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Role of *narK2X* and *narGHJI* in Hypoxic Upregulation of Nitrate Reduction by *Mycobacterium tuberculosis*

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***Mycobacterium tuberculosis* is one of the strongest reducers of nitrate in the genus *Mycobacterium*. Under microaerobic conditions, whole cells exhibit upregulation of activity, producing approximately eightfold more nitrite than those of aerobic cultures of the same age. Assays of cell extracts from aerobic cultures and hypoxic cultures yielded comparable nitrate reductase activities. *Mycobacterium bovis* produced only low levels of nitrite, and this activity was not induced by hypoxia. *M. tuberculosis* has two sets of genes, *narGHJI* and *narX* of the *narK2X* operon, that exhibit some degree of homology to prokaryotic dissimilatory nitrate reductases. Each of these were knocked out by insertional inactivation. The *narG* mutant showed no nitrate reductase activity in whole culture or in cell-free assays, while the *narX* mutant showed wild-type levels in both assays. A knockout of the putative nitrite transporter *narK2* gene produced a strain that had aerobic levels of nitrate reductase activity but failed to show hypoxic upregulation. Insertion of the *M. tuberculosis narGHJI* into a nitrate reductase *Escherichia coli* mutant allowed anaerobic growth in the presence of nitrate. Under aerobic and hypoxic conditions, transcription of *narGHJI* was constitutive, while the *narK2X* operon was induced under hypoxia, as measured with a *lacZ* reporter system and by quantitative real-time reverse PCR. This indicates that nitrate reductase activity in *M. tuberculosis* is due to the *narGHJI* locus with no detectable contribution from *narX* and that the hypoxic upregulation of activity is associated with the induction of the nitrate and nitrite transport gene *narK2*.**

The ability of *Mycobacterium tuberculosis* to persist in a host for long periods despite acquired immunity without producing obvious symptoms, along with its capacity to reactivate and cause clinical tuberculosis, has been an impediment to the complete control and eradication of this disease. The grim result is that approximately one third of the world's population is asymptomatically infected with *M. tuberculosis* and thus carries latent disease (14, 16).

Information has recently begun to emerge on the physiological state of the tubercle bacilli during latent infection. In the course of early infection, they are surrounded in granulomas by activated macrophages which limit access to some nutrients including oxygen (7, 35, 52). Among the various systems that have been used to model disease latency in vitro, one involves the cultivation of tubercle bacilli under conditions leading to the controlled gradual depletion of oxygen (49). This model has been used to study dormancy in both *Mycobacterium tuberculosis* and the closely related *Mycobacterium bovis* BCG (24). Both species are obligate aerobes unable to grow without oxygen, but when subjected to gradual oxygen depletion, they can survive for extended periods. The growth of cultures in sealed, slowly stirred tubes results in the gradual and uniform controlled depletion of oxygen until the bacilli enter a microaerobic stage of nonreplicating persistence (NRP-1) and then proceed into the anaerobic state (NRP-2), in which they may survive for many months (24, 49).

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In an anaerobic environment, many bacteria are able to use nitrate as a final electron acceptor in place of oxygen for the maintenance of a proton motive gradient to continue growing. Historically, *M. tuberculosis* has been differentiated from *M. bovis* by the fact that only *M. tuberculosis* can reduce significant amounts of nitrate (NO_3^-) to nitrite (NO_2^-) (44, 48). Nitrate reductase activity occurs at a low level during the aerobic growth of *M. tuberculosis* and increases significantly upon entry into the microaerobic NRP-1 stage (50). This, along with the fact that neither *M. tuberculosis* nor *M. bovis* reduce nitrite (44, 48), suggests that the reduction of nitrate serves as an alternate energy source rather than nitrogen source during the adaptation to hypoxic conditions.

The best characterized nitrate reductase system is that of *Escherichia coli* where there are two membrane-bound respiratory enzymes. The four-gene *narGHJI* operon is induced 4-fold under anaerobic conditions and an additional 19-fold by nitrate (40) and permits anaerobic growth in the presence of nitrate. The second very similar operon, *narZYWV*, encodes a nitrate reductase enzyme expressed at a low level aerobically and induced during stationary phase but not regulated by either oxygen or nitrate levels (8).

M. tuberculosis has an *narGHJI* locus (Fig. 1) able to produce nitrate reductase activity when cloned into *Mycobacterium smegmatis* (53). Also identified in the *M. tuberculosis* H37Rv genome during sequencing was a gene designated *narX*, which has been proposed to code for a "fused nitrate reductase" (11). This proposal was made because the predicted product of *narX* would be a protein with homology to parts of the NarG, NarJ, and NarI proteins, although its actual function is unknown.

Here we further characterize the nitrate reducing system of

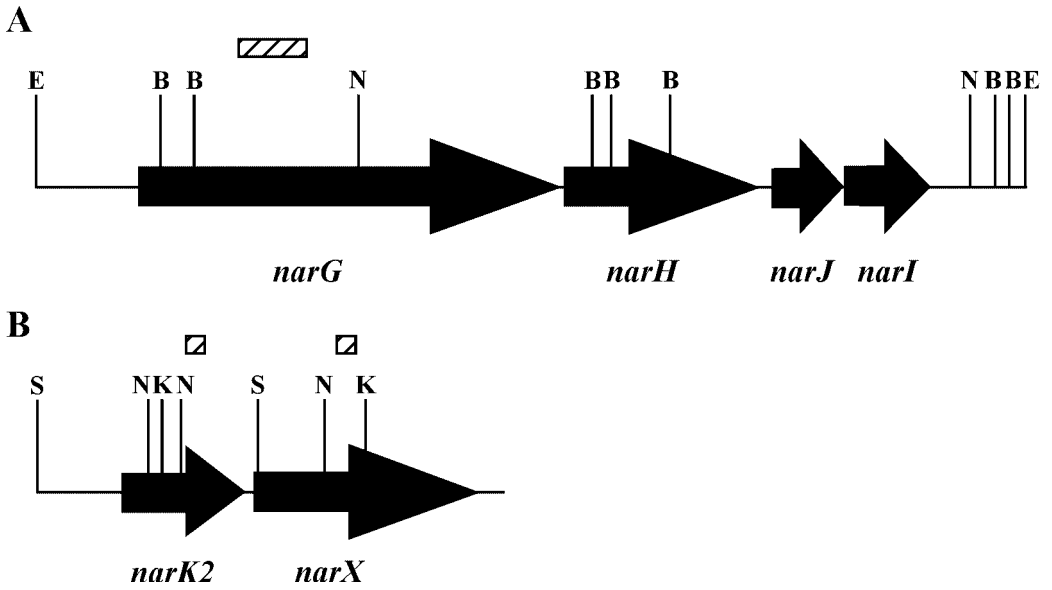

FIG. 1. Genes possibly involved in nitrate reductase activity. Schematic diagram of both the *narGHJI* (A) and *narK2X* (B) loci in *M. tuberculosis*. Arrows show the open reading frames with the gene names below the arrows. Relevant restrictions sites are shown: B, *Bam*HI; S, *Sma*I; E, *Eco*RV; N, *Nco*I; K, *Kpn*I. The locations of probes used in Southern blots (hatched boxes) are indicated. The diagram is not drawn to scale.

M. tuberculosis and compare it to that of *M. bovis*. To determine the role of *narG* and *narK2X* in the reduction of nitrate by *M. tuberculosis*, each gene was inactivated. Nitrate reduction and mRNA levels of the various genes were measured during aerobic growth and shiftdown into NRP-1 as well as stationary phase.

MATERIALS AND METHODS

Strains and media. *M. tuberculosis* H37Rv and avirulent *M. bovis* BCG Pasteur from the culture collection of this laboratory were used. Virulent *M. bovis* Ravenel was provided by Frank Collins. *M. smegmatis* mc^{2}155 was from William Jacobs (38). The Nar$^-$ *E. coli* mutant JCB4023 [*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 gyrA219 non-9 narG*::*erm* Δ*napAB narZ*::Ω] (9, 31) and JCB4018 (Δ*narAB* Δ*nar*::Ω Δ*narK* Δ*narU*::*kan*) were provided by Jeff Cole (9, 31).

Liquid mycobacterial cultures were grown at 37°C in Dubos Tween-albumin broth (DTA) (Difco, Detroit, Mich.). The cultures were plated on either Dubos oleic albumin agar (DOA) or DTA with the addition of 1.6% agar (DTA agar). Aerobic cultures were incubated on a model G24 rotary shaker-incubator (New Brunswick Scientific, Edison, N.J.). For NRP cultures, conditions included slow magnetic stirring in sealed tubes with a headspace ratio (HSR) of 0.5 as previously described (46, 49). Nitrate, when used, was added at a final concentration of 5 mM unless indicated otherwise. *E. coli* cultures were grown in Luria-Bertani (LB) or M9 medium. Anaerobic cultures of *E. coli* were grown in full, tightly sealed tubes containing medium supplemented with Oxyrase (Oxyrase, Inc., Mansfield, Ohio).

Kanamycin was used at 25 μg/ml for *M. tuberculosis* and at 50 μg/ml for *E. coli*. Gentamicin was used at 5 μg/ml, and apramycin was used at 30 μg/ml. All antibiotics and chemicals, including sodium azide, sodium molybdate, and tungstic acid, were from Sigma (St. Louis, Mo.). All oligonucleotide primers were from Ransom Hill Bioscience (Ramona, Calif.) and are listed in Table 1.

Sonication. To make cell-free mycobacterial extracts, cultures were centrifuged and the cells were washed with an equal volume of phosphate buffer (pH 7.2) containing 0.02% Tween 80 and then with phosphate buffer (pH 7.2) without Tween 80. The cell suspensions were sonicated for 15 min in the cup-horn of a W-380 sonicator (Heat Systems-Ultrasonic, Farmingdale, N.Y.) at 4°C and filtered through 0.22-μm-pore-size Spin-X filters (Corning Costar Corp., Cambridge, Mass.). Approximately 30% of the nitrate reductase activity was recovered in the filtrate from both aerobic and NRP cultures. Glycerol was added to the filtrate to 10%, and the protein concentration was determined by a standard Bradford assay. The samples were frozen at −70°C until use.

Nitrate reductase assay. For the cell-free assay, 100 μg of protein was added to a 0.6-ml tube. Then 50 μl of 1 M $NaNO_3$ and 50 μl of methyl viologen (in 100 mM phosphate buffer [pH 7.2]) were added to the tube, and the volume was adjusted to 450 μl with phosphate buffer, pH 7.2. Argon was bubbled over the samples, which were placed at 37°C for 2 min. To start the reaction, 50 μl of freshly made 29 mM sodium hydrosulfite (dithionite) in 10 mM NaOH was added. At 20-min intervals, 100-μl samples were removed, and the nitrite concentration was determined. All assays were performed in triplicate and replicated at least once with independent cell sonicates.

For whole-cell assays, the nitrite concentration was determined in 200 μl of culture from aerobic cultures or 20 μl from NRP and senescent stationary cultures. Nitrite concentration was determined by the Griess reaction (48).

Reporter analysis. To create the reporter plasmid, the *E. coli lacZ* gene was amplified by PCR with primers p19 and p20 (Table 1). These primers created a *Kpn*I site upstream and a *Sma*I site downstream of the gene. After digestion with these enzymes, the fragment was cloned into *Dra*I-*Kpn*I-digested pMH94 (23) to create pMP100. pMP100 exists as a plasmid in *E. coli*, but in *Mycobacterium* species, it integrates into the chromosome as a single copy.

The upstream promoter regions of *narGHJI* and *narK2X* were amplified by PCR. A 376-bp fragment directly upstream of the *narG* start site was amplified with primers p46 and p47. For *narK2X*, a 282-bp fragment was generated with primers p48 and p49 based on previous promoter analysis of *narK2X* (21). After digestion with *Xba*I and *Kpn*I, these fragments were cloned into pMP100 to create pMP101 (*narGHJI*) and pMP102 (*narK2X*).

pMP100, pMP101, and pMP102 were electroporated into *M. tuberculosis* to create strains PMP100, PMP101, and PMP102, respectively. The insertion of the plasmid into the chromosome as a single copy was verified by Southern blot analysis. β-Galactosidase activity was determined by the method of Timm et al. (42) and calculated as follows: 200 × optical density at 420 nm (OD_{420})/mg of protein/min.

RNA isolation. To isolate RNA, cultures were quickly mixed with 4 volumes of 5 M GTC lysis solution 1 (5 M guanidine thiocyanate, 0.5% sodium *N*-lauryl sarcosine, 25 mM trisodium citrate, 0.1 M 2-mercaptoethanol, 0.5% Tween 80 [pH 7.0]) (25) and immediately pelleted by centrifugation at 20,000 × *g* for 30 min. The pellet was resuspended in 1 ml of Trizol (InVitrogen, Carlsbad, Calif.) and transferred to a 2-ml tube approximately one-third filled with 0.1-mm-diameter zirconia-silica beads. Cells were disrupted by three 1-min pulses at full speed in a Mini-Bead beater (Bio Spec Products, Bartlesville, Okla.). Samples were centrifuged for 5 min at 10,000 × *g*, and the supernatants were extracted once with $CHCl_3$ and precipitated with isopropanol. After resuspension, DNA was removed by treatment with DNase (Boehringer Mannheim) for 2 h at 37°C. RNA was purified by chromatography with an RNeasy column (Qiagen, Chats-

TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5′→3′)[a]	Purpose in this study[b]
p11	ACTACGCCGACAACACCAAGTTCG	Probe for *narG*
p12	TGTTCTGCTGGGTTCGGTCGTGGTA	Probe for *narG*
p13	TGCTGCTGGTGGCGACCGCGCTGGTCGCGT	Cloning *narX*
p14	GCGCGGACTTGTTCGACGAGTAGACGTGT	Cloning *narX*
p19	GTGAGCGGGTACCAATTTCACACAGG	Cloning *lacZ*
p20	GGCCTGCCCGGGTATTATTATTTTTG	Cloning *lacZ*
p23	GGTGATCGCCATGGTCGACCTGCAGG	Cloning *aphI*
p24	TAATGCTCTGCCATGGTTACAACCAA	Cloning *aphI*
p27	ATGCGCCTCGGTGCTGCTGACCTACCCGAA	Probe for *narX*
p28	GGATCGGCACGGCGCAGCTCAGAGACCGTG	Probe for *narX*
p46	CTGGATTGCGGATCTAGAGCGAACCCTCAA	Construction of pMP101
p47	ACGGTCACGGGGGTACCCTCCTCGTCATGA	Construction of pMP101
p48	TCCCCTTTCTAGAGGCGACCAGGCTCAGCT	Construction of pMP102
p49	TCATCGACAGGTACCGGGGTCTCGGACTCC	Construction of pMP102
p51	CAGCTCCTGGATCCGGCTGCCGGTCCGTGG	Cloning *narK2*
p58	ACTCGAGTGGCGAACGGGTGAGTAACACGT	RT-PCR of 16S rRNA
p59	AGGCCGTCACCCCACCAACAAGCTGATAGG	RT-PCR of 16S rRNA
p60	ACTACGCCGACAACACCAAGTTCGCCGACG	RT-PCR of *narG*
p61	AGCGGCGCACATAGTCGACAAAGAACGGAA	RT-PCR of *narG*
p62	ATTGGTGGGACGTGGTGTGGCAATGCGCCT	RT-PCR of *narX*
p63	GACCGTCGATGTGGGCCAGCAATTCCTCTG	RT-PCR of *narX*
p66	TGCTTCGTGATGCACCCTACTTTCGGCCCA	RT-PCR of *narK2*
p67	CCGCCGAACACGATCGCGTACAGAAACGAC	RT-PCR of *narK2*
p68	CCAAGTCGGACAAGCTTCGGGCGACCGAGA	Cloning *narK2*

[a] Mismatches used to create restriction sites are indicated by underlining.
[b] RT-PCR, reverse transcriptase PCR.

worth, Calif.) and treated again with DNase, which was then inactivated at 70°C for 5 min.

Quantitation of mRNA levels. For each sample, 500 μg of RNA was added to a mixture of antisense primers (total concentration of 1 μM) and all four deoxynucleoside triphosphates (total concentration of 500 μM) in a total volume of 16 μl. The resulting mixture was heated to 80°C for 3 min. Subsequently, 2 μl of 10× PCR buffer, 1 μl of Moloney murine leukemia virus reverse transcriptase (RT), and 1 μl of placental RNase inhibitor from a RETROscript kit (Ambion, Austin, Tex.) were added. In every case, a duplicate sample was prepared without Moloney murine leukemia virus RT for the no-RT control. The reaction was stopped, and the enzyme was inactivated by heating at 92°C for 10 min.

Real-time quantitative PCR was performed with the Brilliant SYBR green QPCR Master Mix kit (Stratagene, La Jolla, Calif.). Reactions were performed in a volume of 50 μl, and the reaction mixtures consisted of a 0.1 μM concentration of both primers (Table 1), 25 μl of 2× master mix, and 5 μl of cDNA. The control with no RT was included in each run. An additional sample with RNA diluted 1:10 was also included to measure 16S rRNA. Amplification was performed in the ICycler (Bio-Rad, Hercules, Calif.) with sampling during elongation. The samples were subjected to PCR as follows: (i) an initial denaturation step of 10 min at 95°C; (ii) 30 cycles, with 1 cycle consisting of 30 s at 95°C and 1 min at 68°C; (iii) an extension step of 7 min at 68°C. A melting curve analysis was then performed. All samples were run on a 2% agarose gel to verify that only a single band was produced. Each gene was analyzed from three independent RNA samples.

Construction of knockouts. Knockouts were created by cloning either *narG*, *narX*, or *narK2* into pJQ200KS (32) followed by the insertion of *aphI*, a kanamycin resistance marker. For *narG*, a *Bam*HI-*Apa*I fragment was cloned from cosmid I65 (6) into the same sites of pJQ200KS. For *narX* and *narK2*, the entire genes were amplified by PCR performed with the Advantage-GC 2 PCR kit with primers p13 and p14 (*narX*) or p51 and p68 (*narK2*) and cloned with the TA cloning kit (InVitrogen) to produce pNarX2 or pNarK2. *narX* was subcloned into pJQ200KS by cutting both plasmids with *Xho*I and *Sac*I. *narK2* was subcloned with *Bam*HI and *Hind*III to make pTSJ1.

The *aphI* gene from Tn*903* was amplified with primers p23 and p24, which created *Nco*I sites at both ends of the 946-bp fragment. This fragment was cloned into the unique *Nco*I sites of the *narG* fragment to create plasmid pNarG3.1 or into *narX* to make pNarX3.1. Due to the presence of two *Nco*I sites, insertion of *aphI* to create pTSJ2 resulted in a small deletion in the *narK2* gene.

pNarG3.1, pNarX3.1, and pTSJ2 were electroporated into *M. tuberculosis* followed by selection on DTA agar plates with 25 μg of kanamycin per ml and 5 μg of gentamicin per ml. (DTA agar produced soft colonies that were easy to pick.) Colonies that were the results of single-crossover events having either pNarG3.1, pNarX3.1, or pTSJ2 inserted into the chromosome copy of *narG*, *narX*, or *narK2*, respectively, were identified by Southern blot analysis. This confirmed the presence of both the wild-type gene and the additional gene with the *aphI* insertion. Digoxigenin-labeled probes for Southern blot analysis were created by PCR (Roche Diagnostics, Indianapolis, Ind.) using p11 and p12 as primers for *narG*, p27 and p28 for *narX*, and p66 and p67 for *narK2*.

Three single-crossover mutants were chosen and replated on DTA agar with 2% sucrose and kanamycin. Possible double-crossover mutants, containing *aphI* inserted into the *Nco*I site of the chromosome copy of *narG* or *narX*, were initially identified by sensitivity to gentamicin and confirmed by Southern blot analysis. This showed both the presence of the gene with the *aphI* insertion and the loss of the wild-type gene.

Cloning of *narGHJI*, *narX*, and *narK2*. The *M. tuberculosis narGHJI* operon was cloned as an *Eco*RV fragment from the I65 cosmid (6) into the *Dra*I sites of pPE207 (28). This was electroporated into *M. smegmatis*, and after selection for apramycin resistance, clones were screened for nitrate reductase activity. The plasmid obtained was named pNarGHJI1. This plasmid was electroporated into *E. coli* JCB4023 and *M. smegmatis* and maintained aerobically with apramycin but without nitrate.

To clone *narX*, the *narX* gene was cut from pNarX2 with *Bam*HI and cloned into the same site of pPE207 to create pNarX5.

To clone *narK2, Bam*HI and *Hind*III were used to subclone the *narK2* gene into the same sites in pPE207 to create pTSJ3. To make pTSJ4, *narK2* was removed from pTSJ1 with *Bam*HI and *Sac*I and cloned into the same sites in pBluescript SK+ (pSK).

RESULTS

Nitrate reductase activity in culture. To determine the nitrate reductase activity of cultures, nitrite concentrations were assayed during growth. Measurements were made on both aerobic cultures and in microaerobic NRP-1 phase cultures. In addition to the controlled oxygen depletion model, we also used some tubes of vigorously agitated, loosely capped cultures that were incubated long enough to exhibit the late plateau of

TABLE 2. Production of nitrite in whole-cell culture by *M. tuberculosis* and *M. bovis*

Mycobacterium	Treatment[a]	Nitrite concn[b]		
		Aerobic[c]	NRP-1[d]	SSP[e]
M. tuberculosis		130 ± 23	1,100 ± 80	2,600 ± 430
	400 μM Mo	120 ± 6	780 ± 55	ND
	100 μM W	9 ± 1	120 ± 9	ND
	400 μM Mo + 100 μM W	23 ± 3	500 ± 5	ND
	40 μM azide	3 ± 1	16 ± 7	ND
M. bovis		13 ± 1	5 ± 2	180 ± 52
M. bovis BCG		7 ± 1	2 ± 1	11 ± 5

[a] Treatment with the ions molybdate (Mo) and tungstate (W) and sodium azide.
[b] Mean nitrite concentration (micromolar) ± standard deviation.
[c] After 112 h of growth (OD_{580} of ~0.4).
[d] After 112 h, approximately 45 h in NRP-1 (OD_{580} of 0.1).
[e] After 280 h of growth ($OD_{580} > 1.0$). ND, not done.

TABLE 3. Nitrate reductase activity in cell-free sonicates

Culture treatment	Nitrite reductase activity[a]		
	Aerobic[b]	NRP-1[c]	SSP[d]
No nitrate	20 ± 1	22 ± 5	9 ± 1
5 mM NO_3^-	21 ± 1	18 ± 3	6 ± 1
20 mM NO_3^-	21 ± 2	22 ± 4	ND

[a] Mean nitrite reductase activity (in nanomoles of NO_2^- per minute per milligram of protein) ± standard deviation.
[b] After 112 h of growth (OD_{580} of ~0.4).
[c] After 112 h, approximately 45 h in NRP-1 (OD_{580} of 0.1).
[d] After 280 h of growth ($OD_{580} > 1.0$). ND, not done.

senescent stationary phase (SSP) (47). This phase has been used by some investigators as a model for dormancy, so nitrite concentrations were also determined after the growth of aerated cultures reached an optical density plateau. Since the nitrate reductase system has also been studied in *M. bovis* BCG (18, 53), both virulent and avirulent strains of this species were included for comparison purposes.

Aerobic shaken cultures of *M. tuberculosis*, virulent *M. bovis*, and the avirulent vaccine strain *M. bovis* BCG were started at a concentration of 2.5×10^6 cells/ml in DTA with 5 mM $NaNO_3$, which allowed for approximately 140 h of logarithmic growth before the onset of SSP. After 112 h of aerobic growth, which represented mid-logarithmic phase (OD_{580} of ~0.5), *M. tuberculosis* cultures had produced an average of 130 μM nitrite, while *M. bovis* and *M. bovis* BCG cultures produced only 13 and 7 μM, respectively (Table 2). After 5 days in SSP, the concentration of nitrite in the medium had increased to 2,600 and 180 μM for *M. tuberculosis* and *M. bovis*, respectively, while *M. bovis* BCG had produced only 11 μM.

When the same number of cells was incubated in slowly stirred sealed tubes (0.5 HSR configuration) (49), they grew logarithmically for about 67 h before replication abruptly ceased due to hypoxia, and cells entered microaerobic NRP-1. At 112 h, after approximately 45 h in NRP-1, the nitrite concentration produced by *M. tuberculosis* was 1,100 μM, while the nitrite concentration produced by *M. bovis* was 5 μM and that produced by *M. bovis* BCG was 2 μM (Table 2).

After 112 h of incubation, aerobic cultures of *M. tuberculosis* produced eightfold-less nitrite than those in NRP-1, despite the fact that the aerobic cultures had a cell density approximately fourfold greater. Thus, nitrate reduction in *M. tuberculosis* showed the typical hypoxic induction seen in many other bacteria. Virulent *M. bovis* showed only weak activity, without any increase under hypoxia, while the avirulent *M. bovis* BCG showed only background levels of nitrite production. Cells began to clump and die as they entered SSP, so it was not possible to make a reliable assessment of the induction of nitrate reductase activity only on the basis of nitrite levels.

The effects of several ions on the production of nitrite in culture were also determined (Table 2). The nitrate reductase activity in *M. tuberculosis* was inhibited by the addition of 100 μM tungstate, which is a characteristic response of molybdenoproteins (41). This inhibition could be partially reversed by the addition of 400 μM molybdate in both aerobic and NRP-1 cultures. A low concentration of sodium azide also inhibited activity in *M. tuberculosis*; this is characteristic of the membrane-bound class of nitrate reductase enzymes (1, 2). Nitrate reductase enzymes also use chlorate as a substrate and reduce it to the toxic chlorite. *M. tuberculosis* was unable to grow in 5 mM sodium chlorate. This suggests that *M. tuberculosis* contains a membrane-bound molybdenum-containing nitrate reductase enzyme system.

Cell-free nitrate reductase assay. A cell-free assay was developed to measure the levels of nitrate reductase enzyme. This system utilized cell sonicates rather than whole cells and allowed the measurement of enzyme activity in a measured amount of bacillary protein, independent of nitrate and nitrite transport. Cells of *M. tuberculosis* were grown without or with 5 or 20 mM nitrate and harvested from either aerobic, NRP-1, or SSP cultures (Table 3). There was no significant difference in the specific activity of cell-free sonic extracts from cultures that had been grown with and without nitrate, suggesting that the level of nitrate reductase enzyme in each extract is also equal, and therefore independent of the nitrate concentration in the environment. Furthermore, the activity from extracts of actively growing aerobic cultures and NRP-1 cultures were not significantly different from each other. Cell extracts of SSP cultures showed lower specific activity than extracts from growing aerobic cultures or NRP-1 cultures (Table 3). This could be due simply to a decrease in the number of viable cells in SSP. In summary, the nitrate reductase activity of cell extracts was constant under a variety of conditions, despite strong differences in the apparent activity shown by intact cells.

Expression of *nar* genes. A promoterless reporter plasmid containing *lacZ* was constructed from the integration vector pMH94 (23) and named pMP100. The *narGHJI* upstream promoter region was inserted into this plasmid to create pMP101-*narG*, and that of the *narK2X* region to make pMP102-*narK2X* (21). These plasmids were electroporated into *M. tuberculosis* at a site where the mycobacteriophage *int* gene and attachment site allowed integration of a single copy of the plasmid into the chromosome.

β-Galactosidase levels were determined from samples of each strain taken either during mid-logarithmic phase or after approximately 45 h in NRP-1. The *narGHJI* promoter of *M. tuberculosis* strain PMP101 in aerobic and NRP-1 cultures showed similar levels of activity (Fig. 2). In contrast, the *narK2X* promoter of *M. tuberculosis* strain PMP102 showed

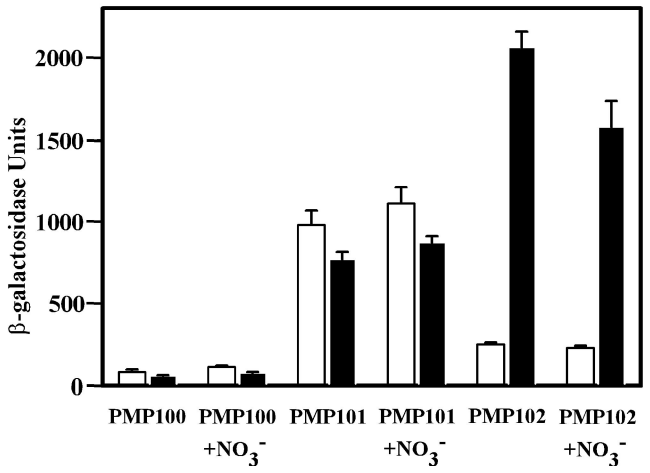

FIG. 2. β-Galactosidase activity in *M. tuberculosis* containing *narGHJI* and *narK2X* promoter constructs. β-Galactosidase assays were performed with cell extracts from aerobic actively growing (open bars) or NRP-1 (filled bars) cultures. Each strain had *lacZ* controlled by the upstream region of *narG* (PMP101) or *narK2X* (PMP102) or no insert (PMP100). The means ± standard deviations (error bars) of three determinations are shown.

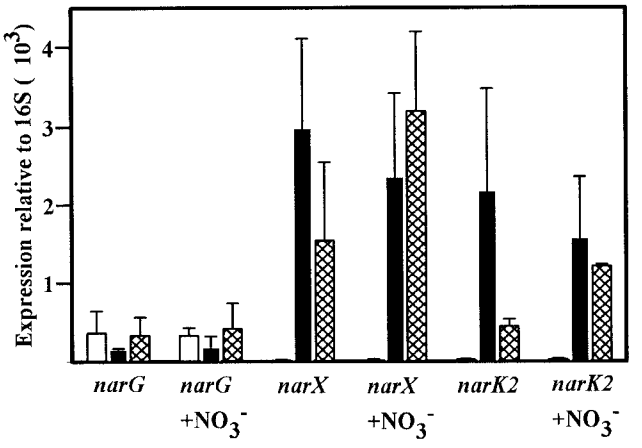

FIG. 3. Quantitative real-time PCR of *narG*, *narX*, and *narK2*. RNA levels are expressed relative to the level of stable 16S rRNA (13, 37). The means ± standard deviations (error bars) of three determinations are shown.

approximately eightfold induction under the controlled hypoxic conditions of NRP-1 in comparison to aerobic levels.

Neither promoter showed any response with the addition of 5 mM nitrate to the growth medium (Fig. 2). Since significant nitrite was also produced from nitrate in all cultures, this implies that the regulation of both the *narGHJI* and *narK2X* promoters are independent of both nitrate and nitrite.

Real-time reverse transcriptase PCR was used to measure mRNA levels quantitatively. The levels of *narG*, *narX*, and *narK2* were determined and compared to the level of the stable 16S rRNA (13, 37). Samples were taken from aerated cultures in mid-logarithmic phase, just after the onset of SSP, and from sealed cultures after approximately 45 h in NRP-1. The levels of *narG* transcripts were similar in aerobic, NRP, and SSP cultures (Fig. 3). In contrast, *narX* and *narK2* mRNA levels were low in aerobic cultures but showed strong induction in both NRP and SSP cultures. Again, the three genes did not show a significant response to the addition of 5 mM nitrate to the growth medium (Fig. 3).

Inactivation of *narG and narX*. To help determine the role of *narGHJI* and *narX* of *M. tuberculosis* in the reduction of nitrate under different culture conditions, the selected genes were inactivated. A kanamycin resistance marker, *aphI*, was inserted into a cloned copy of *narG* or *narX* and recombined by single crossover into the chromosome of *M. tuberculosis*. Double crossovers were then isolated in which the chromosomal copy was replaced by the inactive cloned copy, and all plasmid sequences were lost (Fig. 4).

There were no obvious differences in the growth curves (measured by optical density) between *M. tuberculosis* strain RVW1 *narG*::*aphI*, RVW2 *narX*::*aphI*, and wild type, whether the mycobacteria were grown in aerobic, NRP-1, or the truly anaerobic conditions of NRP-2 with or without 5 mM nitrate. To measure hypoxic survival after shiftdown, viable cell counts were determined by plating for a total of 12 weeks after inoculation. There were no obvious differences in survival with or without nitrate for the wild-type and mutant strains (C. D. Sohaskey, unpublished data). However, cultures of RVW1 *narG*::*aphI* did not reduce nitrate, while those of RVW2 *narX*::*aphI* showed wild-type levels of activity (Table 4). In cell-free assays, the *narX* knockout also showed enzyme levels similar to those of the wild type, but only background activity was seen in extracts from the *narG* knockout (Table 5). RVW1 *narG*::*aphI* was able to grow in 20 mM chlorate in contrast to RVW2 *narX*::*aphI*, which was as sensitive as the wild type. Thus, the insertion in *narX* had no effect on nitrate reductase activity, while that in *narG* eliminated the activity, indicating that only *narGHJI* was responsible for nitrate reductase activity during both aerobic and NRP-1 conditions.

Cloning of *narGHJI* and *narX*. The *narGHJI* operon was cloned from an *M. tuberculosis* cosmid into plasmid pPE207 (28) to produce pNarGHJI1, and *narX* was cloned to produce pNarX5. Both plasmids were electroporated into *M. smegma-*

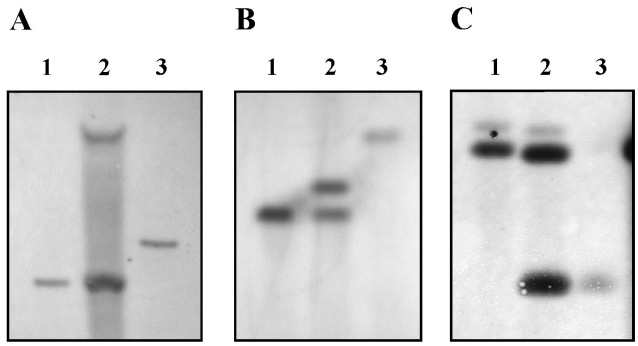

FIG. 4. Southern blot analysis of *narG* and *narX* insertional mutants. Chromosomal DNA from strains used to produce the RVW1 (A), RVW2 (B), and RVW3 (C) mutants was isolated from the wild type (lanes 1), single crossovers containing two copies of the *nar* gene (lanes 2), and double crossovers containing *aphI* inserted into *nar* (lanes 3). DNA was cut with *Kpn*I (A and B) or *Sma*I (C) and probed with a probe specific for either *narG* (A), *narX* (B), or *narK2* (C).

TABLE 4. Nitrate reductase activity of *narG*, *narX*, and *narK2* mutants

Strain	Genotype	Nitrite concn[a]		
		Aerobic[b]	NRP-1[c]	SSP[d]
Wild type	Wild type	140 ± 10	1,400 ± 49	1,900 ± 410
RVW1	Δ*narG*	3 ± 1	2 ± 1	4 ± 2
RVW1(pNarGHJI1)[e]	Δ*narG* + *narG*	160 ± 12	1,700 ± 72	1,000 ± 150
RVW2	Δ*narX*	150 ± 7	1,400 ± 34	2,600 ± 350
RVW3	Δ*narK2*	110 ± 8	35 ± 5	910 ± 80

[a] Nitrate reductase activity as measured by the nitrite concentration (micromolar). Values are means ± standard deviations.
[b] After 112 h of growth (OD_{580} of ~0.4).
[c] After 112 h, approximately 45 h in NRP-1 (OD_{580} of 0.1).
[d] After 280 h of growth (OD_{580} > 1.0).
[e] Strain RVW1 (Δ*narG*) with plasmid pNarGHJI1 (*narG*) regained Nar activity.

tis. *M*. *smegmatis* strains carrying either plasmid were tested for their ability to produce nitrite from nitrate. During aerobic growth at mid-logarithmic phase, wild-type *M*. *smegmatis* produced only 0.7 μM nitrite. *M*. *smegmatis* complemented with pNarGHJI1 produced 27 μM at the same stage of growth, but with pNarX, it produced only 2 μM. In NRP-1, wild-type *M*. *smegmatis* produced 2 μM nitrite; when it was complemented with pNarGHJI1, it produced 14 μM, and when it was complemented with pNarX5, it produced 2 μM. Thus, *M*. *tuberculosis narGHJI* was able to introduce nitrate reductase activity to *M*. *smegmatis* but not the characteristic hypoxic increase in activity. When this plasmid was electroporated into *M*. *tuberculosis* RVW1 *narG*::*aphI*, the complementation restored the lost Nar activity (Table 4).

Ability to support anaerobic growth of *E. coli*. Anaerobically induced nitrate reductase enzymes support the anaerobic replication of many bacteria. Nevertheless, neither *M*. *tuberculosis* nor *M*. *smegmatis* with pNarGHJI1 could be induced to grow under anaerobic conditions either with or without nitrate. Therefore, the ability of the *M*. *tuberculosis narGHJI* operon to complement a *nar E*. *coli* mutant was investigated. *E*. *coli* JCB4023 lacks all three of the nitrate reductase enzymes of *E*. *coli* and consequently does not grow anaerobically with glycerol as the sole carbon source (31). JCB4023 that was complemented with pNarGHJI1 from *M*. *tuberculosis* acquired the ability to grow anaerobically but only in the presence of nitrate (Fig. 5). JCB4023 complemented with the vector pPE207 alone showed no anaerobic growth.

Knockout of *narK2*. Surprisingly, the transcription of *narGHJI* does not increase during hypoxia and enzyme levels do not change, despite a strong increase in nitrate reductase activity in whole cells. We hypothesized that this induction could be due to an increase in nitrate and nitrite transport under hypoxic conditions rather than an increase in nitrate reductase enzyme levels. The role of nitrate and nitrite transport in nitrate reduction was next addressed using an *E*. *coli* mutant. In *E*. *coli*, NarK and NarU are both proposed to be responsible for the transport of nitrate and nitrite (27, 34). In *M*. *tuberculosis*, four genes, *narK1* to *narK3* and *narU* are homologous to *narK* and *narU* (11). One of these genes, *narK2*, is located upstream of *narX* (Fig. 1) and is upregulated during hypoxia (Fig. 2 and 3).

To determine whether *narK2* of *M*. *tuberculosis* can function as a transporter of nitrate and nitrite, *E*. *coli* JCB4018 was used (9). In this mutant, the sole nitrate-reducing enzyme is located intracellularly and requires transport of nitrate into the cell for activity. This mutant also contains knockout mutations in both of the transporters genes (*narK* and *narU*) and therefore lacks nitrate reductase activity in culture despite functional NarGHJI enzyme (9). Under anaerobic conditions, JCB4018 with only vector pSK did not produce nitrite (Fig. 6). *narK2* from *M*. *tuberculosis* was cloned into pSK to produce pTSJ4. JCB4018 with pTSJ4 was able to reduce nitrate, showing that *narK2* can function in nitrate and nitrite transport. Interestingly, nitrite production from JCB4018 with *narK2* from *M*.

TABLE 5. Nitrate reductase activity of *narG*, *narX*, and *narK2* mutants in the cell-free assay

Strain	Genotype	Nitrate reductase activity[a]		
		Aerobic[b]	NRP-1[c]	SSP[d]
RVW1	Δ*narG*	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02
RVW2	Δ*narX*	18 ± 2	21 ± 2	8 ± 2
RVW3	Δ*narK2*	25 ± 4	23 ± 2	6 ± 1

[a] Mean nitrite reductase activity (in nanomoles of NO_2^- per minute per milligram of protein) ± standard deviation.
[b] After 112 h of growth (OD_{580} of ~0.4).
[c] After 112 h, approximately 45 h in NRP-1 (OD_{580} of 0.1).
[d] After 280 h of growth (OD_{580} > 1.0).

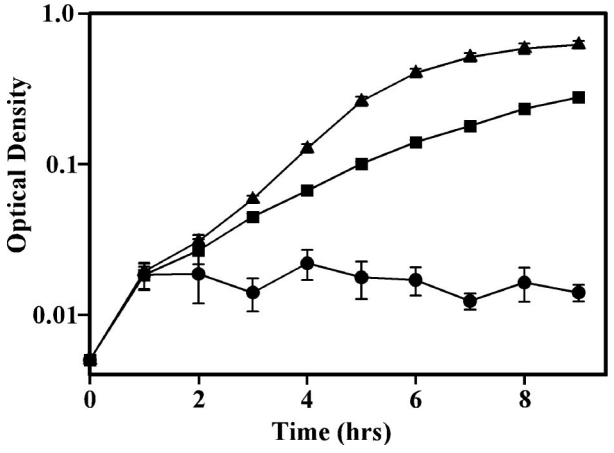

FIG. 5. Anaerobic growth of an *E*. *coli nar* mutant with *M*. *tuberculosis narGHJI1*. *E*. *coli* was grown anaerobically in M9 medium containing glycerol and 20 mM $NaNO_3$. Symbols: ▲, wild type; ■, *E*. *coli* JCB4023 with pNarGHJI1 (complementation mutant); ●, *E*. *coli* JCB4023 with pPE207 (mutant with only the vector).

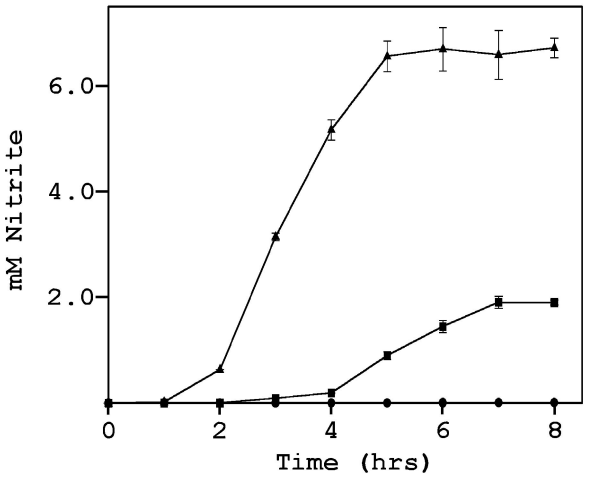

FIG. 6. Nitrite production by an *E. coli* mutant with *M. tuberculosis narK2*. *E. coli* was grown anaerobically in LB medium containing 20 mM $NaNO_3$. Symbols: ▲, wild type; ■, *E. coli* JCB4018 pTSJ4 (complementation mutant); ●, *E. coli* JCB4018 with pSK (mutant with only the vector).

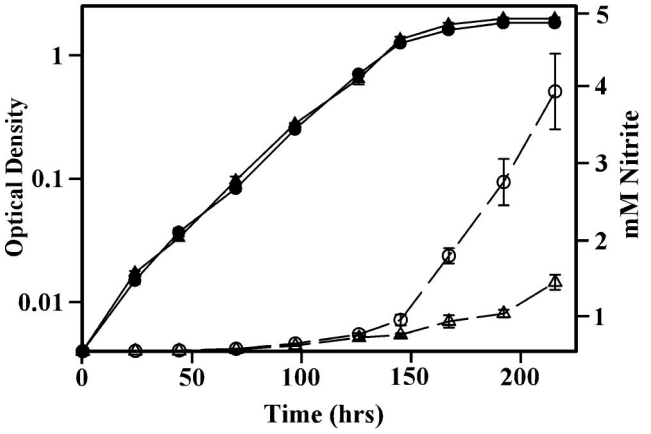

FIG. 7. Induction of nitrate reductase levels in SSP. *M. tuberculosis* was grown aerobically in DTA medium with 5 mM $NaNO_3$, and nitrite concentrations were determined at intervals. Growth (measured by OD_{580}) (solid symbols) and nitrite levels (open symbols) of the wild-type strain (circles) and strain RVW3 *narK2*::*aphI* (triangles) are shown.

tuberculosis ceased at approximately 1.9 mM, while the wild-type strain with *E. coli narK* and *narU* genes continued to 6.5 mM. *M. tuberculosis* under hypoxic, but not aerobic, conditions ceases nitrite production at a level of approximately 2.5 mM (50). This suggests the possibility that this plateau in nitrite production may be due to *narK2*.

To determine the role of *narK2* in the hypoxic induction of nitrate reductase activity in *M. tuberculosis*, this gene was inactivated. A kanamycin resistance marker was inserted into *narK2* to create *M. tuberculosis* RVW3 *narK2*::*aphI* (Fig. 4). RVW3 produced essentially identical optical density growth curves to the wild type under aerobic and NRP conditions (Sohaskey, unpublished). This mutant showed wild-type levels of nitrite production in aerobic cultures (Table 4). However, there was no increase in activity in NRP-1 cultures, although low levels of nitrite were still produced. These levels were well above those of the *narG* knockout strain RVW1.

Induction of nitrate reductase activity in SSP. With the creation of the *narK2* knockout mutant, the question of induction of nitrate reductase activity in SSP could again be addressed. Transcription of *narGHJI* is not induced in stationary phase, but *narK2* is (Fig. 3). However, it was difficult to detect changes in nitrate reductase activity in the face of decreasing cell viability. If strain RVW3, in which the *narK2* gene has been deleted, also lacks this induction, it should be easily detected by comparison to the wild type. The wild-type strain and RVW3 *narK2*::*aphI* were grown aerobically in parallel, and nitrite production was monitored (Fig. 7). The growth of both strains reached a plateau after 145 h of incubation, but the rate of nitrite production from the wild type continued to increase, while that of RVW3 decreased. This indicates that nitrate reducing activity is induced upon entry into SSP and that this upregulation is due to induction of *narK2*.

DISCUSSION

There are two sets of genes in *M. tuberculosis*, *narGHJI* and *narX*, that show homology with prokaryotic nitrate reductase genes, but only *narGHJI* is responsible for nitrate reducing activity in culture. Insertional inactivation of this locus eliminated the production of nitrite, and this activity could be restored by complementation with a plasmid-borne copy of the genes. Insertion in *narX* had no effect on the reduction of nitrate. The increase in nitrate reductase activity in hypoxic culture was due not to induction of *narGHJI* but to increased levels of the nitrate and nitrite transporter *narK2*.

Nitrate reductase activity of *M. tuberculosis* was sensitive to inhibition by both tungstate and azide, suggesting that this enzyme is a membrane-bound molybdenum-containing complex similar to the corresponding *narGHJI* of *E. coli* (41). It also appeared to be functionally similar to that of *E. coli*, having the ability to complement a defective strain of the latter in terms of both the ability to reduce nitrate and to support anaerobic growth. *M. tuberculosis*, unlike *E. coli*, does not increase either *narGHJI* mRNA (Fig. 3) or enzyme levels (Table 3) in response to hypoxia or stationary phase. In support, microarray analysis of transcription in *M. tuberculosis* has shown little induction of *narG* and *narH* after a shift from 20 to 0.2% oxygen, while under these same conditions, *narK2X* was induced (36). In *M. bovis* BCG, *narK2X* was induced during hypoxic shiftdown (20). *narK2X*, but not *narGHJI*, are members of the *dosR*-controlled NRP regulon. The *dosR* product induces a set of 48 genes in response to either hypoxia or NO (5, 45). Both of these conditions are thought to exist in granulomas and play a role in triggering nonreplicating persistence. Upstream of the transcription point of *narK2X*, there are two copies of a DosR binding sequence proposed to be important for this regulation (29). No such sequence is found upstream of *narGHJI*. Interestingly, there is a possible FNR box (39) upstream of *narGHJI* (TTGATnnnnATCCAAT [n is any nucleotide]) but not *narK2X*. In *E. coli* and many other bacteria, FNR regulates gene expression in response to hypoxia, but an FNR ortholog has not been identified in *M. tuberculosis*.

In SSP, *narK2X* but not *narGHJI* was induced in *M. tuberculosis* (Fig. 3). In aerobic shaking cultures, it is possible that a large number of respiring cells could deplete oxygen faster

than it could dissolve, creating hypoxic conditions despite the apparent abundance of oxygen.

Unexpectedly, transcription of both *narGHJI* and *narK2X* were independent of nitrate and nitrite levels. Nitrate reductase activity in *M. tuberculosis* appears to be independent of the substrate concentration as determined by levels of mRNA (Fig. 3) and assays of cell extracts (Table 3). In *E. coli*, the transcription factor NarL regulates genes in response to nitrate in the environment. A possible NarL (Rv0844c) has been identified in *M. tuberculosis*. Both the upstream regions of *narGHJI* and *narK2X* lack good NarL binding sites (43).

It is especially interesting that despite the increase in activity of whole cells of *M. tuberculosis* exposed to hypoxic conditions, nitrate reductase does not appear to support actual anaerobic growth of this species. Instead, it shifts down to the state of nonreplicating persistence as microaerobic conditions develop. Shiftdown appears to be an orderly process, and the cessation of replication also appears to be part of the cell's adaptation to hypoxia rather than simply energy starvation (52). Since *M. tuberculosis* does not grow under anaerobic conditions, there may be no requirement for induction of *narGHJI*. The primary role for nitrate reductase in *M. tuberculosis* could be redox balancing, or it may serve only a temporary function to provide energy during shiftdown to NRP.

To be reduced, nitrate must enter the cell where the catalytic site of the enzyme is located. Subsequently, since *M. tuberculosis* is unable to reduce nitrite, which could accumulate to toxic levels, it must then be exported out of the cell. Early work in *E. coli* had suggested that *narK* was involved only in nitrite export (34), and so the homologous *narK2* in *M. tuberculosis* was annotated as a "nitrite extrusion protein" (11). More recent work with an *E. coli narK narU* double mutant indicated that the two proteins could transport nitrate into and nitrite out of the cell (9). We show here that *M. tuberculosis narK2* can complement this *E. coli* double mutant, supporting a role for *narK2* in nitrate reduction by coding for a transporter of nitrate into and nitrite out of the cell.

M. tuberculosis RVW3 *narK2*::*aphI*, which lacks the nitrate and nitrite transporter, behaved like the wild-type strain in its nitrate reductase activity under aerobic conditions (Table 4). This low level of activity reflects the low rate of diffusion of nitrate into the cell, and this conclusion is supported by evidence that the rate of nitrate reduction by *M. tuberculosis* under aerobic, but not hypoxic, conditions is proportional to the nitrate concentration in the medium (50). During shiftdown to hypoxic NRP-1, nitrate reductase activity levels of RVW3 *narK2*::*aphI* lacked the strong induction seen in the wild type but instead continued at aerobic levels. This indicates that NarK2 is responsible for the hypoxic rise in activity by transporting nitrate into the cell.

In most bacteria in which transcription has been characterized, *narGHJI* and *narK* are induced by hypoxia (3, 26, 30, 54). This makes a determination of the role of each in the regulation of nitrate reductase activity difficult. In *M. tuberculosis, narGHJI* is not induced by hypoxia. Instead, *narK2 is* a major factor in the regulation of nitrate reductase activity. Nitrate reductase is regulated by control of the level of transcription of *narK2*, which controls the transport of nitrate into the cell. This is the first report of regulation of nitrate reduction solely by control of transcription of the nitrate transporter.

The gene *narX* has been found only in *M. tuberculosis* and *M. bovis*. It was designated a fused nitrate reductase, because it codes for a single protein that is homologous to sections of three proteins of the NarGHJI complex. The amino terminus from amino acids 1 to 256 is homologous to the same region of NarG, which is the catalytic subunit of the enzyme (10). The three cysteines and one histidine of NarG implicated in the binding of the [4Fe-4S] cluster (33) are present in NarX, but the amino acids thought to be responsible for the binding of the molybdopterin guanine dinucleotide cofactor are missing. Amino acids 257 to 413 of NarX are homologous to NarJ, which is not part of the nitrate reductase enzyme complex but is necessary for maturation of this complex (10). The carboxyl end of NarX from amino acids 417 to 652 is homologous to NarI, a *b*-type cytochrome. NarI is predicted to contain five membrane loops which are all present in NarX. The conserved histidine and glycines that are important for binding and packaging the two heme groups (4) are all found in NarX. Regulation of nitrate reductase activity does not appear to be associated with *narX*, and its function is still unknown. Transcripts of *narX* were detected in a low percentage of granulomas from the lungs of tuberculosis patients, showing that it is expressed in vivo (17).

Virulent *M. bovis* and avirulent BCG showed significantly less nitrate reductase activity than *M. tuberculosis* (Table 2), and this trait has been used to distinguish the two species (44, 48). *M. bovis* was not completely deficient in this activity, as can be seen by comparison to the *narG* knockout (Table 4), but it also did not show the hypoxic induction exhibited by *M. tuberculosis*. *M. bovis* BCG Pasteur lacked nitrate reductase activity in all stages of growth. *M. bovis* BCG Pasteur is reported to have a deletion of *narH*, which could explain this lack of activity (20). Virulent *M. bovis* has the complete *narGHJI* and *narK2X* operons (19). Analysis of the sequences of *M. tuberculosis* and *M. bovis* predicts two amino acid changes in NarG and one in NarI, while NarH, NarJ, and NarK2 are predicted to be identical in the two species. Transcription levels of these genes may also be different in the two species. This emphasizes the often overlooked variations between these species. *M. bovis*, for example, prefers a reduced oxygen tension and causes different disease manifestations (15, 51).

The role of nitrate reductase in the virulence of *M. tuberculosis* has not been investigated. However, immunodeficient SCID mice infected with an *M. bovis* BCG *narG* mutant showed smaller granulomas with fewer bacteria than those infected with the wild-type strain (53). The mutant produced tissue damage in the lungs of immunocompetent mice but was cleared from many organs, unlike the wild-type strain (18). The 50% lethal dose of a *Salmonella enterica* serovar Typhimurium nitrate reductase-deficient mutant was increased in mice relative to the wild-type strain, but its virulence was not completely attenuated (12). The *M. tuberculosis* equivalent, RVW1, may also be attenuated and might be a candidate for a safe and more effective live vaccine against tuberculosis.

It has been proposed that hypoxia may be partly responsible for the plateau in bacillus counts seen after primary infection with *M. tuberculosis*, which results in a latent infection (52). Differences between the avirulent and virulent forms of *M. tuberculosis* have been attributed in the past to enhanced ability of the former to grow at lower O_2 concentrations (51). Hypoxic

conditions within phagosomes of macrophages are probably sufficient to induce *narK2X*. Indeed, activated macrophages produce an oxygen gradient between the phagosomes and the extracellular space (22). The reduction of nitrate in the absence of oxygen may serve to provide an alternative energy source to the cell, as it adapts to decreasing oxygen levels. This adaptation allows tubercle bacilli to survive microaerobic conditions that may exist in granulomas or macrophages.

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