35 cycles.

cultures were then washed three times with phosphate-buffered saline (PBS), and the OD_{620} was adjusted to 0.01 at the beginning of an experimental culture. Growth and reduction of nitrite in the culture were tested under nitrogen-limiting conditions using *Mycobacterium phlei* medium supplemented with $NaNO_3$ (10 mM), $NaNO_2$ (1 mM), or asparagine (7.5 mM) as the sole nitrogen source (13). One liter of this medium contained 5 g KH_2PO_4, 2.5 g sodium citrate, 0.60 g $MgSO_4$, and 20 ml glycerol, and the pH was adjusted to 6.6 ± 0.2.

For aerobic cultivation, bacterial cultures were grown in 30 ml *M. phlei* medium in a 100-ml flask with an initial inoculum of ~10^5 (OD_{620} ~ 0.01) cells per ml. The flask was then kept under aerobic conditions in a shaker incubator (model 481; Thermo Electron Corporation) maintained at 150 rpm and 37°C. For cultivation of anaerobic dormant bacilli, the Wayne 0.5 HSR (headspace ratio) model was used with a starting inoculum of ~10^5 cells/ml (5).

The number of viable bacilli at different time points in both aerobic and Wayne cultures was determined by first sonicating (Sonics VibraCell sonicator; 4 W for 60 s) the cultures to break up clumps and then spreading dilutions on 7H9 solid agar medium.

Estimation of nitrite in whole-cell culture. Nitrite concentration in cultures was measured by the Griess method (15). Briefly, 1 ml of the culture was added to 1 ml of 1% sulfanilic acid (in 20% HCl) and 1 ml of 1% naphthylenediamine dihydrochloride (NEDD) solution. The reaction mixture was incubated for 15 min to develop a pink color. The absorbance of the supernatant was measured at 540 nm, and nitrite concentration was quantified; the values were compared to a standard curve of nitrite.

Generation of *nirBD* mutant of *M. tuberculosis*. The *nirBD* operon was amplified from *M. tuberculosis* with the primers 5′-AGGGTCGAGCTCGACGTTGACGTCCTTGTC-3′ and 5′-GGTGATCTAGACCGCTACCCGCGCGACCTG-3′. The underlined bases indicate mismatches used to create SacI and XbaI restriction sites. The 3,387-bp fragment was cloned into pST-Blue (Novagen) vector, and sequenced. *nirBD* was subcloned into the suicide vector pJQ200SK (16) by cutting both plasmids with XbaI and SacI. Next, *aph*, a kanamycin resistance marker, was inserted into the NcoI site resulting in the deletion of part of *nirB* and *nirD*. This plasmid was electroporated into *M. tuberculosis* H37Rv, and knockout mutants were identified as previously described (10).

Expression analysis and quantification of mRNA levels. To isolate total RNA, the spheroplast-based method was applied to aerobic and hypoxia-induced dormant cultures (17). Briefly, a spheroplast solution consisting of 0.002% lysozyme, 0.006% D-cycloserine, 1.4% glycine, 0.2% EDTA, and 0.1% lithium chloride (wt/vol) in distilled water was aseptically added to an *M. tuberculosis* culture grown aerobically to an OD_{620} of ~1.0 or anaerobically in the Wayne model on the 7th day with different nitrogen sources. The treated cells were then used for total RNA isolation by the TRIzol extraction. A 1-μg portion of total RNA isolated from *in vitro*- and *ex vivo*-grown mycobacteria was treated with DNase I (Sigma) and then incubated at 70°C according to the manufacturers' instruction. DNase I-treated total RNA was used for cDNA synthesis using random primers and enhanced avian reverse transcriptase provided in the first-strand cDNA synthesis kit (Sigma) at 25°C for 10 min followed by incubation at 45°C for 50 min. The resulting cDNA was used as a template for PCR amplification.

PCR was carried out by using *Taq* DNA polymerase provided in the PCR core kit (Sigma) in a total volume of 50 μl. The amplification PCR product was first analyzed on a 2% agarose gel containing 1% (vol/vol) SafeView dye followed by nucleotide sequencing to verify the fragment.

Real-time quantitative PCR was performed with a brilliant SYBR green quantitative PCR (qPCR) master mix kit (Sigma, St. Louis, MO). Reactions were carried out in a volume of 25 μl, and the reaction mixtures consisted of a 0.05 μM concentration of forward and reverse primers, 12.5 μl of 2× master mix, and 2.5 μl of cDNA. Controls with no cDNA template were included in each run. The internal control for each reaction was 16S gene amplification.

The PCR parameters were as follows: (i) an initial denaturation step of 2 min at 95°C; (ii) 40 cycles of 30 s at 95°C, 30 s at the respective annealing temperature, and 30 s for extension at 72°C; and (iii) a final extension step of 7 min at 72°C. A melting curve analysis was then performed. All samples were run on a 2% agarose gel containing 1% (vol/vol) SafeView dye to verify that only a single band was produced. Each experiment was done three times with independent RNA samples isolated from similar conditions (primer sequences are given in Table 1).

Preparation of cell extracts and Nir enzyme assay. For the nitrite reductase enzyme assay, spheroplast solution was added to *M. tuberculosis* cultures grown aerobically (up to an OD_{620} of ~1.0) and anaerobically in the 0.5 HSR (headspace ratio) Wayne model (up to 7 days) (5). After incubation for 1 h, 50 ml of bacterial cells were concentrated using centrifugation at 10,000 rpm for 10 min at 4°C. The pellet obtained was washed twice with 2 ml of potassium phosphate buffer (50 mM, pH 6.6 ± 0.2) and resuspended in 2 ml of potassium phosphate buffer (50 mM, pH 7.0) containing protease inhibitor (protease cocktail; Sigma). The prepared mixture was sonicated in a water bath for 5 min at 50 kHz. The lysate was obtained after removal of unbroken cells by centrifugation at 10,000 rpm for 5 min at 4°C. The lysate was transferred to another tube and ultracentrifuged at 100,000 rpm for 1 h at 4°C. After centrifugation, the supernatant was again transferred to another tube while the pellet, including the membrane fraction, was resuspended in 2 ml of potassium phosphate buffer containing protease inhibitor. After the total protein concentrations in the supernatant and membrane fractions had been determined using the Bradford method, the nitrite reductase assay was done

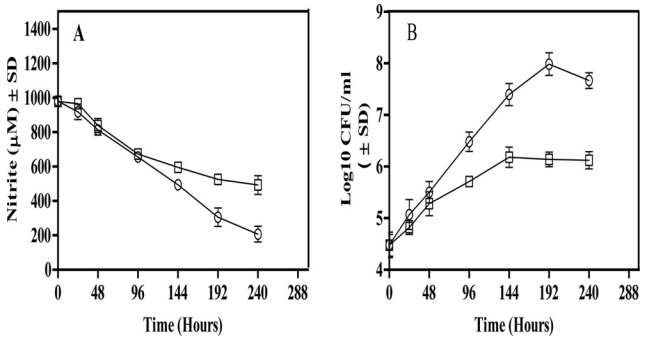

FIG 1 Nitrite reduction and CFU of *M. tuberculosis* H37Ra during aerobic growth and in the Wayne model. Bacteria were cultured with 1 mM nitrite as the sole nitrogen source. (A) Nitrite reduction was determined by testing aliquots at the indicated time points for depletion of nitrite from the medium. (B) Growth of the organism was determined by testing aliquots at the indicated time points by plating on the 7H11 agar plates to enumerate CFU. Circles represent the aerobic cultures, whereas rectangles represent the hypoxic cultures. The results are averages ± SD from three identical experiments.

as described previously (18, 19). Briefly, the final assay mixture contained 1.6 ml of sodium phosphate buffer (50 mM, pH 7.2), 100 μl of methyl viologen (0.01%), 50 μl of enzyme (50 μg/ml), 50 μl of sodium nitrite (1 mM), and 200 μl sodium dithionite-sodium bicarbonate solution (0.8%). The tube was sealed with Parafilm and kept in a 37°C water bath incubator for 30 min. After 30 min of incubation, the assay reaction was stopped by vigorous shaking till the dark blue color disappeared. The amount of nitrite remaining as the substrate in the enzyme assay mixture was measured using the Griess method and compared with that in a control assay where whole-cell extract was not added.

THP-1 monocytes based *in vitro* model of intracellular dormancy of tubercle bacilli. Human THP-1 monocytoid cell line (TIB3456; ATCC, Manassas, VA) was maintained at 37°C and 5% CO_2 in HEPES-buffered RPMI 1640 (Sigma-Aldrich, St. Louis, MO) medium with 10% heat-inactivated fetal bovine serum (FBS), 50 μg/ml gentamicin, and 100 μg/ml penicillin (pH 7.2). Cells were seeded at 10^6/well in a 24-well plate free of antibiotics and were activated with 1 μM retinoic acid and vitamin D (cholecalceferol) (RAVD). After 72 h treatment with RAVD, cells were infected with *M. tuberculosis* or the *nirBD* mutant at a multiplicity of infection (MOI) of 1:10. At 24 h postinfection, the cells were washed with medium to remove unphagocytosed bacteria. Cells were then incubated in a CO_2 incubator at 37°C and treated with RAVD every 72 h. The intracellular bacterial burden was determined at different time points by lysing the macrophages with a 0.05% SDS solution, plating dilutions on 7H11 agar plates, and counting the colonies after 21 days of incubation at 37°C. A stable number of viable bacilli along with the formation of multinucleated giant cells (MNGC) was used as an indicator of onset of intracellular dormancy of *M. tuberculosis* (20).

Data and statistical analysis. Data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Results are expressed as means ± standard deviations (SD) unless otherwise stated. Student's *t* test and standard one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test were used to determine statistical significance. A P value of <0.05 was considered significant.

RESULTS

Reduction of nitrite by *M. tuberculosis* during aerobic growth and hypoxic dormancy *in vitro*. An assimilatory function of nitrite reductase (NirBD) of *M. tuberculosis* during its active replicating stage was previously reported, when WT *M. tuberculosis* but not the *nirBD* mutant grew on nitrite as the sole nitrogen source (14). However, the reduction of nitrite by nitrite reductase (NirBD) remained unknown during the dormant stage of the pathogen, when it does not multiply. We used the Wayne model of dormancy, which uses gradual oxygen depletion *in vitro*, to analyze the reduction of nitrite during the nonreplicating persistence of *M. tuberculosis* (5). Since nitrite reduction was detected when nitrate or nitrite was used as the nitrogen source in the medium, we used an *M. phlei* medium having nitrite as the sole nitrogen source to cultivate *M. tuberculosis* (13). We used avirulent *M. tuberculosis* H37Ra in this experiment, whose NirBD protein has 100% amino acid similarity with the NirBD of *M. tuberculosis* H37Rv.

The time-dependent reduction of nitrite was measured along with the CFU in the Wayne model as well as in the aerobic culture (Fig. 1). In the aerobic culture, the growth of *M. tuberculosis* on nitrite as the sole nitrogen source, with a concurrent time-dependent depletion of nitrite from the medium, confirmed that the organism reduced nitrite for assimilation (Fig. 1). The observed growth is due to assimilation of nitrite and not because of formation of ammonia or other contaminants in the medium.

In the Wayne model, reduction of nitrite was clearly visible in the replicating phase (0 to 144 h) of *M. tuberculosis* H37Ra, with a steady depletion of nitrite from the culture medium. However, a slow depletion of nitrite was also evident during the nonreplicating phase (144 to 240 h). Although the rate of nitrite depletion during the nonreplicating phase seemed lower, the number of cells was also more than 10-fold lower than that in the aerobically grown culture. Interestingly, when nitrite reduction was calculated in terms of micromoles of nitrite depleted per 10^6 cells per day, the nonreplicating dormant cultures had an 8- to 12-fold-higher rate of nitrite reduction at hours 144, 192, and 240 than the aerobic culture (Table 2). Reduction of nitrite by nonreplicating *M. tuberculosis* confirmed that nitrite reductase (NirBD) was active during dormancy. Second, the increased reduction rate of nitrite by the hypoxic culture of *M. tuberculosis* compared to the aerobic culture also suggested a potentially vital role of the NirBD in hypoxic metabolism of the pathogen.

Comparative expression of *nirB* at the transcriptional and translational level during aerobic and hypoxic conditions by *M. tuberculosis*. Since we observed an increased rate of nitrite reduction by nonreplicating cultures compared to the aerobic replicating cultures, we investigated whether the expression of nitrite reductase (NirBD) at the transcriptional and translational levels also differed between these two cultures.

To analyze nitrite reductase gene expression, RT-PCR was done using cDNA synthesized from total RNA isolated from bacilli grown in different nitrogen sources (Fig. 2A). The expression of nitrate reductase MRA_1172 (*narG*), nitrite reductase MRA_261 (*nirB*), and glutamine synthetase MRA_2230 (*glnA1*) in aerobic as well as hypoxic dormant cultures was seen when nitrate

TABLE 2 Rate of nitrite reduction by *M. tuberculosis* H37Ra under aerobic and Wayne hypoxic culture conditions

Time (h)	Reduction rate (mean ±SD)[a]	
	Aerobic (+O_2)	Hypoxic (−O_2)
144	2.37 ± 0.28	19.57 ± 2.13
192	1.89 ± 0.17	21.73 ± 1.68
240	1.74 ± 0.13	17.38 ± 2.021

[a] Micromoles nitrite reduced/48 h/10^6 cells of *M. tuberculosis*.

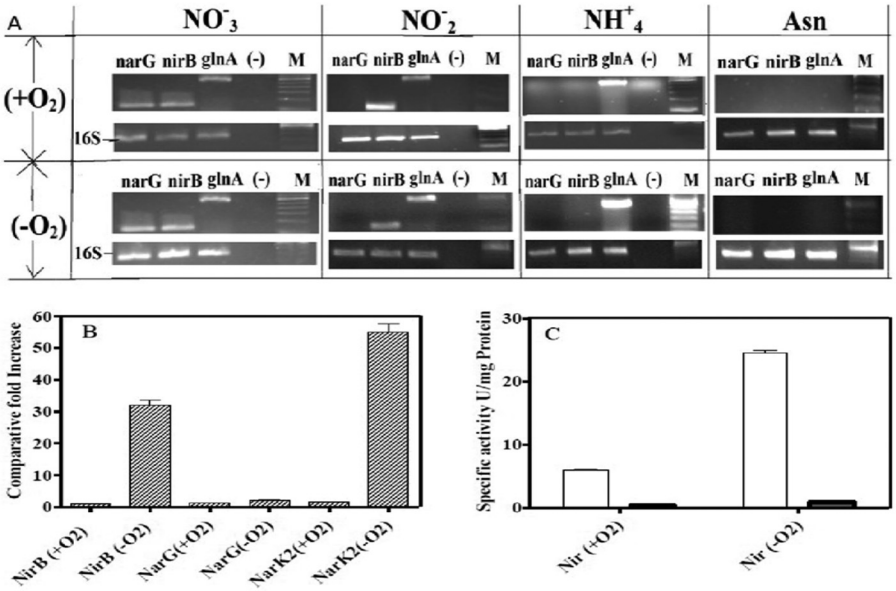

FIG 2 (A) Expression of *M. tuberculosis* genes involved in nitrate/nitrite metabolism under aerobic and Wayne hypoxic conditions with different nitrogen sources. cDNA was prepared from total RNA isolated from bacilli grown in the presence of nitrate, nitrite, ammonium, or asparagine either aerobically ($+O_2$) or in the Wayne model ($-O_2$). cDNA was used as the template for PCR amplification using gene-specific primers for *narG* (nitrate reductase), *nirB* (nitrite reductase), and *glnA1* (glutamine synthetase), and the amplified products are shown after electrophoresis on agarose gels. Lane (-), negative control without cDNA; lane M, 100-bp marker (Invitrogen). (B) Quantitative PCR analysis of *nirB*, *narG*, and *narK2* expression in bacilli grown in the presence of nitrite as the nitrogen source under different conditions. cDNA was prepared from the *M. tuberculosis* culture grown under aerobic ($+O_2$) or hypoxic ($-O_2$) conditions. The relative levels of the transcripts are shown as the difference from that obtained under aerobic conditions, while the 16S gene was used as an internal control. (C) Specific activity of nitrite reductase enzymes in the cytoplasm and membrane fractions of *M. tuberculosis* cells grown in the presence of nitrite as the nitrogen source. Specific enzyme activity was measured during mid-logarithmic phase for aerobic cultures ($+O_2$) and on the 7th day in Wayne model cultures ($-O_2$). White bars represent the Nir activity measured in cytoplasmic fractions, whereas gray bars represent Nir activity measured in membrane fractions. One unit of specific activity of enzyme was defined as 1 μM NO_2^- depleted per min/mg of total protein during the *in vitro* Nir enzyme assay.

was used as the sole nitrogen source. Expression of *nirB* and *glnA1* but not *narG* was detected when nitrite was used as a nitrogen source, indicating that substrate-dependent transcription of the genes is required for nitrate/nitrite metabolism. In the presence of ammonium, only *glnA1* expression was detected, while none of the nitrate- or nitrite-metabolizing genes were expressed when asparagine was the nitrogen source. This further confirmed that in both aerobic and dormant cultures of *M. tuberculosis* H37Ra, the transcription of *nirB* is seen only when nitrite is present.

Quantitative PCR of *nirB* was done to compare the transcript levels of *nirB* gene between aerobic replicating and hypoxic dormant cultures of *M. tuberculosis* (Fig. 2B). It was observed that the level of *nirB* expression during the nonreplicating dormancy was ~32-fold higher than that during aerobic growth. The transcript levels of *narK2* were increased 62-fold during the nonreplicating stage and served as positive controls for this study due to their known induction during dormancy (10).

The expression of *nirB* was also measured at the translational level by determining the specific activity of nitrite reductase in cell extracts obtained from aerobic and hypoxic cultures of *M. tuberculosis* H37Ra (Fig. 2C). The cytoplasmic fraction of cell extract obtained from the Wayne model showed a 4-fold increase in the specific activity of nitrite reductase compared to that in the extracts from aerobically grown *M. tuberculosis* H37Ra. The membrane fraction of cell extracts did not show any discernible nitrite reductase activity, which also confirmed the cytoplasmic location of NirBD. The increased expression of *nirB* at both the transcriptional and translational levels correlated with the increased rate of nitrite reduction during the nonreplicating stage and suggested that the nitrite reductase (NirBD) could have an important function during dormancy of *M. tuberculosis*.

Growth and survival of a *nirBD* mutant of *M. tuberculosis* in the Wayne model. The inability of *nirBD*-deficient *M. tuberculosis* to grow and assimilate nitrite was previously observed only under aerobic culture condition (14). Since the assimilation of nitrite may not be required when the organism is not multiplying, it was of interest to examine whether *M. tuberculosis* could survive without a functional nitrite reductase during hypoxia. Survival of the *nirBD* mutant compared to WT *M. tuberculosis* was therefore evaluated in the Wayne model. Given that the *nirBD* mutant was unable to grow when nitrite was used as the sole nitrogen source, asparagine was also added to the medium to allow growth.

The reduction of nitrite and the viability of the *nirBD* mutant were then compared to those of WT *M. tuberculosis* H37Rv in the Wayne model (Fig. 3). The genetic similarity of nitrite reductases in virulent and avirulent *M. tuberculosis* was corroborated at the functional level when H37Rv showed a pattern of growth and nitrite reduction similar to that in the Wayne model with nitrite as the sole nitrogen source (Fig. 1; Fig. 3A and B). The *nirBD* mutant was unable to assimilate nitrite with no indication of growth or nitrite reduction in the Wayne model when nitrite was used as the sole nitrogen source.

Nonetheless, in a medium where asparagine was added with nitrite, the mutant showed a growth rate comparable to that of WT *M. tuberculosis* H37Rv in the replicating phase of the Wayne hypoxic culture (Fig. 3C). Reduction of nitrite by either the *nirBD*

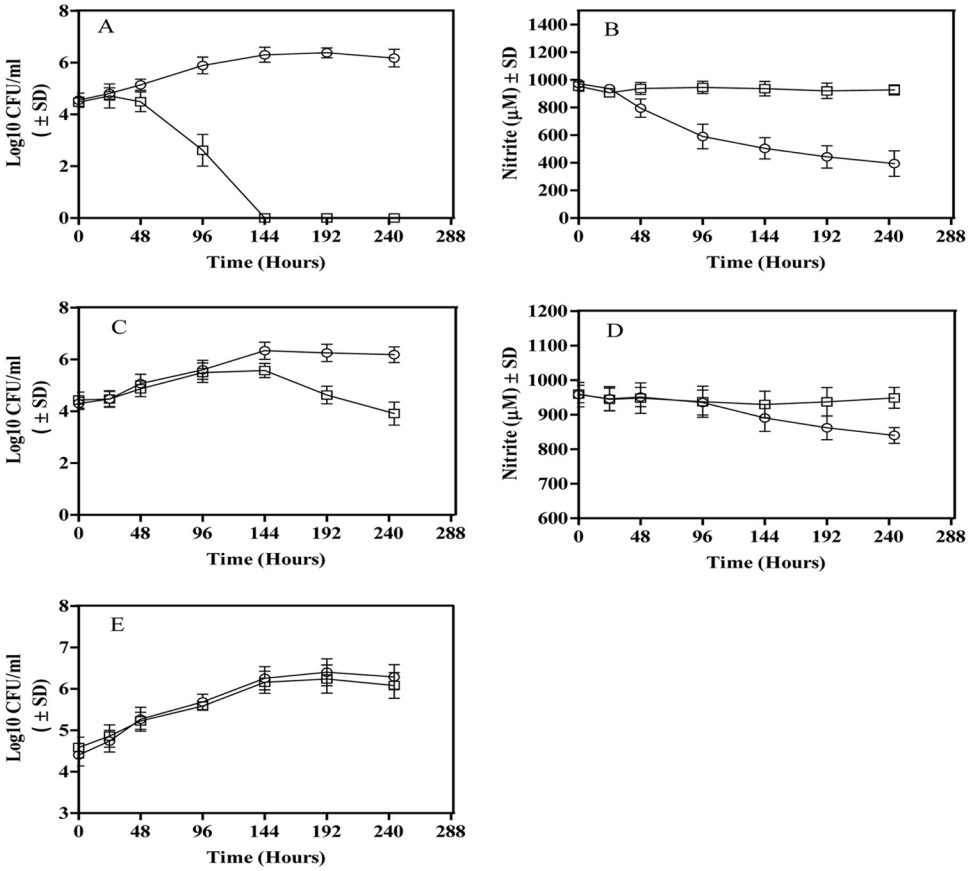

FIG 3 Viability and nitrite reduction by the *nirBD* mutant and WT *M. tuberculosis* in the Wayne model with different nitrogen sources. Viability (A, C, and E) and nitrite levels (B and D) in *M. tuberculosis* H37Rv (○) and the *nirBD* mutant (□) when nitrite (A and B) or nitrite and asparagine (C and D) or asparagine only (E) was the nitrogen source. The results are averages ± SD from three identical experiments.

mutant or WT *M. tuberculosis* was not detected during the replicating phase of Wayne cultures, when nitrite and asparagine were present in the medium (Fig. 3D). However, once cultures shifted down into the nonreplicating phase in the Wayne model, a slow reduction of nitrite by WT was detected. The *nirBD* mutant did not reduce any discernible amount of nitrite and was unable to maintain viability during this nonreplicating phase in the Wayne model. The viability of the *nirBD* mutant was reduced by more than 2 logs compared to that of the WT *M. tuberculosis* at hour 240 in the Wayne hypoxic culture (Fig. 3C). The results thus indicated that the function of NirBD of *M. tuberculosis* under hypoxic conditions not only could be essential but also could be different from that during the aerobic stage of growth. However, it remained unknown whether NirBD is essential for the survival of *M. tuberculosis* during the dormant stage when nitrite is not present in the medium. In order to address this question, asparagine was used as the sole nitrogen source in the Wayne cultures, and the survival of the *nirBD* mutant was examined. With asparagine as the nitrogen source, the viability of *nirBD* mutant remained comparable to that of WT *M. tuberculosis* in the Wayne model for at least 240 h (Fig. 3E). Thus, it could be concluded that the *nirBD* is indispensable when nitrite as a substrate for the enzyme is available.

Survival of the *nirBD* mutant of *M. tuberculosis* during intracellular dormancy. Evidence from the earlier experiment suggested that the expression and functional role of nitrite reductase of *M. tuberculosis* are dependent on the nitrogen source (Fig. 2 and 3). Since the nitrogen source(s) present in the axenic dormant culture may not mimic what is available to *M. tuberculosis* during its actual persistence within a cell, we examined the role of nitrite reductase in an intracellular model of dormancy.

A recently developed human macrophage (THP-1)-based model of intracellular dormancy of tuberculosis was employed to analyze the survival of the *nirBD* mutant (20). Briefly, in this model, the dormancy of *M. tuberculosis* in THP-1 macrophages is induced by constant treatment with retinoic acid (RA) and vitamin D_3 (VD). Combined treatment with RA and VD enhances the expression of DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin) and mannose receptors on THP-1, which inhibits the subsequent intracellular growth of *M. tuberculosis* and induces the dormancy phenotype of the pathogen. After treatment of infected macrophages with RA and VD for a certain period of time, formation of multinucleated giant cells (MNGCs) confirms the establishment of dormancy of *M. tuberculosis*.

The viability of the *nirBD* mutant and WT *M. tuberculosis* was monitored in this model to determine the contribution of nitrite reductase during intracellular dormancy (Fig. 4). After the initial time point, both the WT and the *nirBD* mutant showed decreasing viability. After 15 days, the tubercle bacilli entered dormancy (as evidenced by the formation of MNGC), and the viable count re-

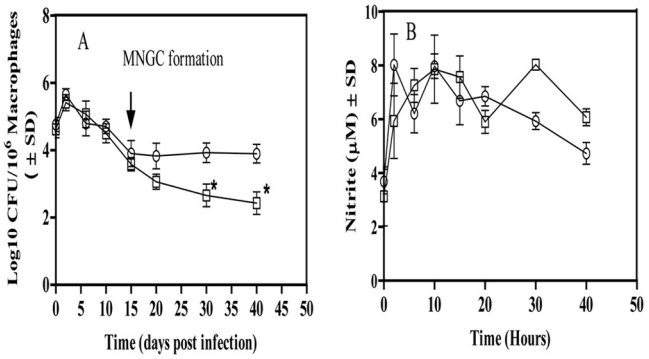

FIG 4 (A) Survival of the *nirBD* mutant and WT *M. tuberculosis* H37Rv in THP-1 macrophages treated with retinoic acid and vitamin D (RAVD). Macrophages were lysed at the indicated time points and plated on 7H11 agar to determine the intracellular burden of bacilli. *, $P < 0.05$. ↓, MNGC formation, which indicates the induction of dormancy in intracellular *M. tuberculosis*. (B) Presence of nitrite within THP-1 macrophages infected with the *nirBD* mutant (□) and WT *M. tuberculosis* H37Rv (○). The results are averages ± SD from three identical experiments.

mained constant for WT for the next 25 days (Fig. 4A). In contrast, the number of viable *nirBD* mutants continued to decrease, although at a reduced rate. At day 25 after the onset of dormancy, the number of viable bacilli of *nirBD* mutant was reduced by more than 1.5 logs compared to the WT *M. tuberculosis*. The inability of *nirBD* mutant to maintain its viability in this intracellular dormancy model of tuberculosis further strengthened the idea that nitrite reductase is involved in supporting the survival of *M. tuberculosis* during latency. More interestingly, the dependence of *M. tuberculosis* on nitrite reductase could be seen even without external addition of nitrite in this intracellular dormancy.

In order to know whether the substrate for nitrite reductase is made available by endogenous production of nitrite by *M. tuberculosis*-infected macrophages, we examined the nitrite levels in the cells harboring dormant bacilli (Fig. 4B). Macrophages infected with the *nirBD* mutant as well as those infected with WT *M. tuberculosis* maintained a stable level of ~5 μM nitrite per 10^6 cells during the intracellular dormancy. Although it was expected that if nitrite reductase (NirBD) was functional during this intracellular dormancy, the amount of nitrite produced by macrophages should have decreased with time for WT *M. tuberculosis*, a constant production of nitrite by intrinsic nitric oxide generation could be the possible reason for stable levels of nitrite detection (21).

DISCUSSION

One of the key factors in the success of *M. tuberculosis* as a pathogen is its ability to persist for extended periods within the human host in a dormant state. The understanding of metabolic pathways operative during latent tuberculosis is an active area of research. Inhibition of aerobic respiration by either hypoxia, nitric oxide, or carbon monoxide is proposed to be the key signal driving *M. tuberculosis* into a nonreplicating persistent state. The discovery of microbial factors which allow the pathogen to adapt to this stress could identify vital drug targets.

The response of *M. tuberculosis* to low oxygen is one of the best characterized of all responses to environmental triggers which induce dormancy. The reduction of nitrate to nitrite increases during hypoxia as the bacteria use nitrate as an alternative terminal electron acceptor (9, 10). The enzyme responsible, NarGHJI, is representative of the bacterial respiratory nitrate reductases. However, it can also play an assimilatory role during aerobic condition and allow *M. tuberculosis* to replicate when nitrate is the sole nitrogen source (14, 22). In this case, nitrite is further reduced to ammonium by the NirBD nitrite reductase, as was confirmed when the *nirB* mutant failed to grow on either nitrate or nitrite (14). Although it was known that NarGHJI could play a respiratory and assimilatory role, whether the same is true of for NirBD was not known.

In this study, we discovered that NirBD-mediated reduction of nitrite also occurred during the hypoxic dormant stage. Indeed, nitrite reduction was at a higher rate than in the aerobic growth phase when nitrite was the nitrogen source (Fig. 1 and Table 2). The addition of asparagine to the medium resulted in a decrease in the rate of nitrite reduction (Fig. 3).

The fact that the reduction in the viability of *nirBD* mutant compared to the WT was seen only during the hypoxic stage suggested that the function of NirBD of *M. tuberculosis* might be different under aerobic and anaerobic conditions. The high level of asparagine (7.5 mM) in comparison to nitrite (1 mM) suggests that the asparagine was not depleted as the cultures entered dormancy, which is further supported by the persistence of both strains in the Wayne model with asparagine only (Fig. 3E).

The *nirBD* mutant had reduced survival in macrophages (Fig. 4). This was analyzed by utilizing a macrophage cell culture system that allowed longer experimental times, up to 40 days (20). The indispensability of NirBD could indicate that *M. tuberculosis* is subjected to hypoxia in the macrophage. Indeed, prior studies have shown that the intraphagosomal oxygen levels in *M. tuberculosis*-infected macrophages are lower than the cytosolic oxygen concentration, supporting the notion that hypoxia could be associated universally with dormancy (23, 24).

There are several possible roles that nitrite reduction may play. One is the assimilation of nitrite as a nitrogen source. This occurs *in vitro* in culture with nitrite or nitrate as the only nitrogen source. The reduction of nitrite to ammonium consumes 6 electrons and is energetically costly, which may explain why *M. tuberculosis* prefers asparagine over nitrite. There was no induction of *nirB* in the presence of ammonium or asparagine (Fig. 2A).

There is very little information on the nitrogen sources available to *M. tuberculosis* within macrophages. Amino acid biosynthesis mutants of *M. tuberculosis* and *M. bovis* do not replicate in macrophages (25–27) and are attenuated in mice (28, 29), suggesting that the phagosome is limiting for amino acids. Free amino acids and peptides are not thought to be the main source of nitrogen. Nitrite and nitrate, the main breakdown products of nitric oxide, may serve as significant nitrogen sources for *M. tuberculosis* at times. However, the medium used to grow macrophages *in vitro*, RPMI, contains high levels of glutamine, which results in abnormally high levels of glutamine in the phagosome (27). This makes the detection of an assimilatory role for NirBD difficult.

The increased rate of nitrite reduction when the pathogen is not replicating suggests a possible role in respiration. The attenuation of the *M. tuberculosis nirBD* mutant during hypoxic dormancy even when other nitrogen sources besides nitrite were available further supports a nonassimilatory function. Under these conditions, the expression of *nirBD* was dependent on the presence of nitrite and lack of oxygen (Fig. 2). The *M. tuberculosis nirBD* gene product is not predicted to be membrane bound and

hence was not detected in the membrane (Fig. 2B), unlike the Nrf nitrite reductase system of *Escherichia coli*, and thus would not contribute to the proton gradient (30). Nitrite reduction is a NAD(P)H-dependent process, and a role for NirBD in this process to maintain the NADH/NAD balance during an interruption in aerobic respiration is possible. The higher cost in electrons for the reduction of nitrite would be a benefit under these conditions. A similar role has been proposed for nitrate reductase in *M. tuberculosis*, which is also required for survival in human macrophages (10, 31).

A role in nitrite and nitric oxide detoxification is also possible. Nitrite has been shown to be toxic to *M. tuberculosis* and therefore must be exported or converted to ammonium, and under hypoxia conditions, it is exported (32). The nitrite reductase NirBD of *E. coli* is involved mainly in detoxification of internal nitrite (33). Nitric oxide can also be produced from the dismutation of nitrite. Nitric oxide is bactericidal at high levels and more stable in the absence of oxygen (34). This nitric oxide may be responsible for the decreased viability seen with the *nirBD* mutant in the Wayne model (Fig. 3C).

The production of ammonium to reduce the acidity of the environment of the phagosome is another possible role for nitrite reductase. It has been reported that secretion of ammonium by *M. tuberculosis* inhibits the phagosome-lysosome fusion (35). NirBD could be one source of this ammonium production from nitrite.

Nitrite could be readily available by the spontaneous degradation of nitric oxide in mammalian tissues (21). Organs where the infection of *M. tuberculosis* is most common, including lungs, liver, and kidneys, can produce 0.1 to 1 mM nitrate and nitrite. Tuberculosis patients have higher levels of these NO breakdown products (nitrate and nitrite) in their serum than control patients (36). Here, a steady level of nitrite was detected in macrophage cultures, suggesting that nitrite would be available during the persistent stage (Fig. 4B).

Nitrite reductase is an important virulence factor for other intracellular pathogens. NO production by macrophages, while toxic in the short term, actually increased long-term survival for the intracellular pathogen *Brucella abortus* (37). This was proposed to be due to the production of nitrate and nitrite, which were subsequently used as terminal electron acceptors. Both nitrate and nitrite reductase mutants of *Campylobacter jejuni* showed reduced colonization of chickens (38).

With the development of animal models representing a true latent infection which closely resembles human tuberculosis, it should now be possible to confirm whether the *in vitro* role of nitrite reductase (NirBD) correlates with the function of the enzyme during *in vivo* persistence of *M. tuberculosis*.

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