Ribonucleotide Reduction in *Mycobacterium tuberculosis*: Function and Expression of Genes Encoding Class Ib and Class II Ribonucleotide Reductases

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Mycobacterium tuberculosis, the causative agent of tuberculosis, possesses a class Ib ribonucleotide reductase (RNR), encoded by the *nrdE* and *nrdF2* genes, in addition to a putative class II RNR, encoded by *nrdZ*. In this study we probed the relative contributions of these RNRs to the growth and persistence of *M. tuberculosis*. We found that targeted knockout of the *nrdF2* gene could be achieved only in the presence of a complementing allele, confirming that this gene is essential under normal, in vitro growth conditions. This observation also implied that the alternate class Ib small subunit encoded by the *nrdF1* gene is unable to substitute for *nrdF2* and that the class II RNR, NrdZ, cannot substitute for the class Ib enzyme, NrdEF2. Conversely, a Δ*nrdZ* null mutant of *M. tuberculosis* was readily obtained by allelic exchange mutagenesis. Quantification of levels of *nrdE*, *nrdF2*, *nrdF1*, and *nrdZ* gene expression by real-time, quantitative reverse transcription-PCR with molecular beacons by using mRNA from aerobic and O_2-limited cultures showed that *nrdZ* was significantly induced under microaerophilic conditions, in contrast to the other genes, whose expression was reduced by O_2 restriction. However, survival of the Δ*nrdZ* mutant strain was not impaired under hypoxic conditions in vitro. Moreover, the lungs of B6D2/F$_1$ mice infected with the Δ*nrdZ* mutant had bacterial loads comparable to those of lungs infected with the parental wild-type strain, which argues against the hypothesis that *nrdZ* plays a significant role in the virulence of *M. tuberculosis* in this mouse model.

Mycobacterium tuberculosis is a formidable human pathogen that is estimated to infect one-third of the world's population (2). The success of this pathogen is attributable to its remarkable ability to persist for prolonged periods in a clinically latent state from which it is able to reactivate and cause disease. During the course of infection in humans, the tubercle bacillus is likely to encounter environments where there is limited O_2 availability, most notably the fibrous granulomas (9). This notion has underpinned efforts to identify the metabolic changes that occur in *M. tuberculosis* in response to hypoxia as a means of modeling the changes that may be associated with clinical latency (13, 37, 44). The value of this approach was underscored by a recent report (38) suggesting that during stationary infection in mice, the bacilli may be in a physiological state that approximates the nonreplicating persistence achieved in the widely used model of adaptation of *M. tuberculosis* to hypoxia in vitro developed by Wayne and coworkers (44, 45).

An important class of enzymes that includes both O_2-dependent and O_2-independent forms is the ribonucleotide reductases (RNRs), which catalyze the reduction of ribonucleotides to deoxyribonucleotides. These enzymes perform an essential role in the cycling of nucleotides in the cell and during replication of the chromosome in all organisms and provide attractive targets for antiproliferative drugs (40) and subunit vaccines (12). Although all RNRs contain an active free radical that is critical for catalytic activity, the essential metal cofactors have not been evolutionarily conserved. The oxygen-dependent, class I RNR enzymes are subdivided into classes Ia and Ib based on allosteric regulation and utilization of different electron donors (19). The class I RNRs consist of two homodimers in a $\alpha_2\beta_2$ subunit structure, and the *nrdAB* and *nrdEF* genes encode the large α-chain (NrdA or NrdE) and small β-chain (NrdB or NrdF) subunits of class Ia and class Ib RNRs, respectively. In contrast, *nrdJ*-encoded class II RNRs are O_2-independent α or α_2 forms and use adenosylcobalamin as a radical generator, whereas the *nrdDG*-encoded class III RNRs contain an O_2-sensitive glycyl radical generated by using *S*-adenosylmethionine. Despite these differences, common catalytic and allosteric mechanisms, as well as retention of critical residues in the protein sequence, suggest that the tertiary structures of all RNRs are similar and that all RNRs had a common evolutionary origin (reviewed in reference 34).

It has been proposed that the division of the classes based on O_2 sensitivity and the presence in many organisms of more than one RNR may allow adaptation to different O_2 levels in the environment (32, 34). Consistent with this notion is the
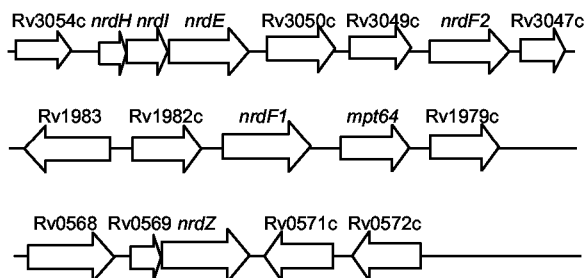
FIG. 1. Genomic organization of RNR-encoding genes in *M. tuberculosis* H37Rv. The gene notation is that of TubercuList (http://genolist.pasteur.fr/TubercuList/).

documented responsiveness of expression of certain bacterial RNR-encoding genes to O_2 availability (14, 24, 43); the regulation of this is poorly understood, but in the case of *Pseudomonas stutzeri nrdD*, expression appears to be controlled by an FNR-type regulator (43). However, the simultaneous presence of more than one class of active RNR in some organisms suggests that this may be a simplistic approach; for example, *Streptomyces clavuligerus* and *Pseudomonas aeruginosa* use both their class I and class II RNRs during aerobic growth (4, 21). Further complexity can also be provided by the presence of more than one enzyme belonging to the same class or subclass, such as the class Ia and class Ib RNRs of *Escherichia coli* (27), or by the presence of more than one large or small RNR subunit with nonoverlapping functions (17).

M. tuberculosis possesses a class Ib RNR encoded by *nrdE* (Rv3051c) and *nrdF2* (Rv3048c) (5, 46), as well as a putative alternate small subunit encoded by *nrdF1* (Rv1981c), which contains key catalytic residues but cannot associate with NrdE to form a functional RNR (46). In addition to these class Ib RNR-encoding genes, *M. tuberculosis* also contains a gene, *nrdZ* (Rv0570), which encodes a putative class II RNR (7) (Fig. 1). Significantly, transcription of this gene was shown recently to be dependent on DosR/DevR, the primary regulator of the hypoxic response in *M. tuberculosis* (31), suggesting that this organism might be capable of modulating deoxynucleoside triphosphate (dNTP) biosynthesis in response to changes in O_2 tension. To investigate this possibility, we analyzed the contributions of the class Ib and class II RNRs to growth of *M. tuberculosis* by targeted knockout of the *nrdF2* and *nrdZ* genes, *nrd* gene expression analysis by real-time, quantitative reverse transcription (RT)-PCR, and phenotypic characterization of a *nrdZ* mutant strain in a murine infection model.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are shown in Table 1. The vectors pNRDF2KO and pΔNRDZ were used for allelic replacement experiments, and pNRDF2 was used for genetic complementation. pNRDF2KO comprised a 5,263-bp *Eco*RI-*Not*I fragment derived from an *Eco*R1 library of *M. tuberculosis* Erdman (46) that was subcloned into p2NIL (30). The *nrdF2* allele was inactivated by insertion of the hygromycin B resistance cassette (*hyg*) from pIJ963 (1) into the unique *Bgl*II site 334 bp downstream from the start codon of the *nrdF2* gene. The markers *sacB* and *lacZ* were cloned into this construct as a *Pac*I cassette from pGOAL17 (30). A PCR-generated *nrdZ* amplicon was used to probe a gridded plasmid library of the *M. tuberculosis* genome (kindly provided by M. Everett, GlaxoSmithKline, Stevenage, United Kingdom). The pDWZ04 clone was found to contain the entire 2,076-bp *nrdZ* gene plus a 3,286-bp sequence downstream of the stop codon and 1,260 bp upstream of the start codon. A 1,568-bp *Pst*I-*Sal*I fragment and a 2,205-bp *Sal*I-*Kpn*I fragment were excised from pDWZ04 and cloned into the corresponding sites of p2NIL to obtain a vector containing a Δ*nrdZ* allele lacking the internal 871-bp *Sal*I fragment that encodes three of the five cysteine residues shown to be essential for RNR activity in *Lactobacillus leichmannii* (3). The *hyg-lacZ-sacB* cassette from pGOAL19 (30) was cloned into the *Pac*I site to obtain pΔNRDZ. The complementing plasmid, pNRDF2, was constructed by cloning a 1,876-bp *Sal*I-*Hin*dIII fragment containing *nrdF2* plus a flanking sequence consisting of 586 bp 5′ of *nrdF2* and 331 bp 3′ of *nrdF2* between the *Xho*I and *Hin*dIII sites of pGINT, which is a gentamicin-resistant (Gm^r) derivative of the integrative vector, pHINT (29).

Bacterial culture conditions. *M. tuberculosis* strains were grown in Middlebrook 7H9 medium supplemented with 0.2% glycerol and 0.05% Tween 80 (18) in roller bottles or as stirred cultures. The antibiotic supplements used were kanamycin (25 μg/ml), hygromycin B (50 μg/ml), and gentamicin (10 μg/ml). *E. coli* DH5α was used for all cloning procedures and was grown in Luria broth or Luria agar with 100 μg of ampicillin per ml when necessary. The electroporation conditions used and the method used for selection of merodiploid and allelic replacement strains were the conditions and method described by Gordhan and Parish (15). Wayne model conditions were produced in round-bottom Pyrex tubes (capacity, 30 ml) which were filled with 20 ml of Dubos albumin broth (Difco) with 0.05% Tween 80 as described by Wayne and Sohaskey (44) to generate a headspace-to-volume ratio of 0.5. The Wayne model for adaptation of *M. tuberculosis* to hypoxia induces a sequential shiftdown of the organism through two stages of nonreplicating persistence (NRPI and NRPII) in response to a self-generated, temporal O_2 gradient (45). In this model, the sealed culture replicates in a logarithmic fashion (log), but as O_2 becomes limiting (concentra-

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
DH5α	*supE44 ΔlacU169* (φ*80 lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Promega, Madison, Wis.
H37Rv	Virulent laboratory isolate (ATCC 25618)	Laboratory collection
Δ*nrdZ*	Mutant of H37Rv carrying a deletion in the *nrdZ* gene (Δ*nrdZ*)	This study
Plasmids		
p2NIL	Cloning vector; Km^r	30
pGOAL17	Plasmid carrying *lacZ* and *sacB* genes as a *Pac*I cassette; Ap^r	30
pGOAL19	Plasmid carrying *hyg*, *lacZ*, and *sacB* genes as a *Pac*I cassette; Ap^r	30
pHINT	*E. coli-Mycobacterium* integrating shuttle vector; Hyg^r Ap^r	29
pGINT	Derivative of pHINT carrying gentamicin resistance cassette; Gm^r Ap^r	S. Durbach
pNRDF2KO	Knockout vector carrying *nrdF2::hyg* allele; Hyg^r Km^r	This study
pNRDF2	Integrative complementing vector carrying *nrdF2* cloned in pGINT; Gm^r Ap^r	This study
pDWZ04	Subclone from gridded library of *M. tuberculosis* cloned in pBluescript carrying *nrdZ* gene and flanking sequences; Ap^r	This study
pΔNRDZ	Knockout vector carrying Δ*nrdZ* allele; Hyg^r Km^r	This study

TABLE 2. Oligonucleotides used in this study

Application	Primer	Sequence (5'-3')[a]
RT	nrdE-RT	cggcgtagacacttgcagga
	nrdF2-RT	gcctcatagccgaggttcatc
	nrdF1-RT	acgatcgaatgcaggctggt
	nrdZ-RT	tcggtcacaccaaccgatag
	hspX-RT	aaccgccaccgacacagt
	sigA-RT	ctgacatgggggcccgctacgttg
Amplification primer pairs[b]	nrdE-F1	gatcaaggcacgggagttctt
	nrdE-R1	ttggattagcgcgattgacg
	nrdF2-F1	gtctggcgttggttgacgac
	nrdF2-R1	cgtctgtagaggtcctgggtgt
	nrdF1-F1	gaccaccgcgaatacacctg
	nrdF1-R1	cgcatgtagggcaaaacgtc
	nrdZ-F1	cggaccggtgtgtcgtttctac
	nrdZ-R1	cttggcggtgacgaaatcac
	hspX-F1	ccgagcgcaccgagcagaag
	hspX-R1	ggtggccttaatgtcgtcctcgtc
	sigA-F1	tgcagtcggtgctggacac
	sigA-R1	cgcgcaggacctgtgagcgg
Molecular beacons	nrdE-MB	CCTCGCgagtccggctacccctatatcatgGCGAGG
	nrdF2-MB	GCAGCGccgagctcaaggactacacctaCGCTGC
	nrdF1-MB	GCTCCCatcgatcatgcgcacgacttgtacGGGAGC
	nrdZ-MB	GGACCCctgtatggctgtgctgtttgatgtgtcGGGTCC
	sigA-MB	CCTCGCgtcgaagttgcgccatccgaGCGAGG

[a] Lowercase letters indicate bases complementary to the *M. tuberculosis* sequence, whereas uppercase letters indicate bases added to form the stem of the molecular beacon.

[b] F1, forward primer; R1, reverse primer.

tion, <1%), replication ceases, and although the optical density continues to increase, the CFU counts do not (NRPI). The culture enters NRPII at O_2 concentrations less than 0.06%, when no further increase in optical density is observed. The 21 tubes used were inoculated with a 1/100 dilution of a log-phase culture in fresh Dubos medium and sealed with Parafilm internally and externally. One tube contained 0.5 μg of methylene blue per ml as an indicator. These cultures were stirred slowly with 5-mm magnetic stir bars (Fisher Scientific, Pittsburgh, Pa.). Tubes were harvested in duplicate or triplicate and chilled on ice prior to measurement of the optical density at 600 nm and harvesting of cells for RNA isolation. MICs of hydroxyurea (HU) for parental and Δ*nrdZ* strains were determined by using BACTEC 460-TB methodology (33) and BACTEC 12B vials (Becton-Dickinson, Towson, Md.) containing cyanocobalamin (Sigma-Aldrich, St. Louis, Mo.) at a concentration of 1 μg/ml and concentrations of HU (Sigma-Aldrich) ranging from 1 to 50 mM.

Isolation of RNA. RNA was isolated from 20-ml aliquots as described by Manganelli et al. (23) and was resuspended in 100 μl of diethyl pyrocarbonate-treated water. RNA was diluted approximately 10-fold prior to RT.

Quantification of RNA levels. Primers and molecular beacons were designed by using the Primer3 software (35), the virtual PCR amplification software AMPLIFY (11), and the DNA M-fold program (36). All primers (Table 2) were tested for the ability to amplify 10^2 to 10^5 genome equivalents in a concentration-dependent manner. Molecular beacons (Eurogentec, Seraing, Belgium) were synthesized with a 5' B-FAM group and a 3'-[4-(4-dimethylaminophenylazo)benzoic acid]succinimidyl ester group. RT primers (2.5 pmol each) were annealed to 4 μg of RNA by incubation at 94°C for 90 s, followed by 65°C for 3 min and 57°C for 3 min. RNA was reverse transcribed at 60°C for 30 min by using a *Carboxydothermus hydrogenoformans* two-step RT kit (Roche Molecular Biochemicals, Mannheim, Germany), and the enzyme was inactivated by incubation at 95°C for 5 min. One-tenth of the cDNA was used for real-time PCR analysis (duplicate 20-μl reaction mixtures) with each molecular beacon and amplification primer pair by using the Roche LightCycler system and FastStart DNA polymerase (both obtained from Roche Molecular Biochemicals) according to the manufacturer's instructions, and the amount was quantified by using genomic standards containing from 10^5 to 10^2 genome equivalents. HspX was quantified by using a LightCycler FastStart DNA Master SYBR Green 1 kit (Roche Molecular Bio-

chemicals). The cycle conditions were as follows: 95°C for 10 min, followed by 15 cycles of 95°C for 0 s, 65°C for 10 s, and 72°C for 10 s and then 25 cycles of 95°C for 0 s, 57°C for 10 s, and 72°C for 10 s.

Infection of mice, bacterial load, and survival. Ten-week-old female B6D2/F$_1$ mice (Charles River Laboratories, Wilmington, Mass.) were infected via the respiratory route as described previously (28). Mice were inoculated by exposure to an aerosolized suspension of the parental or Δ*nrdZ* strains of *M. tuberculosis* by using a nose-only exposure system (In-Tox Products, Albuquerque, N.M.) (42). This procedure resulted in implantation of approximately 100 organisms into the lungs of each mouse, which was confirmed by plating lung homogenates on Middlebrook 7H11 plates 3 h postinfection. At different times four mice from each group were sacrificed, and organs were harvested. Lungs, livers, and spleens were homogenized and plated to determine bacterial loads. A portion of the upper right lung was used for histological analysis. Five mice from each group were monitored for survival.

Statistical analysis. Culture tubes from the Wayne model analysis were harvested in triplicate unless otherwise indicated. RT reactions were performed twice with each RNA preparation, and PCRs were performed in duplicate. One-way analysis of variance was used to determine the significance of differences between data sets.

RESULTS

Targeted knockout of the *nrdF2* and *nrdZ* genes. To investigate the physiological role of the NrdEF2 class Ib enzyme, allelic exchange mutagenesis of *nrdF2* was attempted by using an *nrdF2::hyg* allele, which was constructed by insertion of a hygromycin resistance marker 333 bp downstream of the start codon of the gene (Fig. 2A). As expected, delivery of the mutant allele into the chromosome of *M. tuberculosis* H37Rv on the suicide plasmid pNRDF2KO yielded products of site-specific, single-crossover events. However, counterselection against *sacB*-containing single crossovers by plating on sucrose yielded no double crossovers. All sucrose-resistant clones were found to be spontaneous *sacB* mutants based on the following evidence: (i) all 27 clones were positive in a PCR amplification test for the Km^r cassette on the delivery vector (data not shown); and (ii) more than 200 clones produced blue colonies when they were plated on media containing the indicator X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), confirming the presence of a *lacZ* gene from the delivery vector (30). These results suggested that *nrdF2* might encode an essential function. To test this hypothesis, a single functional copy of *nrdF2* under the control of its own promoter was inserted at the *attB* locus. In the presence of the complementing gene, double crossovers were readily obtained at the chromosomal locus, as shown by the loss of the wild-type allele and the retention of the inactivated and complementing alleles in all 24 sucrose-resistant mutants (Fig. 2B). This result confirmed that *nrdF2* is essential for growth of *M. tuberculosis* in vitro and was consistent with previous biochemical evidence suggesting that NrdF1 cannot substitute for NrdF2 to form a functional class Ib RNR in association with the large NrdE subunit (46).

An additional implication of the finding that *nrdF2* is essential is that NrdZ does not provide sufficient RNR activity under standard in vitro culture conditions to allow *M. tuberculosis* to grow in the absence of NrdEF2. The *nrdZ* gene was shown to be dispensable for growth of *M. tuberculosis* under these conditions by the successful recovery of allelic exchange mutants carrying an unmarked deletion allele, Δ*nrdZ* (Fig. 2C and D), in which an internal gene segment spanning the region encoding three of the five cysteine residues identified as important
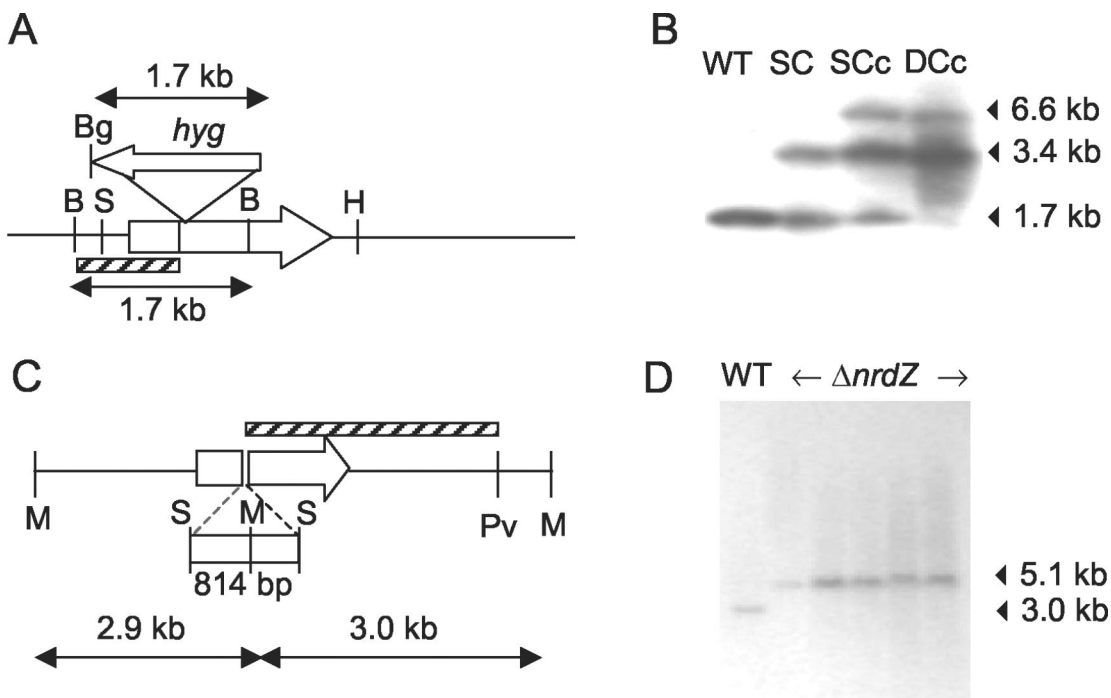
FIG. 2. Targeted knockout of RNR-encoding genes in *M. tuberculosis*. (A) Schematic representation of the inactivated *nrdF2* allele showing the site of insertion of the hygromycin cassette, the position of the 1,126-bp *Bam*HI-*Bgl*II probe (striped box) used for Southern blot analysis, and the extent of homologous DNA used in the knockout construct. (B) Southern blot analysis of *nrdF2* recombinant strains. Chromosomal DNA from wild-type *M. tuberculosis* strain H37Rv (WT), a single-crossover recombinant carrying tandem copies of the inserted and wild-type alleles (SC), a single-crossover recombinant carrying a functional *nrdF2* gene integrated at the *attB* locus (SCc), and a double-crossover recombinant with a crossover at the chromosomal locus of the *nrdF2* allele arising from an SCc background (DCc) were digested with *Bam*HI and probed with the *Bam*HI-*Bgl*II probe containing the 5′ portion of the *nrdF2* gene. (C) Schematic representation of the inactivated Δ*nrdZ* allele showing the 814-bp *Sal*I deletion and the positions of the 2,011-bp *Sal*I-*Pvu*II probe (striped box) and the *Mlu*I sites used for Southern blot analysis. (D) Southern blot analysis of five Δ*nrdZ* allele replacement strains. Chromosomal DNA from wild-type *M. tuberculosis* H37Rv (WT) and five Δ*nrdZ* isolates were digested with *Mlu*I and probed with the *Sal*I-*Pvu*II probe shown in panel C. Abbreviations: Bg, *Bgl*II; H, *Hin*dIII; S, *Sal*I; M, *Mlu*I; Pv, *Pvu*II.

for catalysis in *Lactobacillus leichmannii* class II RNR (3) was deleted. The growth rates of the Δ*nrdZ* mutant and its parental wild type were indistinguishable in all phases under standard culture conditions (data not shown).

Comparative sensitivities of Δ*nrdZ* and wild-type strains to HU. Class II RNRs require adenosylcobalamin as a cofactor. Several mycobacterial species, including *Mycobacterium smegmatis*, have been reported to synthesize cobalamin (22). Although bioinformatic analysis of the *M. tuberculosis* genome sequence has revealed the presence of homologues of most of the genes required to make this complex cofactor, it is not known if *M. tuberculosis* can actually synthesize cobalamin. To further investigate the contribution, if any, of *nrdZ* to growth and survival of *M. tuberculosis* under aerobic conditions, we compared the growth kinetics of the *M. tuberculosis* Δ*nrdZ* mutant to that of its parental wild type under conditions in which the class Ib activity was inhibited by the potent class I RNR inhibitor HU and cyanocobalamin was included in the medium in an attempt to ensure that there was sufficient adenosylcobalamin cofactor. The growth kinetics in the presence of HU (1 to 50 mM) were assessed radiometrically by using the BACTEC system. This analysis confirmed that HU inhibits the growth of *M. tuberculosis* H37Rv, with an MIC of 5 mM (Fig. 3), which is consistent with the susceptibility of the *M. tuberculosis* NrdEF2 enzyme to inhibition by this drug (46). How-

ever, no differential susceptibility to HU was observed for the two strains.

Quantification of *nrd* RNA levels. Since the O_2 requirements of the class I and II RNRs are different, we examined the
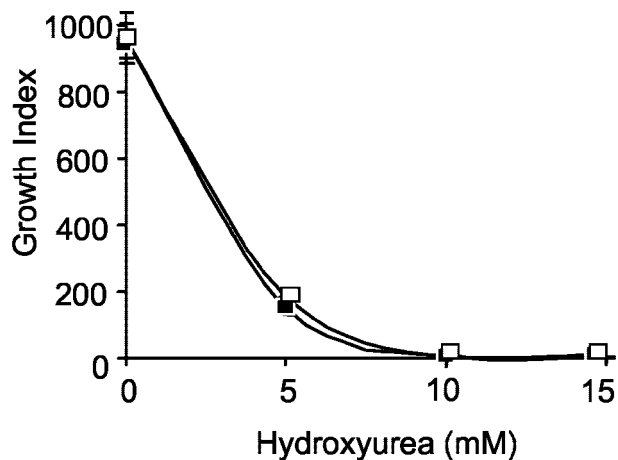

FIG. 3. Inhibition of *M. tuberculosis* strains H37Rv and Δ*nrdZ* by HU, as determined by BACTEC susceptibility testing. Cultures were inoculated into Middlebrook 7H12 test medium supplemented with cyanocobalamin containing different concentrations of HU. Growth (which was directly proportional to the growth index) was monitored for 10 days. Symbols: ■, H37Rv; □, Δ*nrdZ*.
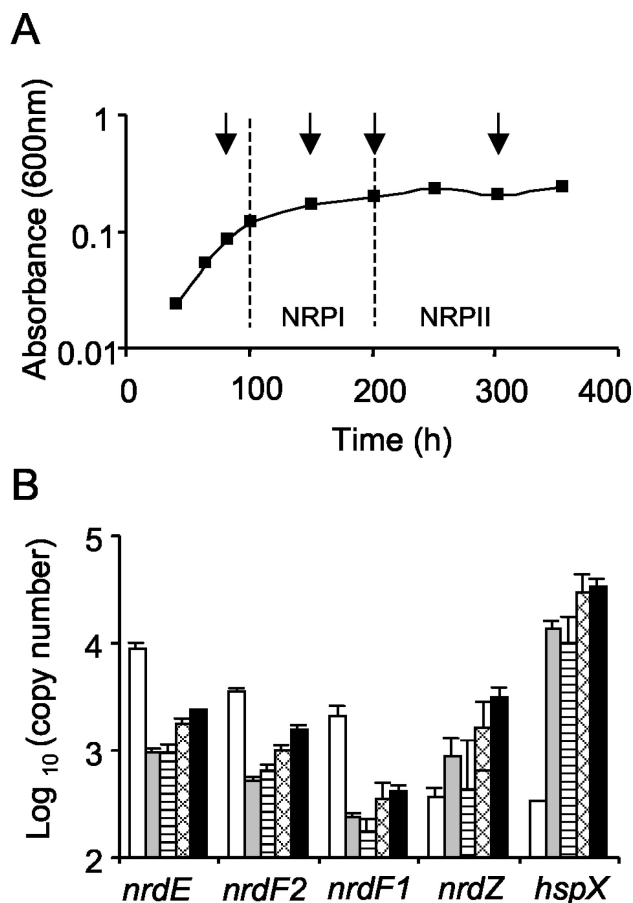
A

B

FIG. 4. Quantitative analysis of *nrd* gene expression in *M. tuberculosis* cultured under the Wayne model conditions (44, 45). (A) Growth of *M. tuberculosis* H37Rv in slowly stirred sealed tubes, showing a gradual cessation of growth as available oxygen becomes limiting. Three independent tubes were harvested at most of the times indicated by arrows; the exception was the 202-h time point, when two tubes were harvested. (B) Relative abundance of message as determined by quantitative RT-PCR by using molecular beacons in aerated (stirred) Dubos medium (open bars) and under Wayne model conditions. Copies of mRNA were normalized to 1,000 copies of *sigA* mRNA and the values shown are the means and standard errors. Log-phase samples were taken at 83 h (grey bars), NRPI samples were taken at 151 h (striped bars), NRPI/II samples were taken at 202 h (cross-hatched bars), and NRPII samples were taken at 302 h (solid bars).

FIG. 5. Growth of the *M. tuberculosis* H37Rv and Δ*nrdZ* strains in B6D2/F$_1$ mice. Mice were infected with *M. tuberculosis* by using an aerosol, and bacillary loads in the lungs were determined over a 14-month infection period, as described in Materials and Methods. Symbols: ■, mice infected with the parental strain; □, mice infected with the Δ*nrdZ* strain. Each point represents the mean for four mice, and the error bars indicate the standard deviations.

responses of expression of the various *nrd* genes to changes in the O$_2$ tension of the culture media. Baseline levels of *nrd* expression were obtained from cultures of *M. tuberculosis* H37Rv grown with agitation (shaking) to the mid-log phase. RNA from such a mid-log-phase culture was subjected to real-time RT-PCR analysis with molecular beacons for all four *nrd* genes (Table 2). The *hspX* gene was included as a positive control for induction of message under low-O$_2$-tension conditions (8, 10, 37). *nrdF2* and *nrdF1* were expressed at similar levels, but the levels were almost 2.5-fold lower than the levels of *nrdE* expression, whereas the level of *nrdZ* expression was 10-fold lower than the levels of expression of *nrdF2* and *nrdF1*. RNA was then isolated at various stages from cultures grown under the conditions of the Wayne model (44, 45) (Fig. 4A). The mRNA levels were normalized by comparison with the
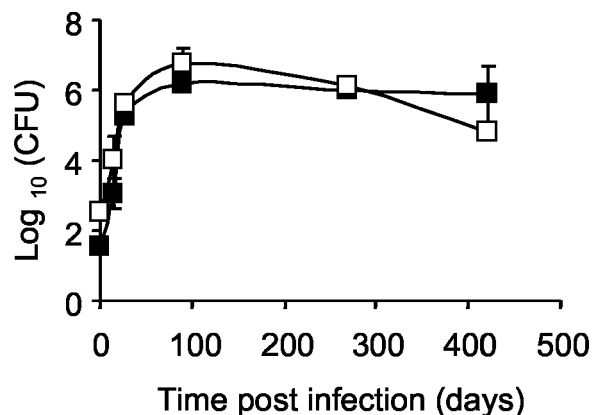
levels of transcription of *sigA*, a housekeeping gene that has been shown to be constitutively transcribed under a number of environmental stress conditions, including O$_2$ deprivation (16), and the significance was analyzed by using one-way analysis of variance (Fig. 4B). Interestingly, the levels of *nrdE*, *nrdF1*, and *nrdF2* in the log phase and NRPI were markedly lower than the levels obtained with well-aerated cultures (ninefold, ninefold, and sevenfold lower, respectively; $P < 0.001$). In contrast, *nrdZ* transcription was induced such that the *nrdE* and *nrdZ* levels were approximately equal. Interestingly, despite an initial drop, transcription of *nrdF2* and *nrdE* increased significantly, albeit slightly, as the O$_2$ in the culture became progressively depleted through NRPI/II and NRPII (NRPI versus NRPII, $P < 0.001$), and the ratios of *nrdE*, *nrdF2*, and *nrdZ* remained constant. Although there were net reductions in the levels of *nrdE*, *nrdF1*, and *nrdF2* in NRPII compared with the unstressed levels, *nrdZ* was upregulated eightfold compared with the unstressed levels. As expected, transcription of *hspX* responded positively to O$_2$ depletion.

Survival of the Δ*nrdZ* mutant under microaerophilic conditions. The induction of *nrdZ* under low-oxygen conditions led us to examine whether the Δ*nrdZ* mutant was impaired for survival under the conditions described for the stirred or settled Wayne model. However, the mutant displayed no impairment of survival compared with the parental wild-type strain in either model, as judged by plating efficiencies of aliquots taken 1 week after entry into NRPII in the stirred Wayne model or 6 months after entry into NRPII in the settled model (data not shown).

Survival of the Δ*nrdZ* mutant in mice. We next investigated whether loss of *nrdZ* had implications for survival of *M. tuberculosis* in mice infected by the aerosol route. B6D2/F$_1$ mice, which are known to be resistant to infection with *M. tuberculosis* (26), were used in order to amplify a possible survival defect in Δ*nrdZ*. Approximately 100-CFU portions of the parental or Δ*nrdZ* strain of *M. tuberculosis* were implanted into the lungs of mice (Fig. 5). The numbers of CFU in the lungs of

mice in both groups increased progressively until day 90 postinfection, and the levels reached about 6 $\log_{10}$ CFU. High numbers of viable mycobacteria persisted in the lungs for up to 14 months. No significant difference in the bacillary loads in the lungs was seen when we compared mice infected with the Δ*nrdZ* strain and mice infected with the wild-type *M. tuberculosis* H37Rv strain up to 14 months postinfection. Similarly, in both groups there were no significant differences in the numbers of CFU in the spleens and livers of the mice (data not shown), and histological analysis of lung samples obtained after 90 days and 9 months revealed comparable pathologies (data not shown).

DISCUSSION

In this study we used a combination of gene knockout and expression analysis to functionally characterize RNR-encoding genes in *M. tuberculosis*. The inability to inactivate *nrdF2* in the absence of a complementing copy of this gene confirmed that the class Ib enzyme NrdEF2 is essential for growth of *M. tuberculosis* and thus represents a valid target for novel antitubercular drug development. The finding that *nrdF2* is essential in *M. tuberculosis* is consistent with previous biochemical data for recombinant proteins, which suggested that NrdF1 could not substitute for NrdF2 to form a functional class Ib enzyme in association with NrdE (46). It has been shown that in *Saccharomyces cerevisiae* a second small subunit of the class Ib RNR, RNR4, although apparently without catalytic activity, is nonetheless essential for viability (17). Huang and Elledge postulated that this subunit has a structural or nonenzymatic regulatory role and suggested that the holoenzyme has a $\alpha_2\beta\beta'$ form. However, an insertion in *nrdF1* has been identified in a transposon library of *M. tuberculosis* (25), suggesting that the situation in *M. tuberculosis* does not parallel that in *S. cerevisiae*. The *nrdF1* gene is expressed in *M. tuberculosis* at a level comparable to that of *nrdF2*, suggesting that it may serve a cellular function, albeit not a function directly associated with ribonucleotide reduction. Interestingly, although the genes encoding the two subunits of the class Ib RNR are not arranged in an operon, they appear to be coordinately regulated in response to increasing oxygen depletion, in contrast to *nrdF1*, which did not show the same response. This observation is analogous to the coordinate regulation of the nonoperon class Ib RNR-encoding genes that has been found to occur in *Corynebacterium ammoniagenes* (41).

The putative class II RNR found in *M. tuberculosis* (NrdZ) is more closely related to the cobalamin-dependent class II enzymes found in archaeal organisms, such as *Archaeoglobus fulgidus* (52% identity over 562 amino acids), than to eubacterial class II enzymes. The *nrdZ* gene also has a notable distribution within the genus *Mycobacterium*; genome sequencing has revealed identical genes in *M. tuberculosis* and *M. bovis* but no homologs in *M. leprae*, *M. smegmatis*, or *M. avium*. Comparison of the amino acid sequence of *M. tuberculosis* NrdZ with the amino acid sequences of functionally characterized archaeal and eubacterial class II enzymes revealed conservation of residues essential for catalysis, but in spite of its predicted activity as a functional RNR enzyme, NrdZ was unable to compensate for the loss of NrdEF2 in *M. tuberculosis* under normal in vitro growth conditions. This finding contrasts with

the results obtained for *S. clavuligerus* (4), in which the class II enzyme NrdJ supplies most of the RNR activity during vegetative growth. Similarly, the class II RNR provides most of the activity in *Deinococcus radiodurans*, which also possesses a class Ib RNR (20). The expression level of the *M. tuberculosis* *nrdZ* gene under aerobic, in vitro growth conditions was 10-fold lower than that of the *nrdF2* gene, suggesting that the failure of NrdZ to compensate for a loss of NrdEF2 might be due to inadequate production of a functional RNR enzyme. Alternatively, the *M. tuberculosis* *nrdZ* promoter might be responsive to signals other than changes in the dNTP pool, by analogy with the situation in *E. coli*, in which the *nrdAB* and *nrdEF* operons are regulated differently and in response to different environmental signals (27).

The differences in the expression levels of the *nrd* and *hspX* genes observed in stirred, continuously aerated cultures and in the log phase in the Wayne model indicate that *M. tuberculosis* modulates gene expression in response to even small changes in oxygen availability. Interestingly, after the initial precipitous drop in the *nrdE* and *nrdF2* levels in the log phase of the Wayne model, slight but significant upregulation of both *nrdE* and *nrdF2* was observed during passage from NRPI to NRPII, suggesting that the enzyme may still contribute activity even under extreme O_2 limitation conditions. In contrast, *nrdZ* was highly and progressively upregulated in all stages of the Wayne model, with the highest level of *nrdZ* expression occurring in NRPII as the culture approached anaerobiosis. The levels of *nrdZ* remained elevated in NRPII and returned to normal after exit from this stage (data not shown). The *nrdZ* gene is located 29 bp downstream of a gene encoding a putative transcriptional regulator, Rv0569, and both genes are highly induced at an O_2 tension of 0.2% (31, 37), suggesting that the two genes may form a hypoxia-inducible operon.

In the Wayne model, DNA synthesis ceases in *M. tuberculosis* as the O_2 tension drops below 1% (45). Although DNA synthesis in the obligate anaerobe *Bacteroides fragilis* ceases in an analogous way after exposure to O_2, this organism possesses a class I RNR that is involved in survival during exposure to O_2 and that plays a role in maintaining dNTP pools for DNA repair and recovery following reintroduction into anaerobic growth conditions (39). We hypothesized that the reverse may be true in the obligate aerobe *M. tuberculosis*, with the O_2-independent RNR playing an analogous dNTP maintenance role under hypoxic conditions generated in vitro in the Wayne model or encountered in an in vivo infection. During nonreplicating persistence of *M. tuberculosis*, DNA replication is predicted to occur intermittently (if at all), which should reduce the demand for dNTPs. However, there is evidence which suggests that repair synthesis occurs during persistent infections (5), implying that under these conditions, dNTP pools must be maintained in *M. tuberculosis*, possibly even at elevated levels (6), to serve the repair function. The *M. tuberculosis* Δ*nrdZ* gene exhibited no survival phenotype in the Wayne model, suggesting that anaerobiosis per se, in the absence of any external stress such as that imposed by the immune response of the host, may not damage DNA to a sufficient extent to result in a survival phenotype. We therefore challenged a relatively *M. tuberculosis*-resistant strain of mice with the Δ*nrdZ* mutant and assessed its ability to proliferate and survive under immune surveillance conditions. However, the mutant

displayed little or no in vivo growth phenotype during the acute and chronic stages of infection, suggesting that NrdZ does not contribute significantly to the pathogenesis of mouse tuberculosis infection under the conditions employed in this study.

There are several possible explanations for the lack of a phenotype in the ΔnrdZ mutant. On the one hand, nrdZ might not encode a functional class II RNR. Alternatively, NrdZ may be functional, but its activity could be compromised by insufficient levels of cobalamin in *M. tuberculosis* under the growth conditions employed in this study. We have not excluded the possibility that in addition to the possible inability of *M. tuberculosis* to synthesize adenosylcobalamin, this bacterium cannot transport and/or convert cyanocobalamin to adenosylcobalamin. Alternatively, the NrdEF2 enzyme alone may provide sufficient activity for maintaining dNTP pools even under severely O_2-limiting conditions (19, 24), thus obscuring any possible contribution made by NrdZ. Work is currently under way to investigate these various possibilities.

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