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NADH Dehydrogenase Defects Confer Isoniazid Resistance and Conditional Lethality in *Mycobacterium smegmatis*

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Isoniazid (INH) is a highly effective drug used in the treatment and prophylaxis of *Mycobacterium tuberculosis* infections. Resistance to INH in clinical isolates has been correlated with mutations in the *inhA*, *katG*, and *ahpC* genes. In this report, we describe a new mechanism for INH resistance in *Mycobacterium smegmatis*. Mutations that reduce NADH dehydrogenase activity (Ndh; type II) cause multiple phenotypes, including (i) coresistance to INH and a related drug, ethionamide; (ii) thermosensitive lethality; and (iii) auxotrophy. These phenotypes are corrected by expression of one of two enzymes: NADH dehydrogenase and the NADH-dependent malate dehydrogenase of the *M. tuberculosis* complex. The genetic data presented here indicate that defects in NADH oxidation cause all of the mutant traits and that an increase in the NADH/NAD$^+$ ratio confers INH resistance.

Mycobacterium tuberculosis is exquisitely sensitive to the drug isoniazid (INH), and this sensitivity has made INH the core of tuberculosis chemotherapy and prophylaxis since the early 1950s (1, 37). However, mutant strains that are INH resistant (INH^r) frequently arise, such that as many as 30% of tuberculosis isolates are INH resistant in many large cities (10, 25, 46). In our effort to develop better tuberculosis drugs, we are trying to understand how INH kills sensitive mycobacteria and how mycobacteria become resistant.

INH inhibits the synthesis of mycolic acids, which are long-chain fatty acids that constitute a major structural component of the mycobacterial cell envelope (48, 52). Inhibition of mycolic acid biosynthesis most likely causes the bactericidal activity of the drug (47). The InhA enzyme catalyzes an essential step in fatty acid elongation and mycolic acid synthesis and is a target for INH action in *M. tuberculosis* and the soil saprophyte *Mycobacterium smegmatis* (3, 36). Structural studies have shown that a reactive form of INH attacks the NAD(H) cofactor that is bound to the InhA active site and generates a covalent INH-NAD adduct (39). Mutations that lead to overproduction of InhA are found in roughly 15 to 30% of resistant tuberculosis isolates (15, 29, 31, 49); several *inhA* missense mutations have also been identified (30). Mutations in *inhA* also confer resistance to a second drug, ethionamide (ETH), that is a closely related chemical analog of INH (3, 15).

INH is a prodrug which is converted to an active form by the KatG catalase-peroxidase (51). Mutation of the *katG* gene is the most common mechanism of INH resistance in *M. tuberculosis* (15, 27, 31, 49, 55). KatG is thought to catalyze the oxidation of INH, generating an electrophilic species that reacts with targets such as InhA (18). INH activation may occur by the peroxidase activity of the KatG enzyme (20, 41).

Many clinical INH^r isolates of *M. tuberculosis* (16%) have mutations that increase expression of the *ahpC* homolog, which is thought to encode one subunit of the NAD(P)H-dependent alkyl hydroperoxidase AhpCF (49). In some clinical strains, the increased expression of alkyl hydroperoxidase may compensate for a *katG* defect which causes peroxide sensitivity and reduced virulence (16, 40). A large fraction of the clinical isolates (13%) have *ahpC* promoter mutations and do not have *katG* mutations (49). The mechanism for INH resistance in these clinical isolates is unknown. Perhaps the increased expression of AhpC lowers the intracellular peroxide concentration and thereby prevents KatG-mediated peroxidation of INH (50, 56). A role for peroxides in INH sensitivity has been proposed (38).

This report describes a new mechanism for INH resistance in *M. smegmatis*. A survey of spontaneous INH^r mutants shows that at least half are defective for NADH dehydrogenase (Ndh type II), which oxidizes NADH and transfers electrons to quinones of the respiratory chain. Many of the *ndh* mutants have multiple phenotypes, including thermosensitivity (T^s), auxotrophy, and resistance to ETH. Some of the Ndh mutants have a T^s lethal phenotype, which indicates that Ndh is essential for the viability of *M. smegmatis*. Our genetic data indicate that INH^r and all of the other mutant phenotypes are due to defects in NADH oxidation which increase the intracellular NADH/NAD$^+$ ratio. We propose that the increased NADH concentration causes INH resistance by two mechanisms: it may interfere with KatG-mediated peroxidation of the drug (like the *ahpC* promoter mutations), and/or it may displace the INH-NAD adduct from the InhA active site.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1, and the plasmids employed are presented in Table 2. All *M. smegmatis* strains were derived from the laboratory strain mc^{2}155, which is proficient for transformation (42). All plasmids were maintained in *Escherichia coli* STBL2 (Gibco BRL), which allows stable propagation of plasmids containing mycobacterial genes.

Media. The liquid medium used for *M. smegmatis* cultures was Mueller-Hinton broth (Difco); Tween 80 was added to 0.2% (vol/vol). The rich medium employed was Mueller-Hinton medium (Difco). The minimal medium used was Middlebrook 7H9 broth (Difco) supplemented with 0.2% (vol/vol) glycerol or

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Source or reference
E. coli STBL2	*mcrA* Δ(*mcrBC-hsdRMS-mrr*) *recA1 endA1 gyrA96 thi supE44 relA1*	Gibco BRL
M. smegmatis		
mc^{2}155	*ept-1* (laboratory wild-type strain)	42
mc^{2}651	*ept-1 inhA1*	3
mc^{2}1390	*ept-1 ndh-4*	This study
mc^{2}1406	*ept-1 ndh-14*	This study
mc^{2}1407	*ept-1 ndh-15*	This study
mc^{2}1412	*ept-1 ndh-21*	This study
mc^{2}1416	*ept-1 ndh-29*	This study
mc^{2}1419	*ept-1 ndh-4*/pYUB801	This study
mc^{2}1420	*ept-1 ndh-4*/pYUB806	This study
mc^{2}1421	*ept-1*/pMV261	This study
mc^{2}1422	*ept-1*/pYUB805	This study
mc^{2}1423	*ept-1*/pYUB807	This study
mc^{2}1424	*ept-1 ndh-4*/pMV261	This study
mc^{2}1442	*ept-1 ndh-4*/pYUB805	This study
mc^{2}1443	*ept-1 ndh-4*/pYUB808	This study

0.2% glucose. Anitimicrobial agents (Sigma) were used at the following concentrations: INH, 25 μg/ml; kanamycin A monosulfate, 10 μg/ml; hygromycin B (Boehringer Mannheim), 150 μg/ml; and streptomycin sulfate, 100 μg/ml. *E. coli* strains were grown in Luria-Bertani broth containing the appropriate antibiotics—ampicillin, hygromycin B, and kanamycin (50 μg/ml)—to maintain selection for plasmids.

Isolation of spontaneous INH^r mutants. Spontaneous INH^r mutants were isolated from 25 independent cultures (30°C incubation; nonmutagenized) and plated onto selective medium containing INH at 25 μg/ml. The numbers of CFU in 10 of these cultures were determined by plating 10-fold serial dilutions onto nonselective medium. The plates were incubated at 30°C for 7 days and scored for the number of CFU. Plates used for isolation of INH^r mutants were incubated at 37°C for 5 days.

Selection for INH^r following EMS mutagenesis. Ethyl methanesulfonate (EMS) mutagenesis was used to isolate one INH^r T^s mutant, the *ndh-4* mutant. The protocol of Miller, involving a 45-min exposure to the mutagen, was followed (28). Mutagenesis increased the frequency of INH^r and Sm^r mutants ~10-fold and caused no detectable decrease in culture viability.

Measurement of INH sensitivity. Three independent cultures were grown to an optical density at 600 nm of 0.7, and 10-fold serial dilutions (0.1 ml each) were plated onto nonselective medium as well as medium containing INH; concentrations of the drug varied 2-fold, from 0.5 to 100 μg/ml. The MIC was scored as the concentration of drug that caused at least a 100-fold reduction in the number of CFU.

Tests of sensitivity to ETH, ethambutol, and streptomycin. Sensitivity was tested with a zone of inhibition assay using a top agar plating method. ETH was dissolved in dimethyl sulfoxide (50 mg/ml), and 10 μl was spotted onto the lawn of cells. (Application of dimethyl sulfoxide alone did not inhibit cell growth.) BBL antimicrobial Sensi-Disks were used to test ethambutol (50 μg) and streptomycin (50 μg) sensitivity. Sensitivity was determined by measuring the diameter of the zone in which the drug inhibited growth. These assays were repeated three times for each strain.

Tests for T^s lethal phenotypes. Tenfold serial dilutions of exponentially growing cultures were each spread onto two plates; one was incubated at 30°C to determine the number of CFU for that culture, while the other plate was incubated at 42°C for 2.5 days, scored for the presence or absence of colonies, and then transferred to 30°C. The data reported below are the fractions of cells that survive the 42°C incubation and form colonies upon transfer to 30°C and are the average values for data from three different experiments.

Tests of linkage to *inhA*. Linkage analysis was performed with cosmid pYUB325 as described previously (3). Cosmid pYUB325 contains a 45-kb segment of the *M. smegmatis* chromosome that includes the *inhA*$^+$ allele and a nearby Tn*5-seq1* insertion (encoding kanamycin resistance). Recombination substrates were prepared by linearizing cosmid pYUB325 with *Pac*I (New England Biolabs), which releases the chromosomal sequences as a single fragment. (The mycobacterial chromosome has no *Pac*I sites.) This substrate (~0.5 μg) was introduced into the mutant strains by electrotransformation (9). The entire transformation mixture was plated to kanamycin-containing medium, and the plates were incubated for 7 days at 30°C. Km^r recombinants (~200) were tested for INH sensitivity by patching them onto kanamycin-containing medium with and without INH and were tested for thermosensitivity by patching them to nonselective medium and incubating the plates at 42°C.

Identification of complementing genes by using cosmid pYUB412 genomic libraries. Genes which complement the mutant T^s phenotype were found by using large-fragment (45-kb) libraries of *M. smegmatis* and *Mycobacterium bovis* BCG and the integrative cosmid pYUB412 (Ap^r Hyg^r) (4). The *M. smegmatis* library was a gift from Franz C. Bange, and the *M. bovis* BCG library was a gift from Jordan Kriakov. The cosmid pYUB412 and the use of double *cos-Pac*I cosmids have been described previously (2, 17, 33). The libraries were introduced into one T^s mutant, strain mc^{2}1390, by electrotransformation (9); complementation of the T^s phenotype was selected at 42°C. Temperature-resistant (T^r) transformants were tested for Hyg^r (vector marker), for sensitivity to INH, and for the ability to grow on minimal medium. Genomic DNA was purified from those transformants which met these criteria (17). The complementing fragments were recovered from the chromosome by *Pac*I digestion, ligation into *Pac*I arms of pYUB412, and in vitro packaging into phage lambda (Stratagene Gigapac II). The recovered cosmids were then tested for complementation of the original mutant, mc^{2}1390; all recovered cosmids complemented the original mutant (six tested). Two of these cosmids, pYUB801 (*M. smegmatis*) and pYUB806 (*M. bovis* BCG), were used for shotgun subcloning into the *E. coli*-mycobacterial shuttle plasmid pMV261 (44).

The insert of cosmid pYUB801 (*M. smegmatis*) was subcloned to a 2.5-kb fragment that retained the ability to complement all the *ndh-4* mutant phenotypes (plasmid pYUB802). Sequence analysis identified one 1.4-kb open reading frame with significant homology to an *M. tuberculosis* gene on a 1.5-kb *Nhe*I-*Pst*I fragment of cosmid MTCY359 (CDS19) (6). This *M. tuberculosis* gene, when subcloned into pMV261 (pYUB805), complemented all of the mutant phenotypes.

A 2.2-kb fragment of *M. bovis* BCG DNA that complemented all of the mutant phenotypes was obtained from shotgun subcloning of cosmid pYUB806 to generate pYUB807. Sequence analysis identified an open reading frame that encodes a protein 60% identical to the *Thermus aquaticus* Mdh enzyme (32). The *M. bovis* BCG *mdh* gene (1.1 kb) cloned into pMV261 (pYUB808) complemented all of the mutant phenotypes.

DNA sequencing methods and PCR amplification. The inserts of plasmids pYUB802 (*M. smegmatis*; 2.5 kb) and pYUB806 (*M. bovis* BCG; 2.5 kb) were sequenced with the Applied Biosystems Prism Dye Terminator Cycle Sequencing Core kit with AmpliTaq DNA polymerase (Perkin-Elmer), using the Applied

TABLE 2. Plasmids used in this study

Plasmid	Description	Source or reference
pMV261	Km^r *E. coli*-mycobacterial shuttle plasmid	40
pYUB325	pYUB328::mc^{2}155 (45-kb cosmid; contains *inhA*, Tn*5-seq1*)	9
pYUB328	Ap^r cosmid with *Pac*I sites flanking the polylinker	37
pYUB412	Ap^r Hyg^r *E. coli*-mycobacterial shuttle cosmid (derivative of pYUB328); ColE1 origin; phage L5 *int attP* integrates into the *attB* site of the *M. smegmatis* chromosome	36
pYUB801	pYUB412::mc^{2}155 (45-kb cosmid; contains *ndh*)	This study
pYUB802	pMV261::mc^{2}155 (2.5-kb *Stu*I subclone from pYUB801; contains *ndh*)	This study
pYUB803	pMV261::*ndh* (1.6-kb *Pst*I-*Hin*dIII subclone of pYUB802)	This study
CY359	pYUB328::*M. tuberculosis* H37Rv (45-kb cosmid; contains *ndh*)	6
pYUB805	pMV261::*ndh* (1.5-kb *Nhe*I-*Pst*I subclone of CY359)	This study
pYUB806	pYUB412::*M. bovis* BCG (45-kb cosmid; contains *mdh*)	This study
pYUB807	pMV261::*M. bovis* BCG (2.5-kb *Eco*RV subclone of pYUB806; contains *mdh*)	This study
pYUB808	pMV261::*mdh* (1.1-kb PCR product of pYUB807; contains *mdh*)	This study

TABLE 3. Growth phenotypes of the different INH^r T^s mutants

Allele	Mutant class[a]	INH susceptibility (MIC, μg/ml)[b]	Thermosensitivity[c]	% Viability after 42°C incubation[c]	Nutrient supplement required[d]	ETH susceptibility (diam of inhibition zone ± SD)[e]	Relative Ndh activity[f]
ndh$^+$		5	T^r	100	None	4.3 ± 0.3	1
ndh-4	I	>100	T^s lethal	4	CAA	0	0.04
ndh-14	I (0.4)	>100	T^s lethal	8	CAA	0.1 ± 0.3	0.04
ndh-15	II (0.4)	>100	T^s	40	Ser or Gly	0.4	0.18
ndh-21	III (0.2)	>100	T^s	40	None	2.7 ± 0.3	0.39

[a] Mutants were isolated from the laboratory wild-type strain mc^2155 by INH^r selection at 30°C. The mutants were grouped according to their ability to grow on minimal medium. The number in parentheses is the fraction of spontaneous INH^r T^s mutants that are of this class. Data for two class I mutants are presented to show that the phenotypes of the *ndh-4* mutant are not an artifact of mutagenesis.

[b] INH susceptibility was determined as the minimum concentration that reduced the number of CFU 100-fold (see Materials and Methods). Susceptibility was tested at the permissive temperature (30°C).

[c] Thermosensitivity (T^s) was scored as the inability to grow at 42°C. The T^s lethal mutants could not survive 42°C incubation for 2.5 days, as indicated by a >90% reduction in viability after 42°C incubation. This viability, measured as the number of CFU that formed after transfer to the permissive temperature divided by the CFU of that culture, is the average of values from three different experiments.

[d] Many of the mutants failed to grow on minimal medium plus glycerol or glucose at the permissive temperature. Some mutants required supplementation of Casamino Acids (CAA); others grew on minimal medium supplemented with serine or glycine.

[e] ETH susceptibility was determined as the diameter of the zone (in millimeters) in which growth was inhibited by the drug; 500 μg was applied in a 10-μl volume.

[f] Ndh activity is the rate of NADH oxidation with menadione as an electron acceptor (see the Fig. 3 legend and Materials and Methods).

Biosystems model 377 automated DNA sequencer. The sequences of both strands were determined by primer walking.

The complementing gene of pYUB802 (*M. smegmatis*) was PCR amplified from chromosomal DNA of representative INH^r mutants and the INH-sensitive (INH^s) parent, mc^2155; the nucleotide sequence was determined, and the presence of a mutation was verified by repeating the sequencing with different primers.

Preparation of cell extracts and membrane fractions. Cultures (700 ml) of each strain were grown to an optical density at 600 nm of 0.6 to 0.7; the T^s mutants were grown at 30°C, and the T^r mutants were grown at 37°C. Cells were washed twice in cold buffer (50 mM KPO_4 [pH 7.5], 5 mM $MgSO_4$) and then resuspended in 1 ml of buffer per g of cell paste. DNase (20 μg) was added, and the cells were broken in a French pressure cell (16,000 lb/in^2; 10 pressure applications). Cell debris was removed by centrifugation (12,000 × *g*, 30 min), and membrane particles from the clarified extracts were enriched by centrifugation (100,000 × *g*, 2 h) on a sucrose step gradient (20% and 60% [wt/vol]). Membrane fractions were recovered at the interface of the 20% and 60% sucrose layers. Membrane fractions of *E. coli* LE392 were prepared to ensure that this method did not destroy the unstable type I NADH dehydrogenase activity; large amounts of this activity were detected. The methods used for the preparation of cell extracts and membrane fractions were modified from previously reported techniques (13, 22).

Enzyme assays. All assays were performed at room temperature in 50 mM KPO_4 (pH 7.5)—5 mM $MgSO_4$ in a total volume of 1 ml. Substrates were used at 100 μM (final concentration) unless otherwise stated. Reagents were purchased from Sigma Chemical Co. unless otherwise noted. NADH dehydrogenase activity in membrane fractions was measured spectrophotometrically by following the rate of NADH oxidation at 340 nm (ε_{340} = 6.2 mM^{-1} cm^{-1}) in the presence of menadione (5 mM stock solution in acetonitrile) (22). NADH dehydrogenase activity was also determined by measuring the rate of DCIP (2,6-dichlorophenol-indophenol, sodium salt) reduction at 610 nm (ε_{610} = 16.5 mM^{-1} cm^{-1}) in the presence of NADH (22). Type I NADH dehydrogenase activity (Nuo) was determined by measuring the rate of NHDH (reduced nicotinamide hypoxanthine dinucleotide) oxidation at 340 nm (22). Assays for succinate dehydrogenase activity were performed with disodium succinate (30 mM final concentration), measuring DCIP reduction. Since NADH dehydrogenase and succinate dehydrogenase are membrane-associated enzymes, assays were performed with membrane fractions in order to enrich these activities. Succinate dehydrogenase activity was measured to test for variation among the extracts prepared from the different strains. All strains showed similar succinate dehydrogenase activities. Malate dehydrogenase activity was measured in clarified cell extracts by following NADH oxidation in the presence and absence of oxaloacetic acid; activity was calculated as the oxaloacetate-dependent oxidation of NADH. KatG peroxidase activity was determined in clarified cell extracts by measuring the rate of *o*-dianisidine oxidation at 460 nm (ε_{460} = 11.3 mM^{-1} cm^{-1}) in the presence of H_2O_2 (0.06%) (21). Protein concentrations were determined with the Pierce BCA protein assay. Extract preparation and assays were performed on the same day. Assays for each mutant strain were always performed in parallel with those for an INH^s *ndh*$^+$ control strain; the data presented are the averages for three repetitions of each assay.

Nucleotide sequence accession numbers. The accession number for the *ndh* sequence of *M. smegmatis* is AF038423; the accession number for the *mdh* sequence of *M. bovis* is AF038422.

RESULTS

Isolation and phenotypic characterization of INH^r T^s mutants. We have taken a genetic approach that specifically identifies essential enzymes involved in INH action. Our initial hope was to identify T^s mutations in *inhA* in order to define the biochemical role of this enzyme in the growth of *M. smegmatis*. This problem was approached by isolating INH^r mutants at 30°C (using INH at 25 μg/ml) and testing for those that were thermosensitive and hence unable to grow at 42°C (no INH added). These traits were expected to be caused by a mutation that confers resistance and also causes thermolability of the InhA enzyme. Since INH^r T^s mutants were expected to be rare, resistant mutants were initially isolated from EMS-mutagenized cultures. Surprisingly, of 25 INH^r mutants tested, 12 were temperature sensitive for growth. One of these T^s INH^r mutants, the *ndh-4* mutant, was further characterized (Table 3).

The selections and screenings for INH^r T^s mutants were repeated without mutagenesis. Spontaneous INH^r (25 μg/ml) mutants arose at frequencies expected of single point mutations (10^{-6} to 10^{-7} per CFU). Of 66 different INH^r mutants tested, 50% were temperature sensitive for growth at 42°C. Most of the T^s mutants did not have T^s lethal defects; they tolerated incubation at 42°C (2.5 days) and continued to grow after transfer to the permissive temperature (30°C) (Table 3). Four of the T^s mutants had lethal defects and could not survive the 42°C incubation (e.g., *ndh-4* and *ndh-14*) (Table 3). All of the INH^r T^s mutants were at least 20-fold more INH resistant than the parent strain (Table 3).

Most of the INH^r T^s mutants (26 of 33) grew poorly at 30°C on minimal medium supplemented with glycerol or glucose. The INH^r T^s mutants could be grouped into three classes on the basis of their nutrient requirements (Table 3). The first class grew poorly on all media and required Casamino Acid supplements for growth on minimal medium (40% of the T^s mutants; e.g., the *ndh-4* and *ndh-14* mutants). The second class grew well on rich medium and required serine or glycine for growth on minimal medium (40% of the T^s mutants; e.g., the *ndh-15* mutant). The third class grew well on both minimal and rich media (e.g., the *ndh-21* mutant). We noted that many of the T^r INH^r mutants were also auxotrophs (Ser/Gly auxotrophs

and general auxotrophs), but these mutants were not further characterized.

One possible explanation for the observed INH resistance is that the mutants have a general metabolic defect that interferes with active-transport systems. This type of defect should confer resistance to other drugs as well. The possibility of a general transport defect was excluded by the finding that four representative mutants were equally sensitive to both streptomycin and ethambutol compared to the INH^s parent strain (data not shown).

Mutations in the *inhA* gene are correlated with coresistance to INH and ETH in *M. tuberculosis* and *M. smegmatis*, whereas mutations in *katG* are correlated only with INH resistance (3, 15). The INH^r T^s mutants also exhibited low-level resistance to ETH, which was indicated by the inhibitory zone being relatively smaller than that of the INH^s strain (Table 3).

The mutations conferring INH^r and T^s are not in the *inhA* gene. Linkage analysis was performed to determine whether the newly acquired INH resistance was due to mutations in or near the *inhA* gene (3). This study used a recombination substrate, cosmid pYUB325, which contains a 45-kb segment of the *M. smegmatis* chromosome that includes the *inhA*$^+$ allele and a nearby Km^r-encoding insertion. When linearized pYUB325 is introduced into *M. smegmatis*, Km^r recombinants can form by exchange of the homologous sequences flanking both sides of the Km^r element. This recombination can occur with allelic exchange at the *inhA* gene to incorporate the *inhA*$^+$ allele. A recipient strain with an *inhA* resistance mutation (strain mc^{2}651) forms Km^r transformants with coinheritance of the *inhA*$^+$ allele in 30 to 70% of the transformants; these recombinants are INH^s. Two of the INH^r T^s mutants (mc^{2}1390 and mc^{2}1407) were tested for linkage to *inhA* by linear transformation with pYUB325. None of the Km^r transformants acquired an INH^s or T^r phenotype. (One hundred Km^r recombinants from each mutant strain were tested.) We infer that the mutations causing INH^r and thermosensitivity are not located in or near the *inhA* gene.

The *ndh* genes of *M. smegmatis* and *M. tuberculosis* complement the phenotypes of the INH^r T^s mutants. A 45-kb chromosomal fragment from the *M. smegmatis* genome that complements the T^s phenotype of one INH^r mutant (*ndh-4*; mc^{2}1390) was identified from a large-insert genomic library (see Materials and Methods). The cosmids that conferred T^r growth also conferred INH and ETH sensitivity and prototrophic growth to the *ndh-4* mutant (e.g., cosmid pYUB801). This complementation was not an artifact of gene overexpression, since the cosmid vector (pYUB412) integrates into the mycobacterial chromosome and the complementing gene is expressed from its own promoter. A 1.6-kb fragment that complements the *ndh-4* mutant was isolated from cosmid pYUB801 (plasmid pYUB803). DNA sequence and BLAST analyses (1a) showed that the minimal plasmid pYUB803 has a 1.4-kb gene predicted to encode a 51-kDa protein similar to the type II NADH dehydrogenase of *E. coli* (Ndh; 13% identity and 56% similarity) and an Ndh homolog in a *Synechocystis* sp. (40% identity) (Fig. 1) (54).

The *ndh* homolog from *M. tuberculosis* complements all of the *ndh-4* mutant phenotypes (Fig. 2; Table 4). The *M. tuberculosis ndh* gene was identified on a 1.5-kb *Nhe*I-*Pst*I fragment of cosmid MTCY356 and was subcloned into plasmid pMV261 (pYUB805) (6). The Ndh enzyme of *M. tuberculosis* is 81% identical to the *M. smegmatis* homolog. Eight T^s mutants representing the three different T^s mutant classes (the *ndh-4*, *ndh-14*, *ndh-15*, and *ndh-21* mutants and four other mutants) were tested for complementation by the *M. tuberculosis ndh* gene; all of the phenotypes of each mutant were complemented.

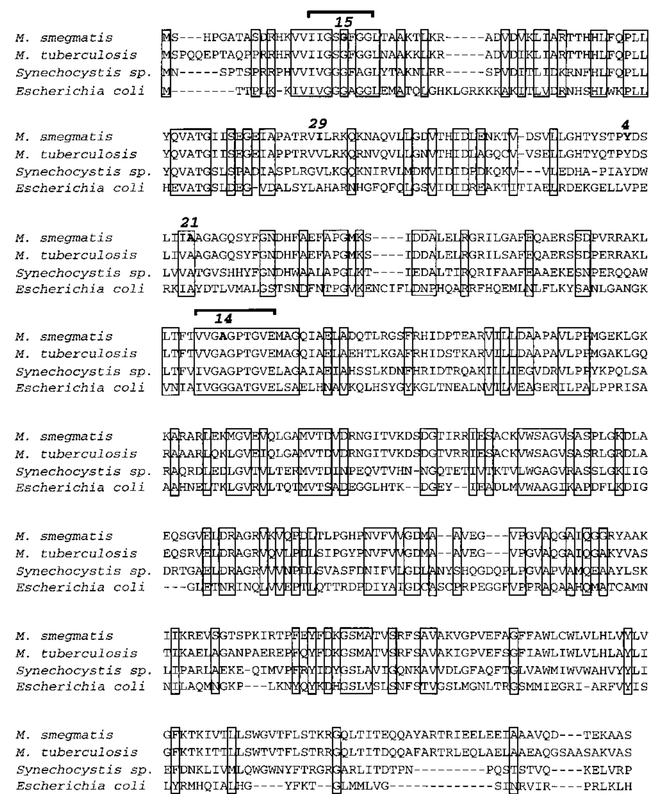

FIG. 1. Protein sequence alignment of the Ndh enzyme of *E. coli* and the putative homologs of *M. smegmatis*, *M. tuberculosis*, and a *Synechocystis* sp. The positions of amino acid substitutions of four different INH^r ETH^r T^s mutants are indicated with boldface print, and the corresponding mutant allele numbers are indicated above the substitution sites. Allele *ndh-15* has a G→S substitution, *ndh-4* has a Y→H substitution, *ndh-21* has an A→T substitution, and *ndh-14* has an A→S substitution. The *ndh-29* mutation (I→F substitution) confers INH^r but does not cause thermosensitivity; this mutant is described in the text. The first bracket indicates the putative FAD binding motif; the second bracket indicates the putative NAD binding motif (23). Conserved residues are boxed. The Ndh sequence of *M. smegmatis* is 81% identical to that of *M. tuberculosis*, 13% identical and 51% similar to the *E. coli* sequence, and 42% identical to the *Synechocystis* sp. sequence.

Identification of mutations in the *ndh* gene. The *ndh* gene was PCR amplified from the chromosomes of four INH^r ETH^r T^s mutants (the *ndh-4*, *ndh-14*, *ndh-15*, and *ndh-21* mutants). Sequence analysis identified a different missense mutation in each mutant, and two of the mutants, the *ndh-14* and *ndh-15* mutants, had substitutions in the highly conserved NAD and flavin adenine dinucleotide (FAD) binding domains (Fig. 1) (23).

A second gene, *mdh*, complements the *ndh* mutant phenotypes. An *M. bovis* BCG genomic library was used to identify genes of the *M. tuberculosis* complex that complement the *ndh-4* mutant. (The *M. tuberculosis* complex includes *M. tuberculosis*, *M. bovis*, other closely related virulent species, and the avirulent *M. bovis* BCG vaccine strain.) Surprisingly, a different complementing gene was identified. Subcloning and sequence analyses identified a gene encoding a 36-kDa protein that is 60% identical to the *T. aquaticus* malate dehydrogenase enzyme (Mdh; EC 1.1.1.37) (32). Mdh catalyzes the NADH-dependent interconversion of oxaloacetate and malate, a reaction of the tricarboxylic acid (TCA) cycle (35).

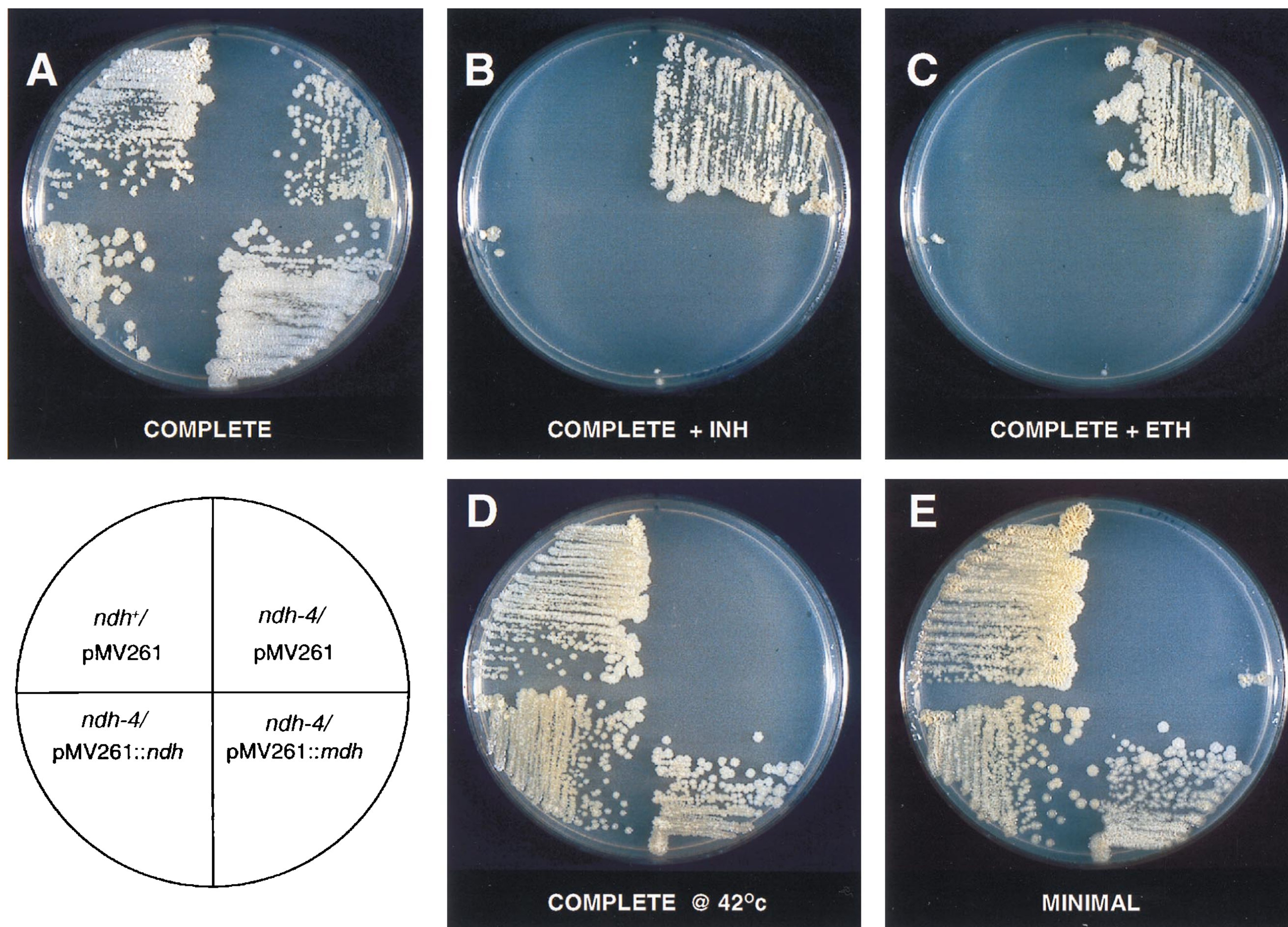

FIG. 2. Phenotypes of one representative *M. smegmatis* INH^r T^s mutant, the *ndh-4* mutant, and complementation with the *ndh* and *mdh* genes. The *ndh*$^+$ parent strain and the *ndh-4* mutant shown here carry the *E. coli*-mycobacterial shuttle plasmid pMV261, which confers Km^R. The lower half of each plate shows the *ndh-4* mutant complemented by the *ndh* gene of *M. tuberculosis* (left; pYUB805) and the *mdh* gene of *M. bovis* BCG (right; pYUB808); these genes are expressed from the *groEL* promoter of plasmid pMV261. The plate in panel D was incubated at 42°C for 4 days, all other plates were incubated 6 days at 30°C. The concentration of both INH and ETH was 50 μg/ml, and all plates contained kanamycin.

A minimal 1.1-kb fragment containing *mdh* fully complements all of the *ndh-4* mutant phenotypes when expressed from the pMV261 vector (pYUB808) (Fig. 2 and Table 4). Furthermore, in both the *ndh-4* mutant and the parent strain, mc^{2}155, *mdh* expression causes hypersensitivity to INH and ETH (Table 4). Eight T^s mutants representing the three different T^s mutant classes (the *ndh-4*, *ndh-14*, *ndh-15*, and *ndh-21* mutants and four other mutants) were tested for complementation by *mdh*; all of the phenotypes of each mutant were complemented. The *mdh* gene was PCR amplified from *M. tuberculosis* H37Rv genomic DNA, and the nucleotide sequence was found to be 100% identical to the *M. bovis* BCG sequence. (PCR amplification and sequencing were performed twice to verify this identity.)

The INH^r T^s mutants are defective for NADH dehydrogenase activity. The Ndh enzyme of *E. coli* catalyzes the first step in the respiratory chain: NADH oxidation and quinone reduction (54). Based on sequence similarity, we predicted that the mycobacterial homolog would have the same activity and that the mutants would be defective. Two different assays were used

TABLE 4. Complementation of the *ndh-4* mutant by minimal plasmids carrying the *ndh* or *mdh* gene[a]

Strain	Relevant genotype	Thermosensitivity	INH susceptibility (MIC, μg/ml)	ETH susceptibility (inhibitory zone diam ± SD)	Supplement required
mc^{2}1421	*ndh*$^+$/pMV261	T^r	5	3.5 ± 0.3	None
mc^{2}1422	*ndh*$^+$/pMV261::*ndh* (*M. tuberculosis*)	T^r	5	3.4 ± 0.1	None
mc^{2}1423	*ndh*$^+$/pMV261::*mdh* (*M. bovis* BCG)	T^r	1	4.6 ± 0.1	None
mc^{2}1424	*ndh-4*/pMV261	T^s lethal	>100	0	CAA
mc^{2}1425	*ndh-4*/pMV261::*ndh* (*M. tuberculosis*)	T^r	2.5	3.3 ± 0.3	None
mc^{2}1426	*ndh-4*/pMV261::*mdh* (*M. tuberculosis* complex)	T^r	1	5.8 ± 0.1	None

[a] All strains were derived from the *ndh*$^+$ parent strain mc^{2}155. Plasmid pYUB805 is pMV261::*ndh* (*M. tuberculosis*); plasmid pYUB808 is pMV261::*mdh* (*M. tuberculosis* complex). See footnotes to Table 3 for explanations of data categories.

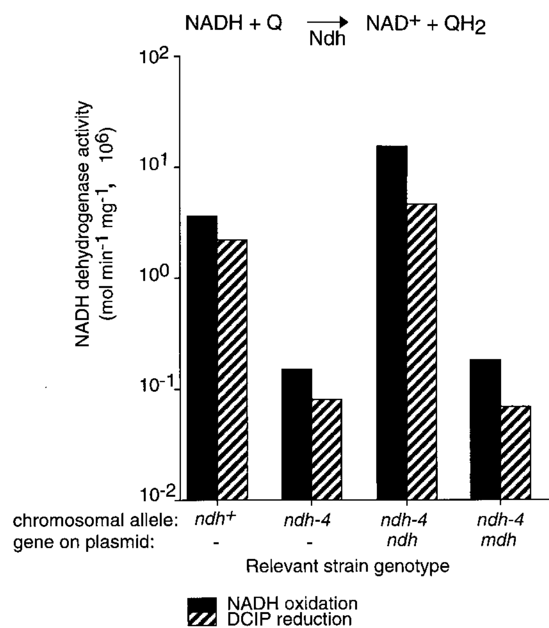

FIG. 3. Comparison of NADH dehydrogenase activities in the INH^s parent strain, the INH^r *ndh-4* mutant, and the *ndh-4* mutant complemented by the *ndh* and *mdh* genes. For each strain, the chromosomal allele and the plasmid-borne gene are indicated below the horizontal axis. The rate of NADH oxidation was measured with menadione as an electron acceptor (solid black bars). The rate of NADH-dependent DCIP reduction is represented by bars with diagonal stripes. Cultures were grown at 30°C, and assays were performed at room temperature. Membrane fractions were used for these assays; reaction conditions are described in Materials and Methods. The error (standard deviation) is less than 10% for all data points and is not shown.

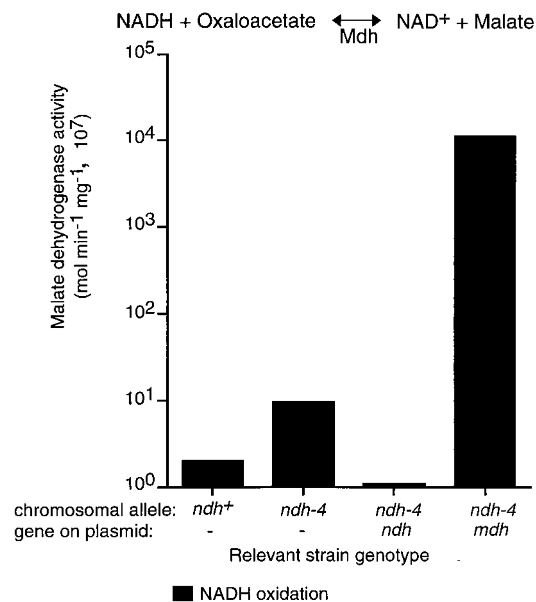

FIG. 4. Comparison of Mdh activity of the INH^s parent strain, the INH^r *ndh-4* mutant, and the *ndh-4* mutant complemented by the *ndh* and *mdh* genes. For each strain, the chromosomal allele and the plasmid-borne gene are indicated below the horizontal axis. These assays measure oxaloacetate-dependent NADH oxidation in clarified cell extracts. Oxaloacetate-independent NADH oxidation (background) is subtracted from these data. Assay conditions are described in the legend to Fig. 3 and in Materials and Methods.

to measure NADH dehydrogenase activity: oxidation of NADH in the presence of a quinone (menadione), and the NADH-dependent reduction of an electron acceptor (DCIP) (Fig. 3). These assays were performed with membrane fractions because the Ndh enzyme of *E. coli* is a peripheral membrane protein.

NADH dehydrogenase activity was measured in the *ndh*$^+$ control, the *ndh-4* mutant, and strains with the *ndh-4* mutation and the complementing plasmids (Fig. 3). The *ndh-4* mutant exhibited reduced NADH dehydrogenase activity relative to the *ndh*$^+$ control in both assays (25-fold reduction), and the complementing *M. tuberculosis ndh* gene restored activity to the *ndh-4* mutant (plasmid pYUB805). NADH dehydrogenase activity is specific for NADH; no oxidation of deamino-NADH (reduced nicotinamide hypoxanthine dinucleotide) or NADPH was detected in our membrane fractions. The complementing *mdh* gene did not restore NADH dehydrogenase activity to the *ndh-4* mutant (pYUB808).

Representatives of the three INH^r T^s mutant classes were tested for Ndh defects by measuring NADH oxidation (Table 3). The defects in Ndh activity correlated with the mutants' growth defects. The largest Ndh defect (25-fold reduction) was observed among mutants (the *ndh-4* and *ndh-14* mutants) with the most severe growth defects: T^s lethality, general auxotrophy, and poor growth under permissive conditions. A Ser-Gly auxotroph (the *ndh-15* mutant) exhibited a sixfold reduction in Ndh activity relative to the ndh$^+$ control, and the prototroph (the *ndh-21* mutant) showed a threefold reduction. Data for five representative mutants are presented; however, the tests were performed on at least two different mutants of each phenotypic class, and the results were consistent with those shown in Table 3. Ndh activity was also measured by the NADH-dependent DCIP reduction assay, and the results were similar to those of the NADH oxidation assay.

Mdh is highly active in oxidation of NADH. Expression of the *M. tuberculosis* complex Mdh enzyme corrected the phenotypes of the *ndh* mutants and conferred hypersensitivity to INH and ETH. The only common function shared by Mdh and Ndh is oxidation of NADH. We therefore reasoned that the Mdh enzyme might complement the mutant defects by oxidizing NADH with oxaloacetate as an electron acceptor. Mdh activity was determined by measuring oxaloacetate-dependent NADH oxidation with clarified whole-cell extracts of the *ndh-4* mutant and strains with the complementing plasmids. The *ndh*$^+$ strains and the *ndh-4* mutant exhibited extremely low Mdh activities (Fig. 4); the Mdh activity of the *ndh-4* mutant was about fivefold higher than those of the *ndh*$^+$ strains. Low Mdh activity was expected since previous studies did not detect this activity in *M. smegmatis*; this species possesses a different malate oxidation system, one that uses the FAD-dependent malate-vitamin K reductase (35). Expression of the *M. tuberculosis* complex *mdh* gene greatly increased NADH oxidation only when oxaloacetate was used as an electron acceptor (1,000-fold increase).

***ndh* mutants are frequently recovered from INH^r selections at 37°C.** It is surprising that Ndh defects are found in such a large fraction of INH^r mutants isolated at 30°C. Perhaps the reduced growth temperature favors the recovery of *ndh* mutants or limits the recovery of *inhA* and *katG* mutants. The selection for INH resistance was repeated at 37°C to determine whether the selection temperature biases the recovery of mutants. Twenty INH^r mutants were isolated from 10 different cultures of strain mc^{2}155. A plasmid with the *M. smegmatis ndh* gene (pYUB802) was introduced into each of the INH^r mutants, and the transformants were tested for INH sensitivity. Expression of the *ndh* gene restored sensitivity to 50% of the

TABLE 5. KatG peroxidase activity of the *ndh* mutant strains

Relevant genotype[a]	INH resistance (MIC, μg/ml)[b]	KatG peroxidase activity[c]
ndh$^+$	5	1
ndh-14	>100	0.5
ndh-15	>100	0.7
ndh-21	>100	0.9
ndh-29	>100	1.4
ndh-4/pMV261	>100	0.14
ndh-4/pMV261::*ndh* (*M. tuberculosis*)	2.5	1.1
ndh-4/pMV261::*mdh* (*M. bovis* BCG)	1	0.4

[a] All strains are derived from the INH^s parent strain mc^{2}155 (Table 1).
[b] These data are from Tables 3 and 4.
[c] KatG activity was measured as the rate of *o*-dianisidine oxidation in the presence of H_2O_2 as described in Materials and Methods. All assays were repeated three times with the same extracts; in all cases, error was less than 10%. All activities are expressed relative to that of the *ndh*$^+$ control, strain mc^{2}155 or mc^{2}1421; the assays were performed in parallel.

INH^r mutants isolated at 37°C. Six of these were tested for complementation by *mdh* of the *M. tuberculosis* complex; all were complemented (pYUB808). Unlike the mutants isolated at 30°C, most (70%) of these putative *ndh* mutants isolated at 37°C grew well on minimal medium and were thermoresistant. Assays of NADH dehydrogenase activity of one T^r prototrophic mutant, *ndh-29*, showed a small Ndh defect (3.7-fold reduction). Sequence analysis identified a missense mutation in the *ndh-29* mutant (Fig. 1). We infer that Ndh defects confer INH^r in about 50% of mutants isolated at 37°C and that this resistance is not typically associated with growth defects.

Resistance of the *ndh* mutants is not due to lower-level KatG expression. One possible explanation for the mutants' resistance is that the Ndh defect reduces *katG* expression by decreasing the amount of H_2O_2 produced from the respiratory chain. Lower *katG* expression has been observed in *ndh* mutants of *E. coli* (14). KatG expression in different *ndh* mutant strains was measured by determining the amount of peroxidase activity.

KatG expression was lower in some *ndh* mutants; however, KatG activity did not correlate with INH susceptibility. Mutants that have small Ndh defects had normal peroxidase activity (e.g., the *ndh-21* and *ndh-29* mutants) (Table 5). These mutants were resistant to INH (MICs, >100 μg/ml). Reduced peroxidase activity was observed in mutants with larger Ndh defects (the *ndh-4*, *ndh-14*, and *ndh-15* mutants), and complementation with the *ndh*$^+$ gene restored peroxidase activity (Table 5). However, Mdh expression did not restore peroxidase activity to the *ndh-4* mutant but conferred hypersensitivity to INH and ETH.

DISCUSSION

Mutants that are defective for NADH dehydrogenase (Ndh; type II) are frequently found among spontaneous INH-resistant mutants of *M. smegmatis*. These mutations often confer low-level resistance to the structurally related drug ETH. Many *ndh* mutants have multiple growth phenotypes, including temperature sensitivity and the inability to grow on minimal medium supplemented with glycerol or glucose. Expression of the malate dehydrogenase enzyme (Mdh) of the *M. tuberculosis* complex restored thermoresistance and prototrophic growth to the *ndh* mutants and also caused increased sensitivity to INH and ETH (Table 4).

We propose that all of the *ndh* mutant phenotypes, INH^r, ETH^r, T^s, and auxotrophy, are due to defects in NADH oxidation which result in NADH accumulation and NAD$^+$ depletion. The increased NADH concentration may inhibit reactions that are sensitive to this nucleotide; a lower NAD$^+$ concentration may reduce the rates of NAD$^+$-dependent reactions. These two factors may impair operation of the TCA cycle and/or inhibit biosynthetic pathways; they also could explain why the most defective Ndh mutants cannot grow on minimal medium. Biochemical studies of *E. coli* have shown that NADH is a potent inhibitor of the first committed reaction in the biosynthetic pathway for serine and glycine, which would explain the specific serine or glycine requirement of many *ndh* mutants (45). Expression of the Mdh enzyme would correct the mutant phenotypes by oxidizing NADH and reducing oxaloacetate—the energetically favorable reaction. This Mdh activity is robust in strains expressing Mdh; these strains show increased sensitivity to INH and ETH (Fig. 4; Table 4).

The thermosensitive lethality of many *ndh* mutations indicates that Ndh is the primary enzyme responsible for NADH oxidation in *M. smegmatis* and is essential for viability. The thermosensitive lethality of some *ndh* mutations may be due to a severe imbalance in the NADH/NAD$^+$ ratio at the nonpermissive temperature that is caused by temperature-sensitive Ndh activity. Oxidation of NADH produced via glycolysis and the TCA cycle is essential for maintenance of metabolic flux. However, the essentiality of Ndh is surprising because most organisms have multiple enzymes and pathways for oxidizing NADH. *E. coli* has two different NADH dehydrogenase enzymes (Nuo and Ndh) and can oxidize NADH by fermentation (8, 12). An *E. coli ndh nuo* double mutant has an impaired ability to oxidize NADH and grows poorly on minimal medium supplemented with glucose, presumably because of an increased NADH/NAD$^+$ ratio (53). From sequence analysis, it appears that *M. tuberculosis* has genes encoding the Nuo system (Sanger database; cosmid SCY03A2). We believe that the Nuo system is not active in *M. smegmatis*, since some *ndh* mutants have very low NADH dehydrogenase activity (~25-fold reduction). These mutants arise spontaneously at a high frequency (10^{-6} to 10^{-7} per CFU), so the defect is not likely to be caused by multiple mutations. Furthermore, assays for Nuo activity (measuring oxidation of deamino-NADH, a Nuo-specific substrate) in our wild-type strain were negative. It is possible that *M. smegmatis* has genes encoding the Nuo system and that *nuo* expression is repressed under the conditions of high aeration and rich medium used in our experiments. Oxygen tension influences *nuo* and *ndh* expression in *E. coli* (7, 43).

Defects in Ndh activity cause resistance to INH and ETH in *M. smegmatis*. Since the *ndh* mutations that confer resistance are always recessive and occur in conjunction with reduced Ndh activity, it seems unlikely that Ndh is an INH target (as proposed in reference 11). Our genetic data indicate that Ndh potentiates the action of INH and ETH by oxidizing NADH and that an increase in the NADH/NAD$^+$ ratio confers resistance. Recent structural studies showed that INH reacts with the NADH located within the active site of the InhA enzyme (39). Enzymatic studies show that NADH is required for INH to inhibit the InhA enzyme and that mutations which reduce the affinity for NADH confer resistance (5, 19, 36). Therefore, it is surprising that mutations which increase the NADH/NAD$^+$ ratio confer resistance. We propose that an increase in the NADH concentration prevents the action of INH and ETH by two different mechanisms which may act in conjunction to confer high-level resistance. First, NADH may displace the active drug from the InhA enzyme, such that an increased NADH concentration may prevent target inhibition. An in-

creased NADH concentration may also prevent peroxidation reactions that are required to activate the drugs. NADH is a substrate for peroxidases such as AhpCF and the KatG catalase peroxidase (21, 34). Increased amounts of NADH may drive peroxide reduction, thereby preventing KatG-mediated peroxidation of INH. NADH may also competitively inhibit peroxidation of INH by KatG.

Mutants with thermosensitive defects in the Ndh enzyme were the most frequent class of INH^r mutants recovered from selections performed at 30°C. One expects that *katG* mutations would be recovered more frequently than *ndh* mutations because any *katG* deletion or point mutation that eliminates or reduces activity should confer INH resistance. In contrast, Ndh is essential, so *ndh* mutations which eliminate activity would be forbidden. The paucity of *katG* deletion mutants recovered in our selections is enigmatic and may indicate that INH can be activated by multiple enzymes in *M. smegmatis*. Another unexpected finding is that a large proportion of the *ndh* mutants isolated at 30°C have a thermosensitive phenotype. This may be due to properties of Ndh, which is a peripheral membrane enzyme. The high frequency of *ndh* T^s mutants has complicated the isolation of the *inhA* T^s mutants, which are expected to be more rare.

The genetic data presented here indicate that NADH oxidation systems influence INH sensitivity. While others have suggested that the exquisite INH sensitivity of *M. tuberculosis* is due to a species-specific target (24), we believe that metabolic functions such as differences in the KatG enzyme, alkyl hydroperoxide reductase expression, peroxide concentrations, and the NADH oxidation systems could fully explain the different sensitivities of *M. tuberculosis* and *M. smegmatis*. Results reported in the early literature of Middlebrook and Cohn suggest that INH resistance in *M. tuberculosis* can occur via metabolic defects that may be similar to the *ndh* defects found in *M. smegmatis* (26). These investigators attempted to culture INH-resistant tubercle bacilli from patients treated with INH monotherapy and found that 8 of 21 patients produced tubercle bacilli exhibiting growth defects. Of these eight isolates, five were auxotrophs and three were not culturable in rich or minimal medium. Perhaps these resistant tubercle bacilli had NADH oxidation defects that prevented their growth on certain types of media.

The isolation of drug-resistant mutants that are thermosensitive for growth provides a means to identify essential enzymes involved in drug action. These types of enzymes may be useful for the development of new antimicrobial agents. This approach should work with any microorganism that is amenable to genetic analysis and should apply to any antimicrobial agent. Our studies unexpectedly found that the *ndh*-encoded NADH dehydrogenase is essential for the viability of *M. smegmatis* and that this enzyme normally promotes INH action by oxidizing NADH.

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