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Novel Selection for Isoniazid (INH) Resistance Genes Supports a Role for NAD^+-Binding Proteins in Mycobacterial INH Resistance

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The genetic basis of isoniazid (INH) resistance remains unknown for a significant proportion of clinical isolates. To identify genes which might confer resistance by detoxifying or sequestering INH, we transformed the *Escherichia coli oxyR* mutant, which is relatively sensitive to INH, with a *Mycobacterium tuberculosis* plasmid library and selected for INH-resistant clones. Three genes were identified and called *ceo* for their ability to complement the *Escherichia coli oxyR* mutant. *ceoA* was the previously identified *M. tuberculosis glf* gene, which encodes a 399-amino-acid NAD^+- and flavin adenine dinucleotide-requiring enzyme responsible for catalyzing the conversion of UDP-galactopyranose to UDP-galactofuranose. The proteins encoded by the *ceoBC* pair were homologous with one another and with the N terminus of the potassium uptake regulatory protein TrkA. Each of the three Ceo proteins contains a motif common to NAD^+ binding pockets. Overexpression of the *M. tuberculosis glf* gene by placing it under the control of the *hsp60* promoter on a multicopy plasmid in *Mycobacterium bovis* BCG produced a strain for which the INH MIC was increased 50% compared to that for the control strains, while similar overexpression of the *ceoBC* pair had no effect on INH susceptibility in BCG. Mycobacterial extracts containing the overexpressed Glf protein did not bind radiolabeled INH directly, suggesting a more complex mechanism than the binding of unmodified INH. Our results support the hypothesis that upregulated mycobacterial proteins such as Glf may contribute to INH resistance in *M. tuberculosis* by binding a modified form of INH or by sequestering a factor such as NAD^+ required for INH activity.

Since its discovery as an antimycobacterial drug, isonicotinic acid hydrazide (isoniazid [INH]) has been one of the first-line antibiotics in the treatment of tuberculosis. Because of its unique toxicity for tuberculocus mycobacteria, it has long been believed that insights into the mechanism of action of INH, as well as into the organism's mechanisms of resistance, might provide clues to the human pathogenicity of *Mycobacterium tuberculosis* complex bacilli (2). Another strong impetus for investigating the mode of action of this drug has been the emergence of resistance to INH and other key antimycobacterial agents (11). Drug resistance poses a significant threat to our ability to control the estimated 8 million new cases of tuberculosis that occur annually.

It appears that *M. tuberculosis* may become resistant to INH by a variety of genetic changes. Alteration or loss of the *katG* gene encoding a catalase-peroxidase enzyme is clearly associated with INH resistance in a high proportion of clinical isolates (48, 49). DNA sequencing or PCR-single-strand conformational polymorphism analyses of INH-resistant strains have demonstrated *katG* alterations in as many as 97% (21) or as few as 18% (27) of strains. Other large studies indicate that about 50 to 75% of INH-resistant *M. tuberculosis* isolates contain at least one mutation in the *katG* gene locus (12, 20, 31). To account for the remaining fraction of strains with normal *katG* genes, other INH resistance genes must exist.

Regulators of *katG*, such as OxyR, a redox-sensitive protein which activates *katG* transcription following oxidative stress in gram-negative bacilli, were hypothesized to participate in INH resistance. However, *M. tuberculosis* complex species contain a defective, vestigial remnant of the *oxyR* gene in spite of the fact that other nontuberculous mycobacteria contain close *oxyR* homologues (6, 35). Another candidate, the alkyl hydroperoxidase gene (*ahpC*), whose expression is OxyR dependent in enteric bacilli, has also been investigated as an INH resistance gene (8, 50). While upregulatory mutations in the *ahpC* gene of *M. tuberculosis* are associated with virulence in *katG* mutant strains, there are conflicting reports as to whether overexpression of *ahpC* leads to INH resistance (13, 36, 43). Finally, mutations in *inhA*, which encodes a fatty acyl enoyl reductase that is a component of fatty acid synthase type II systems, have been shown to cause INH resistance in *Mycobacterium smegmatis* (1, 7). DNA sequencing of the *inhA* locus of INH-resistant *M. tuberculosis* strains has unmasked mutations in the 5′ noncoding sequences upstream of *inhA*, and it has been suggested that these substitutions may correlate with INH resistance (21, 31). Other studies have challenged the role of *inhA* in INH resistance in *M. tuberculosis* altogether (18). In the current model of INH action, KatG is believed to convert INH to an activated form that subsequently inhibits InhA or another enzyme involved in mycolic acid biosynthesis. In view of these uncertainties it is not surprising that most surveys of INH-resistant strains have documented that a high percentage of resistance (25 to 50%) remains unaccounted for by *katG* mutations or other putative resistance loci, such as *inhA* or *ahpC*.

Recently, *Escherichia coli oxyR* mutants were shown to be moderately sensitive to INH (29, 30). This sensitivity was exacerbated by oxidants such as hydrogen peroxide, suggesting that a reduced ability to detoxify reactive oxygen intermediates was associated with susceptibility to INH. Since a major mechanism of drug resistance is the acquisition or upregulation of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
E. coli		
DH5α	ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96*	33
TA4112	*oxyR*Δ*3* [*oxy*Δ(*oxyRbtuB*)*3*] (derived from RK4936)	42
M. bovis BCG	Pasteur strain	ATCC 35734
M. smegmatis mc^26 1-2 C	Transformable variant of mc^26	47
Plasmids		
pUC18 and 19	High-copy-number *E. coli* plasmids	33
pMV261	*E. coli*-mycobacterial shuttle vector with *hsp60* promoter	38
pCJ5	pUC18 containing the original 1,778-bp *M. tuberculosis ceoA* (*glf*) genomic sequence	This study
pCJ6	pUC18 containing the original 1,692-bp *M. tuberculosis ceoBC* genomic sequence	This study
pCJ5-10	pMV261 containing the ORF of *glf* (1,197 bp) cloned in frame with the *hsp60* promoter	This study
pCJ6-10	pMV261 containing the ORF of *ceoB* (681 bp) cloned in frame with the *hsp60* promoter	This study
pCJ6-11	pMV261 containing a 947-bp *ceoBC* sequence cloned in frame with the *hsp60* promoter	This study

detoxification enzymes, we postulated that some strains of *M. tuberculosis* might become INH resistant by overexpressing genes that modify or sequester the drug. In the present study we took advantage of the INH sensitivity of the *E. coli oxyR* mutant to select for *M. tuberculosis* gene products that might play such a role.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The bacterial strains and plasmids used here are listed in Table 1. *E. coli* strains were cultured on Luria-Bertani (LB) agar or broth with or without selective antibiotics. Mycobacterial strains were cultured in Middlebrook 7H9 broth or 7H10 agar (Difco) supplemented with albumin-dextrose complex, Tween 80, and glycerol according to the specifications of the manufacturers and with 50 μg of cycloheximide (Sigma) per ml. The concentrations of selective antibiotics for the pNBV1 vector were 200 μg of hygromycin for *E. coli* and 50 μg of hygromycin for mycobacteria per ml. The kanamycin concentration used for the pMV261 vector was 25 μg/ml for both *E. coli* and mycobacteria.

Screening for INH-resistant TA4112 transformed with a library of *M. tuberculosis* DNA. Genomic DNA from *M. tuberculosis* H37Rv was sheared by nebulization, and size-selected fragments ranging from 1.9 to 2.1 kb were gel purified, end repaired, and cloned into phosphatase-treated pUC18 according to the method developed by H. O. Smith and proven to give highly random insert distributions that are suitable for shotgun genomic sequencing projects (9). Characterization of the library showed that over 98% of transformants contained cloned single inserts. Electrocompetent *E. coli* TA4112 (*oxyR* deletion [see Table 1]) was electroporated with 0.5 μg of the library DNA in a 0.1-cm cuvette at 1.8 kV. Ampicillin-resistant transformants were selected on LB agar plates containing 400 μg of INH per ml, 400 μg of INH per ml–50 μM H_2O_2, or 400 μg of INH per ml–200 μM H_2O_2.

INH resistance assay for *E. coli* strains. Tenfold dilutions of overnight cultures of TA4112 harboring recombinant pUC18 clones were made in LB broth, and 10 μl samples of each dilution were spotted onto LB agar plates containing ampicillin and various concentrations of INH or H_2O_2. We included H_2O_2 along with INH in our selection protocol since it suppressed the growth of minimally INH resistant colonies and produced a much cleaner background. After an overnight incubation at 37°C, the colonies in each spot were counted (up to 50 colonies could be counted accurately). For those spots that had more than 50 colonies the growth was scored in percentage scales compared to untreated controls. The minimal bactericidal concentration (MBC) was the average concentration at which fewer than 10 colonies survived (at least a fivefold reduction in CFU). This method was previously shown to correlate well with classical agar dilution assays (4).

DNA sequencing. Recombinant plasmids of interest were end sequenced by double-stranded dye terminator methods with the M13 forward and reverse primers. To acquire internal sequence information, *Xho*I, *Hae*III, and *Sau*3AI digests of the plasmid inserts were cloned into pUC18 or pUC19 and sequenced with the M13 forward and reverse primers. Primer walking was performed to complete the sequences. The sequences were aligned with the AssemblyALIGN software program (Oxford Molecular Group).

Cloning *glf* and *ceoB* into overexpression vectors. To clone the *glf* open reading frame (ORF) into the mycobacterial overexpression vector pMV261, PCR amplification with the primers MtbrfbP1 (5′-CTGCAGCAACCGCTCGTTTTGACCTTTTCG) and MtbrfbP2 (5′-GAATTCGTTGACTCCTCGAGGTAC) and directional cloning with *Pst*I and *Eco*RI were used. Similar strategies were used to construct pMV261-based expression vectors for *ceoB* and *ceoBC* with the primer pair MtbtrkAP1 (5′-GGATCCAATGCGGGTGGTTGTGATG) and MtbtrkAP2 (5′-GAATTCATGTCGTGTCCGTTTTCC) and the primer pair MtbtrkAP1 and MtbtrkAP3 (5′-CAGGCGTCGTTGAACAGC), respectively. The junctions between the *hsp60* promoter and the beginning of the coding sequence of *ceo* genes were verified by DNA sequencing.

Transformation of mycobacteria. Electrocompetent *M. smegmatis* and BCG were prepared by washing exponentially growing bacteria in sterile ice-chilled deionized water and storing them in 10% glycerol at −80°C until use. Plasmid DNA (100 to 500 ng) was mixed with 50 μl of competent cells on ice and electroporated at 1.8 kV in a 1-mm cuvette. The mycobacteria were rescued in 1 ml of Middlebrook 7H9 broth supplemented with 10% albumin-dextrose complex and 50 μg of cycloheximide per ml at 37°C for 2 to 4 h for *M. smegmatis* and overnight for BCG. The bacterial suspension was then plated in fractions onto Middlebrook 7H10 agar plates containing kanamycin. The plates were incubated at 37°C in 5% CO_2 until the colonies were visible on the plates. The presence of the appropriate plasmid was confirmed in kanamycin-resistant transformants by colony PCR.

INH susceptibility testing of recombinant BCG strains. BCG harboring *glf*, *ceoB*, and *ceoBC* overexpression plasmids or a control plasmid grown on 7H10 agar plates were suspended in phosphate-buffered saline. Clumps were dispersed by vigorous vortexing in the presence of 3-mm glass beads. The remaining unbreakable clumps were removed by gravity. The clump-free suspension was adjusted to the optical density of McFarland standard no. 1. BACTEC bottles (12B medium; Becton Dickinson) were first inoculated with 0.1 ml of INH at different concentrations and 0.1 ml of kanamycin (for transformed BCG only) to a final concentration of 50 μg/ml. Then 0.1 ml of BCG suspension (McFarland standard no. 1) was inoculated into these bottles. The controls included diluted (1:100) and undiluted inocula in the absence of INH. The growth indices (GI) of these bottles were read daily at approximately same time each day for 14 days. Growth of the bacilli in a particular bottle was indicated if positive changes in GI from day to day continued during the incubation period, whereas no growth was noted when there were negative or no changes in GI (see Fig. 4). The INH MICs for the strains tested were defined as the lowest concentrations at which no growth was observed.

In vitro INH binding assay. Cell lysates (100 μg of total protein) from *M. smegmatis* carrying either pMV261 or pCJ5-10 were mixed with 5 μCi of ^{14}C-labeled INH (specific activity, 59 mCi/mmol; AIDS Research and Reference Reagent Program, National Institutes of Health) in a 100-μl reaction mixture. After 30 min at 4°C, the mixture was passed over a Sephadex G-25 (Pharmacia) gel filtration column (1.0 by 20.0 cm) that had been prewashed and equilibrated with 10 mM Tris-HCl (pH 8.0) at 4°C. The flow rate was ca. 0.15 ml per min. Fractions (0.5 ml) were collected and assayed for radioactivity by scintillation counting, and the protein content was determined by the Coomassie blue protein assay (Pierce).

The sequences reported here have been deposited in the GenBank database (accession numbers AF026540 and AF026541).

TABLE 2. MBCs of INH and hydrogen peroxide for *E. coli* TA4112 harboring *glf* and *ceoBC*

Plasmid	MBC of:	
	INH (μg/ml)	H_2O_2 (μM)
pUC19	200	200
pCJ5	>400	150
pCJ6	>400	150

RESULTS

Selection of clones which complement the *E. coli oxyR* mutant to INH resistance. A highly random, size-selected *M. tuberculosis* genomic DNA library constructed in pUC18 was used to transform the INH-sensitive *E. coli oxyR* mutant TA4112. An estimated 24,000 ampicillin-resistant transformants were generated and evaluated in the selection for genes that conferred INH resistance on the *E. coli oxyR* mutant. With the average insert size in this library being 2.0 kb and a narrow distribution range of insert sizes (>99% of inserts were 1.9 to 2.1 kb), our selection tested the equivalent of approximately 4.8×10^7 bp of DNA, more than 10 times the size of the *M. tuberculosis* genome. Seven clones were selected on INH-H_2O_2, three of which showed a consistent phenotype upon retransformation into TA4112. Of these three clones, two nonidentical plasmids were derived for further study: pCJ5 (identified twice) and pCJ6. For TA4112 harboring pCJ5 and pCJ6, the MBCs of both INH and H_2O_2 are listed in Table 2. Each clone conferred at least a twofold increase in resistance to INH alone. In contrast, both TA4112 transformants were more sensitive to the H_2O_2 alone compared to the same strain harboring empty pUC19. This observation suggests that the effect conferred by pCJ5 and pCJ6 was specific to INH and that the recombinant plasmids did not encode peroxide-detoxifying proteins such as KatG.

DNA sequencing identifies two *M. tuberculosis* genes that complement the *E. coli oxyR* mutant: *glf* and *ceoBC*. The inserts carried in pCJ5 and pCJ6 were sequenced. pCJ5 contained a 1,778-bp insert, and pCJ6 carried a 1,692-bp insert (Fig. 1). ORFs in the sequences were evaluated for their adherence to known mycobacterial codon usage, for the presence of appropriate translational start signals, and for their homology to other genes in the database.

The 5′-terminal 216 bp of the 28-kDa antigen gene was present at one end of pCJ5, matching its previously reported sequences (3, 16). A 1,197-bp ORF was present in the center of the cloned DNA and was transcribed divergently from the 28-kDa antigen gene. The gene was originally named *ceoA* for complementing *E. coli oxyR*; later, when it became apparent that *ceoA* was identical to the recently identified *M. tuberculosis glf* gene encoding UDP-galactopyranose mutase, we adopted the *glf* nomenclature (40). The apparent start codon for the *glf* gene is ATG, and 11 bp upstream of the ATG a reasonable ribosome binding site (5′-AAGG-3′) appears.

In pCJ6 two tandem ORFs were identified that appear to be

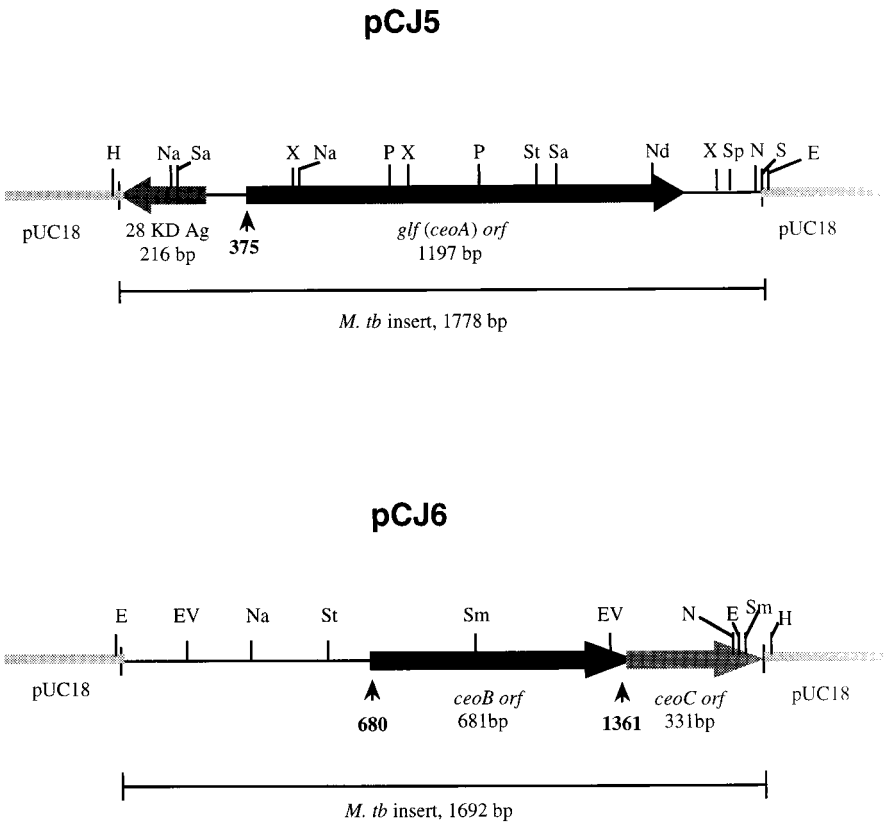

FIG. 1. ORF and restriction analysis of cloned DNA containing the *ceo* genes identified in pCJ5 and pCJ6. The black-boxed regions with arrowheads represent the ORFs of interest. The gray-boxed region with an arrowhead indicates the incomplete ORF of *ceoC* in pCJ6. The small arrows under each ORF indicate the positions of the initiation codons for *ceo* ORFs. The abbreviations for the restriction enzymes are as follows: E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; N, *Nhe*I; Na, *Nar*I; Nd, *Nde*I; P, *Pvu*II; S, *Sal*I; Sa, *Sac*II; Sm, *Sma*I; Sp, *Sph*I; St, *Sty*I; and X, *Xho*I.

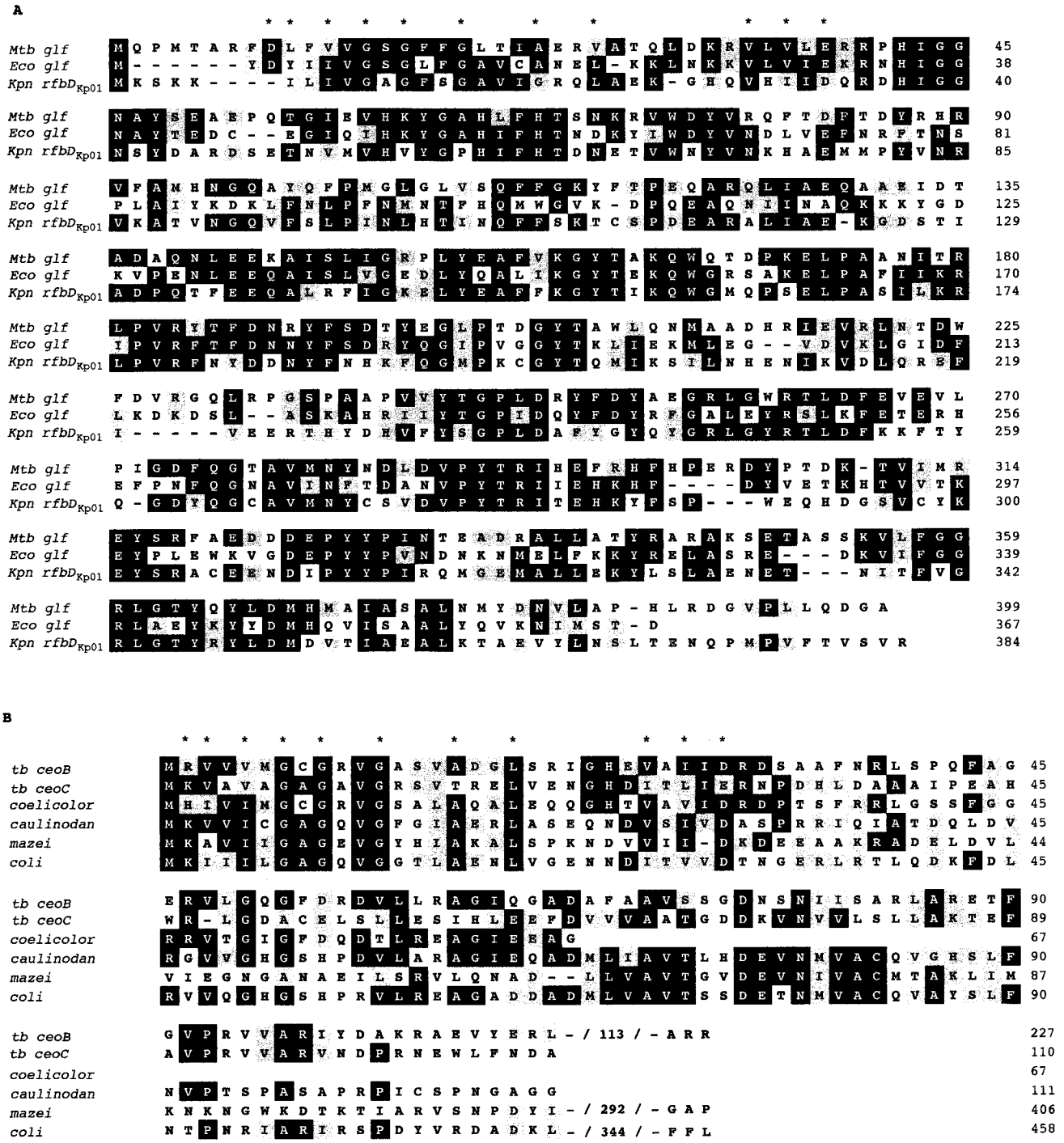

FIG. 2. Amino acid sequence alignments among Glf and RfbD homologues (A) and among CeoB, CeoC, and TrkA homologues (B). Identical amino acid residues are grouped in black boxes, and conserved residues are indicated in gray. Those amino acids involved in the NAD^+ binding motif are identified with asterisks. The alignment was performed by the method of Hein (10) and is available in the DNAStar software package.

translationally coupled since the stop codon of the first overlaps the start codon of the second. We have named this gene pair *ceoBC*. Only the 5′-terminal 331 bp of *ceoC* is contained in clone pCJ6. The deduced protein sequences of CeoB and CeoC share significant homology to one another, with 30% amino acid identity over 110 residues. The apparent start codon of *ceoB* is ATG, and it is preceded by a ribosome binding site (5′-GAGAGGA-3′) spaced 6 bp upstream.

***M. tuberculosis* Glf and CeoBC share amino acid sequence homology to Glf and TrkA, respectively, and each contains an N-terminal NAD^+ binding motif.** The *M. tuberculosis glf* gene encodes a deduced protein of 399 amino acids that is identical to the *M. tuberculosis glf* gene product identified by Weston et al. (40) and shows 43% amino acid identity to gene products from the *rfb* gene clusters of *Klebsiella pneumoniae* (39) and *E. coli* (37, 46), which participate in lipopolysaccharide O-antigen

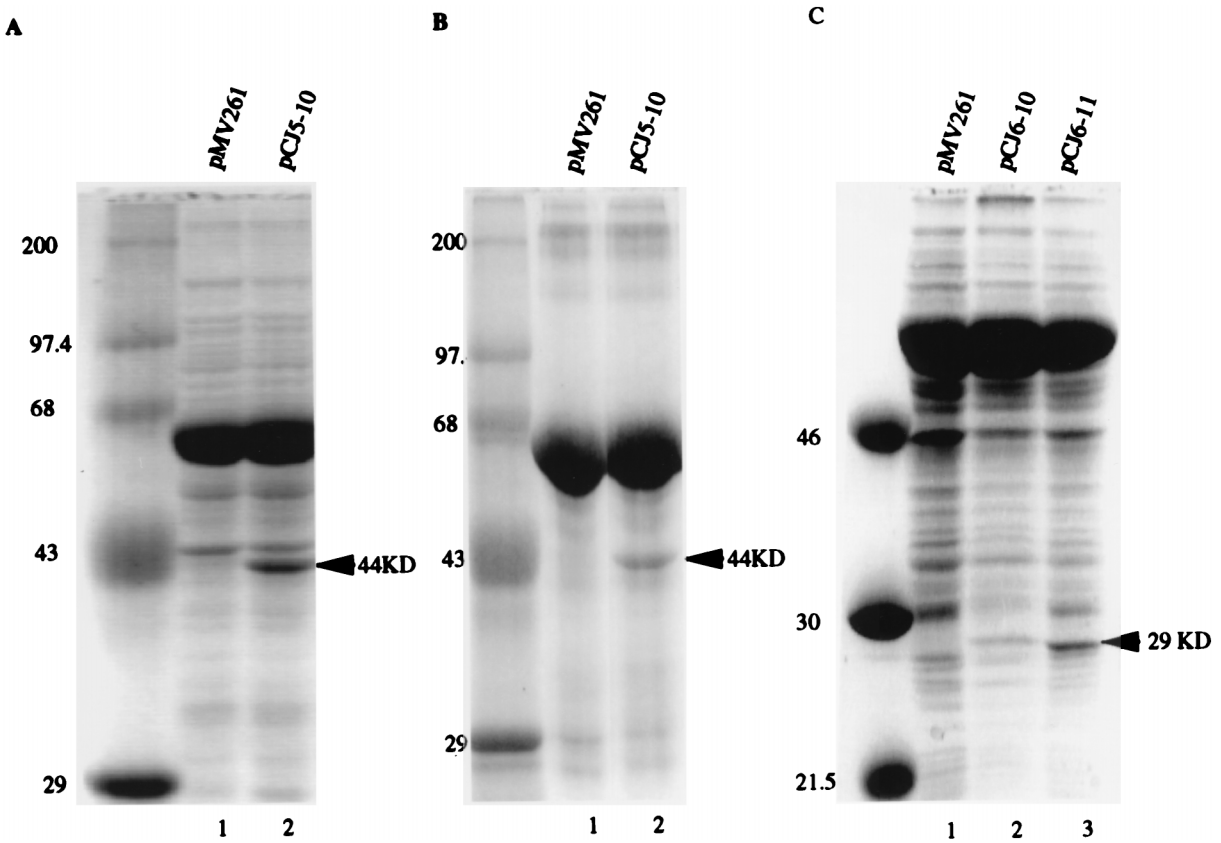

FIG. 3. Overexpression of the *glf* gene in *M. smegmatis* (A) and BCG (B) and of the *ceoB* gene in *M. smegmatis* (C). Whole bacterial SDS lysates equivalent to 10 mg of bacilli (wet weight) or 30 μg of partially purified protein extracts from mycobacteria harboring the *glf* (pCJ5-10), *ceoB* (pCJ6-10), or *ceoBC* (pCJ6-11) overexpression plasmids or the control plasmid (pMV261) were analyzed. Proteins were stained with Coomassie blue after SDS-polyacrylamide electrophoresis. The numbers on the left of each image indicate the sizes of the molecular weight standards, and the arrows on the right indicate the apparent molecular weights of Glf and CeoB.

biosynthesis (Fig. 2A). At the N terminus of these proteins a classical NAD^+ binding motif appears (45). The enzymes encoded by these genes have been biochemically characterized as the UDP-galactopyranose mutases that convert UDP-galactopyranose to UDP-galactofuranose (15, 23, 40).

CeoB shares amino acid sequence homology with the N-terminal sequence of the TrkA protein family, with 52% identity with *Streptomyces coelicolor* TrkA and 25% identity with *E. coli* TrkA (Fig. 2B). TrkA is an essential component of the Trk protein complex that is responsible for the constitutive K^+ uptake system in *E. coli* (5, 34). The same NAD^+ binding motif found in the Glf family is also found in the CeoB and TrkA proteins of various species, including gram-negative bacteria (22, 24), *S. coelicolor* (41), *Azorhizobium caulinodans* (25), and archaebacteria (17). Part of a second gene, which we have named *ceoC*, is present immediately after *ceoB* starting at nucleotide position 1361, and it overlaps *ceoB* by one nucleotide. The CeoC fragment has 30% amino acid identity with CeoB and 24% identity with the TrkA proteins of *S. coelicolor* and *E. coli* (Fig. 2B).

Overexpression of the *M. tuberculosis glf* gene results in INH resistance in BCG. Given that both *glf* and *ceoB* have NAD^+ binding motifs in their primary amino acid sequences and that INH is a nicotinamide analogue, we hypothesized that overexpression of these NAD^+ binding proteins might sequester or modify INH in vivo, leading to INH resistance. To test our hypothesis directly, plasmids overexpressing *glf* and *ceoB* were constructed and introduced into *M. bovis* BCG.

The coding sequences of the *M. tuberculosis glf*, *ceoB*, and *ceoBC* genes were placed under the control of the *hsp60* promoter, which is strongly expressed in mycobacteria, to yield pCJ5-10, pCJ6-10, and pCJ6-11, respectively. Figure 3 shows the levels of Glf and CeoB expression in *M. smegmatis* and *M. bovis* BCG harboring these plasmids. A 44-kDa protein consistent with Glf was observed only in the extracts of pCJ5-10-transformed *M. smegmatis* (Fig. 3A, lane 2) and BCG (Fig. 3B, lane 2). A unique 29-kDa protein consistent with CeoB was found in both pCJ6-10- and pCJ6-11-transformed *M. smegmatis* extracts (Fig. 3C, lanes 2 and 3). The sizes of both proteins agreed with the predicted masses of 45 and 28 kDa for the *M. tuberculosis* Glf and CeoB proteins, respectively. Surprisingly, pCJ6-11, in which both CeoB and the CeoC protein fragment are expressed, appears to give significantly higher levels of the CeoB protein (Fig. 3C, lane 3) than does pCJ6-10, which does not contain CeoC-encoding sequences (Fig. 3C, lane 2).

To assess whether the overexpression of *M. tuberculosis* Glf or CeoB could lead to INH resistance in mycobacteria, we determined the INH MICs of BCG clones harboring pCJ5-10, pCJ6-10, and pCJ6-11 by using the BACTEC radiometric method. Figure 4 shows a panel of BACTEC growth indices for BCG–pCJ5-10 (recombinant *glf* overexpressor) and BCG-pMV261 (vector control) in which matched inocula of each

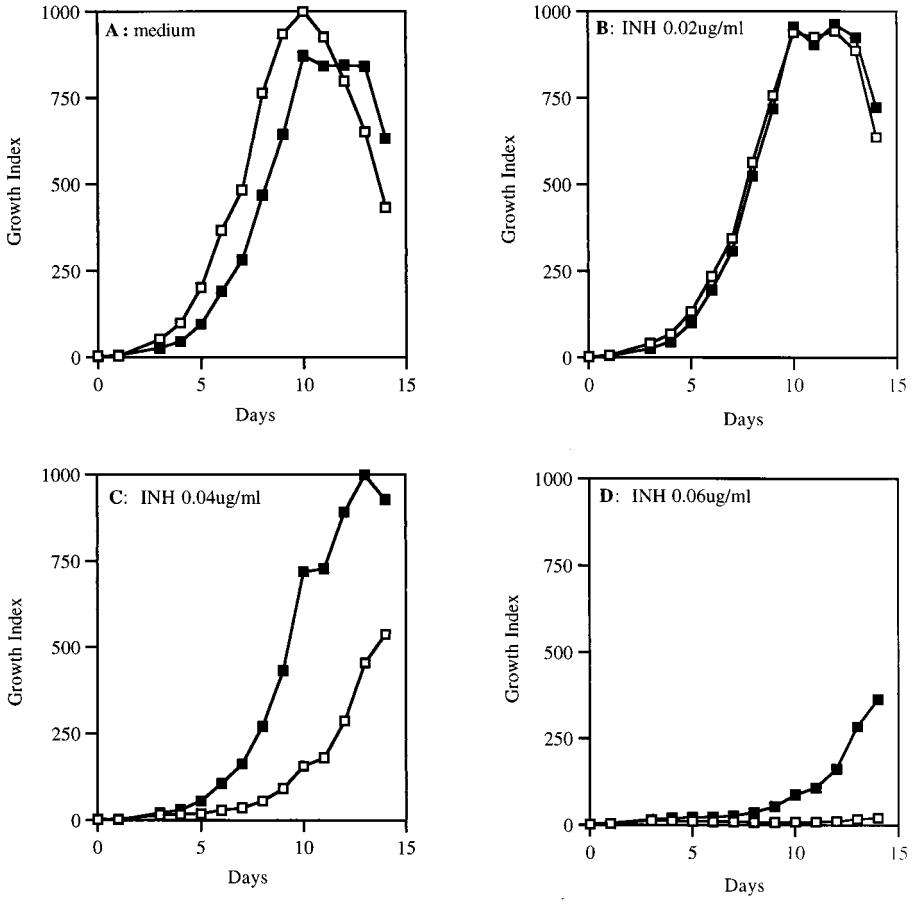

FIG. 4. Overexpression of Glf confers INH resistance on BCG. By using the BACTEC radiometric assay, the growth of BCG harboring either control plasmid pMV261 (□) or the Glf overexpression plasmid pCJ5-10 (■) in the presence of 0 (A), 0.02 (B), 0.04 (C), or 0.06 (D) μg of INH per ml was evaluated daily over a period of 2 weeks.

strain were monitored daily for growth in the presence of different concentrations of INH. As can be seen, the growth of BCG-pMV261 (vector control) was inhibited by INH concentrations of 0.04 and 0.06 μg/ml, while BCG–pCJ5-10 continued to grow exponentially at these concentrations. At a 0.08-μg/ml concentration of INH, both BCG strains did not grow (data not shown). The 50% increase in the INH MIC for BCG overexpressing *glf* was consistently observed and reproduced with another recombinant BCG isolate. No significant differences in catalase activity were detected among these BCG strains. Thus, the overexpression of *glf* in BCG resulted in low-level INH resistance.

The same analysis with BCG-pCJ6-10 (recombinant *ceoB* overexpressor) and pCJ6-11 (recombinant *ceoBC* overexpressor) did not reveal changes in the INH susceptibility compared with the BCG control strain. Similarly, *glf* and *ceoBC* overexpression in *M. smegmatis* did not produce a significant increase in the INH MIC for this species. Since the INH MIC for *M. smegmatis* is higher (5 μg/ml) than for BCG (0.02 μg/ml), it is possible that the low sensitivity of *M. smegmatis* masked the effects of Glf and CeoBC even when they were overexpressed.

Glf-containing extracts do not reveal INH binding in vitro. To evaluate whether the *M. tuberculosis* Glf protein might be acting as an INH-sequestering protein, we tested its ability to bind to INH in vitro. Cell extracts were prepared from *M. smegmatis* strains overexpressing the Glf protein from pCJ5-10, and the extracts were mixed with ^{14}C-labeled INH. The mixtures were then passed through a Sephadex G-25 column to separate the proteins from unbound INH. The elution profiles for total protein and radiolabeled INH obtained from the control extract and the Glf overexpression extract were very similar. No radioactive INH was found to be associated with the protein fractions in the Glf overexpression extract and, in view of the fact that the Glf protein constitutes at least 1% of the soluble protein of this strain (Fig. 3A), our assay should have detected stoichiometric INH binding to Glf. One possible explanation for this observation could be that Glf might only bind to activated INH. It is also possible that certain components were absent in our assay, such as Mn^{2+} and NADH, which were used in the in vitro INH binding assay for InhA (28). To determine whether Glf interacts with activated INH in vivo, we cultured the *M. smegmatis* strain overexpressing the *M. tuberculosis glf* gene in the presence of radiolabeled INH. Cell lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis, and the protein-associated radioactivity was detected by phosphorimaging. This analysis did not reveal in vivo binding of radiolabeled INH to the Glf protein expressed in *M. smegmatis* (data not shown). On the basis of this experiment we cannot exclude the possibility that in association with other mycobacterial proteins, perhaps BCG or *M. tuberculosis*-specific proteins, Glf does in fact bind INH or its activated intermediate. However, it is more likely that Glf

sequesters a factor required for INH activity or that Glf modifies INH but is a relatively poor INH-binding protein.

DISCUSSION

Artificially overexpressing the *glf* gene in *M. bovis* BCG led to a small but reproducible change of the INH MIC from 0.06 to 0.08 μg/ml. Hence, the overexpression of the *M. tuberculosis glf* gene produces a strain that is almost twice as resistant to INH as the parent strain but which is still narrowly within the susceptibility range adopted by most clinical laboratories (0.1 μg/ml in BACTEC 12B medium). A twofold increase in the MIC has also been observed when the *ahpC* gene was overexpressed in *M. tuberculosis* H37Rv (13).

Low-level mycobacterial resistance to INH may be important clinically. Humans metabolize INH by hepatic *N*-acetylation, and population-based studies have shown that individuals are either rapid or slow INH acetylators (26). By 6 h after a 4-mg/kg oral dose of INH, rapid acetylators have an INH concentration in serum of <0.2 μg/ml. In view of the growing trend towards the twice-weekly administration of INH, rapid acetylators infected with *M. tuberculosis* strains with low-level INH resistance may experience prolonged intervals of subtherapeutic drug levels. Low-level resistance mutations might predispose to high-level resistance mutations. By permitting a larger portion of the bacillary population to survive an initial exposure to INH, such mutations might in effect "buy time" for classical *katG* mutations to arise.

The *M. tuberculosis glf* gene which we found in this study encodes the Glf enzyme that catalyzes the conversion of UDP-galactopyranose to UDP-galactofuranose (40). The latter is the substrate for the biosynthesis of arabinogalactan, an essential cell wall component of mycobacteria. The enzyme's unique substrate specificity and pivotal role in arabinogalactan biosynthesis have made Glf a target for developing new antimycobacterial agents. Our results suggest that Glf may also participate in INH resistance. Although our binding assay did not detect Glf sequestration or covalent linkage to INH, it remains possible that Glf is an INH binding protein in the presence of appropriate cofactors or with the activated form of INH.

Alternatively, Glf may confer relative INH resistance by sequestering a cofactor for INH action such as NAD^+ or NADH. An interaction between INH and NAD^+ has been proposed (44) and is supported by a considerable amount of biochemical and genetic literature. Quemard et al. (28) have shown that binding of the radiolabeled, activated INH to InhA only occurred in the presence of NADH, and another biochemical study revealed that NAD^+ or NADH was required for the inhibition of InhA (14). A recent study has shown that InhA binds NADH and that the binary enzyme-nucleotide complex is the target for activated INH, which causes covalent attachment of NAD^+ to InhA (32). Mutations in the NADH binding domain of InhA reduce the affinity of NADH binding to the enzyme and confer resistance to INH (7, 28).

Further evidence suggesting the importance of NADH in INH resistance came from a study by Miesel et al. (19). Temperature-sensitive, INH-resistant mutants of *M. smegmatis* were found to have mutations in the *ndh* gene (type II) encoding NADH dehydrogenase (Ndh). Genes encoding Ndh and malate dehydrogenase (Mdh), another enzyme that utilizes NADH, from *M. tuberculosis* complemented the mutant phenotypes. These investigators propose that the intracellular ratio of NADH to NAD^+ can influence the activation of INH by KatG or the interaction of INH with its target InhA. Thus, Glf, which requires reduced flavin adenine dinucleotide and either NADH or NADPH for activity (40), may cause INH resistance by influencing the NADH/NAD^+ ratio.

While it is clear that *katG* mutations play an important role in INH resistance, the resistance mechanisms involved in catalase-positive, INH-resistant strains have not been fully explained. Further studies of *glf* expression in clinical isolates will be necessary to determine if the findings described here play a significant role in human drug-resistant tuberculosis.

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