JOURNAL OF BACTERIOLOGY, Feb. 1999, p. 1343–1347 Vol. 181, No. 4
0021-9193/99/$04.00+0

Cloning and Characterization of Arylamine *N*-Acetyltransferase Genes from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*: Increased Expression Results in Isoniazid Resistance

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Received 9 September 1998/Accepted 3 December 1998

Arylamine *N*-acetyltransferases (NATs) are found in many eukaryotic organisms, including humans, and have previously been identified in the prokaryote *Salmonella typhimurium*. NATs from many sources acetylate the antitubercular drug isoniazid and so inactivate it. *nat* genes were cloned from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*, and expressed in *Escherichia coli* and *M. smegmatis*. The induced *M. smegmatis* NAT catalyzes the acetylation of isoniazid. A monospecific antiserum raised against pure NAT from *S. typhimurium* recognizes NAT from *M. smegmatis* and cross-reacts with recombinant NAT from *M. tuberculosis*. Overexpression of mycobacterial *nat* genes in *E. coli* results in predominantly insoluble recombinant protein; however, with *M. smegmatis* as the host using the vector pACE-1, NAT proteins from *M. tuberculosis* and *M. smegmatis* are soluble. *M. smegmatis* transformants induced to express the *M. tuberculosis nat* gene in culture demonstrated a threefold higher resistance to isoniazid. We propose that NAT in mycobacteria could have a role in acetylating, and hence inactivating, isoniazid.

Arylamine *N*-acetyltransferases (NATs) are cytosolic enzymes which acetylate arylamines and hydrazines by transfer of the acetyl group from acetyl coenzyme A to the free amino group forming an acetylamide (33). The same enzymes are also able to catalyze the transfer of an acetyl group to the oxygen of an arylhydroxylamine (10, 32). NAT is widespread among eukaryotes (28, 33), and the existence of NAT in prokaryotes was, until recently, thought to be confined to *Salmonella typhimurium* (32). *S. typhimurium* NAT has the ability to *N*-acetylate arylamines and the hydrazine isoniazid (24, 32). In humans there are now known to be two isoenzymes, NAT1 and NAT2 (2). The human enzyme NAT2, whose substrates include sulfonamide-based antibacterial compounds (20), was first identified as the enzyme which inactivates the front-line antitubercular drug isoniazid (9). The sulfonamides sulfamethoxazole and *p*-aminosalicylate are acetylated predominantly by the human isoenzyme NAT1 (4, 13), as is *p*-aminobenzoic acid (18, 30). Both NAT1 (22, 34) and NAT2 (for a review, see reference 5) show polymorphism in human populations. Identification of human NAT2 polymorphisms provides an explanation for the different effective therapeutic doses of isoniazid in fast and slow acetylators (8, 9).

We report here the cloning of the *nat* genes from the eubacteria *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* and show that the *M. smegmatis* NAT is active with a range of substrates, including isoniazid. We demonstrate, using antibodies raised against recombinant *S. typhimurium* NAT, that NAT is present in wild-type *M. smegmatis*. It was reasoned that if isoniazid was acetylated by NAT prior to activation, an elevated resistance to isoniazid would be observed if more NAT were present in *M. smegmatis*. We therefore expressed the *M. tuberculosis nat* gene in *M. smegmatis* using the shuttle vector pACE-1 (21) and observed the effect of induction of NAT protein on the growth of mycobacteria cultured in isoniazid.

Identification of *nat* genes in *M. smegmatis* and *M. tuberculosis*. A radiolabelled DNA probe representing a 264-bp *Hin*dIII fragment of *S. typhimurium nat* (32), corresponding to a region which spans two highly conserved areas in NAT, was used to isolate clones containing *nat* from *M. smegmatis* and *M. tuberculosis* (strain H37Rv). Gridded libraries containing at least two copies of the *M. smegmatis* and *M. tuberculosis* genomes were screened, and double positives were selected. All sequence analyses were performed by an automated (ABI 377) sequencer (The Advanced Biotechnology Centre, ICSM, London) with Fidelase, an enzyme suitable for regions rich in GC. Amino acid sequences representing putative open reading frames of *M. smegmatis* and *M. tuberculosis* NAT are illustrated in Fig. 1 together with a PILEUP comparison of known NAT amino acid sequences. The sequence of *M. tuberculosis* NAT corresponds exactly to the sequence deposited previously in the database (3), assigned as a hypothetical protein. NAT from the genome of *Escherichia coli* is also shown for comparison and was obtained by database searching, again assigned as a hypothetical protein (1). These sequences are illustrated in order to emphasize the common features in all NAT proteins; the conserved PFENL and RGGDC sequences (where D is either W or Y), containing the active-site cysteine and the arginine residues proposed to participate in the reaction mechanism (6, 32), are indicated in Fig. 1. The *M. smegmatis nat* gene open reading frame is >60% identical to *M. tuberculosis nat*, and these genes have GC contents of 69 and 65%, respectively, compatible with the genes being of mycobacterial origin (15). The mycobacterial NATs each show amino acid sequence identities of ~35 and 30% with the NATs from *S. typhimurium* and human NAT2, respectively (Fig. 1).

Expression of mycobacterial *nat* genes in *E. coli*. The predicted open reading frames of *S. typhimurium*, *M. smegmatis*,

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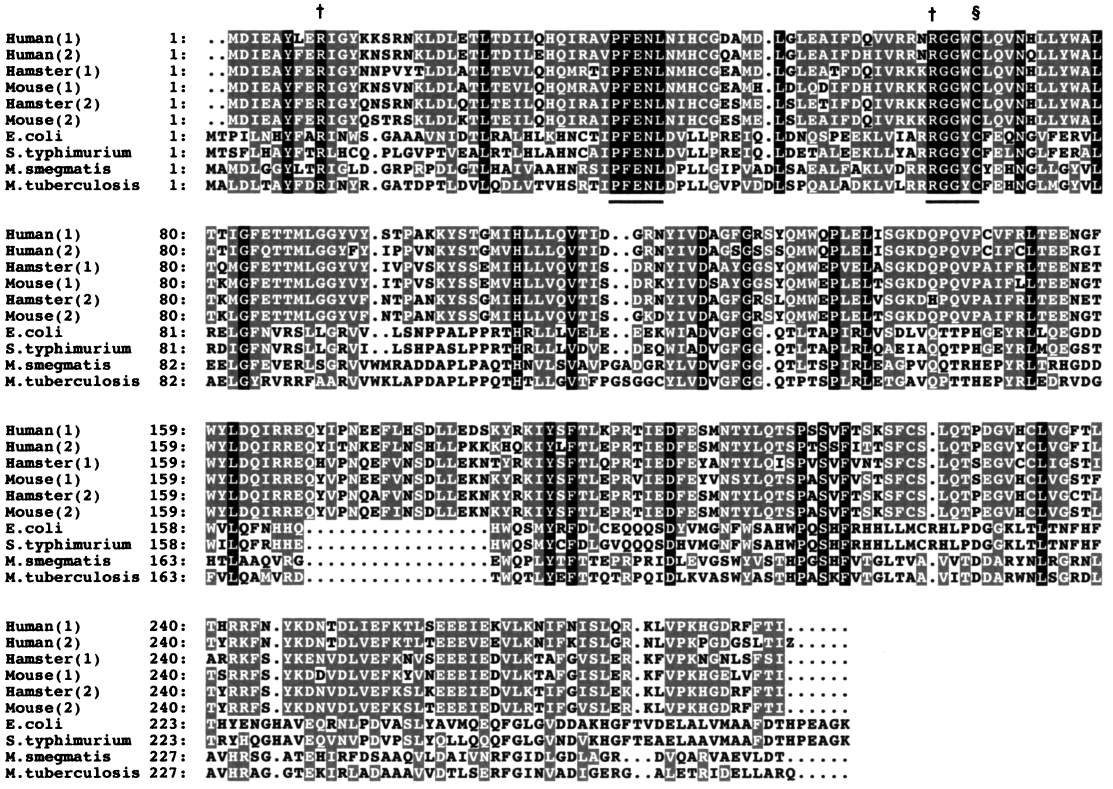

FIG. 1. Amino acid alignment of the predicted *M. smegmatis* and *M. tuberculosis* NAT against NAT homologues of eukaryotic and prokaryotic origins. Amino acids are represented by the single-letter code. Numbers refer to amino acid positions of the aligned sequences, which are ranked in order of decreasing similarity in comparison with human NAT1. Codes on the left identify the relevant NAT sequence. Human(1) is human NAT1, accession no. P18440; Human(2) is human NAT2, accession no. P11245; Hamster(1) is hamster NAT1, accession no. P50292; Mouse(1) is mouse NAT1, accession no. P50294; Hamster(2) is hamster NAT2, accession no. P50293; Mouse(2) is mouse NAT2, accession no. P50295; E. coli is *E. coli* NAT, accession no. P77567; S. typhimurium is *S. typhimurium* NAT, accession no. Q00267; M. tuberculosis is *M. tuberculosis* NAT, accession no. P96848, which codes for a hypothetical protein (3) and has the same sequence as the *M. tuberculosis* NAT described in this report; and M. smegmatis is *M. smegmatis* NAT, accession no. AJ006588. Amino acids showing identity in all NATs are shown in white on a black background, while residues showing conservation and similarity in more than four species of NAT are indicated in white on a grey background. A dot represents a gap introduced to maximize homology. The proposed active site cysteine is indicated (§) (32), as are the arginine residues thought to participate in the reaction (†) (6, 32). Regions of complete identity are underscored.

and *M. tuberculosis nat* genes were cloned into the expression vector pET28b (Novagen). *E. coli* cells (DE23-pLysS) were transformed with the constructs for recombinant protein production, which also encode a 2.1-kDa thrombin-cleavable *N*-terminal histidine tag. Recombinant protein production was carried out as described previously (24). *E. coli* cells were disrupted by sonication on ice, eight times each for 30 s with 15-s intervals between sonications, and centrifuged at 100,000 × *g* for 60 min at 4°C. Supernatant was removed, and the pellet was resuspended in an equal volume of resuspension buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 4 mM dithiothreitol, and 1 mM Pefabloc [protease inhibitor]). It was demonstrated that the *nat* sequence from *M. smegmatis* induces the synthesis of a protein of ~32 kDa, close to the predicted size of 32.3 kDa, corresponding to the open reading frame coding for the NAT protein (275 amino acids) plus the hexahistidine fusion tag (2.1 kDa). The recombinant NAT protein from *M. smegmatis* was found associated predominantly with the insoluble pellet (Fig. 2A, lane 4). The molecular mass of the *M. tuberculosis* NAT protein (275 amino acids) plus the hexahistidine tag is predicted to be 33.5 kDa. The NAT recombinant protein of *M. tuberculosis* has a molecular mass of around 32 kDa and is found exclusively in the insoluble pellet when produced in *E. coli* (Fig. 2A, lane 2). The molecular mass of the *S. typhimurium* NAT protein (283 amino acids) with the hexahistidine tag is predicted to be 34.3 kDa and is found predominantly in the soluble, supernatant fraction (Fig. 2A, lane 3).

An antiserum raised against purified recombinant *S. typhimurium* NAT (24) was used to confirm the identity of the induced protein bands observed in Fig. 2A. The immunization schedule and detection were carried out as previously described (27). The antiserum was used at a dilution of 1:100,000 and did not cross-react with the control *E. coli* supernatant that had been transformed with vector alone under the conditions used (Fig. 2B, lane 1). The antiserum does identify a low level of endogenous *E. coli* NAT when used at a dilution of 1:50,000 (data not shown). The antiserum, at 1:100,000, cross-reacted with a strong band in the supernatant of *E. coli* transformed with *nat* from *S. typhimurium* (Fig. 2B, lane 3) and in the supernatant of *E. coli* which had been transformed with the putative *nat* from *M. smegmatis* (Fig. 2B, lane 4). No band corresponding to *M. tuberculosis* NAT was found in the supernatant fraction (data not shown). However, a protein in the pellet fraction of *E. coli* cells expressing the *M. tuberculosis nat* gene was shown to cross-react with the antiserum to *S. typhimurium* NAT. The degree of recognition of the NAT from *M. tuberculosis* in the pellet (Fig. 2B, lane 2) is less than that expected based on the protein staining intensity (Fig. 2A, lane 2P), suggesting that the antiserum to the *S. typhimurium* NAT

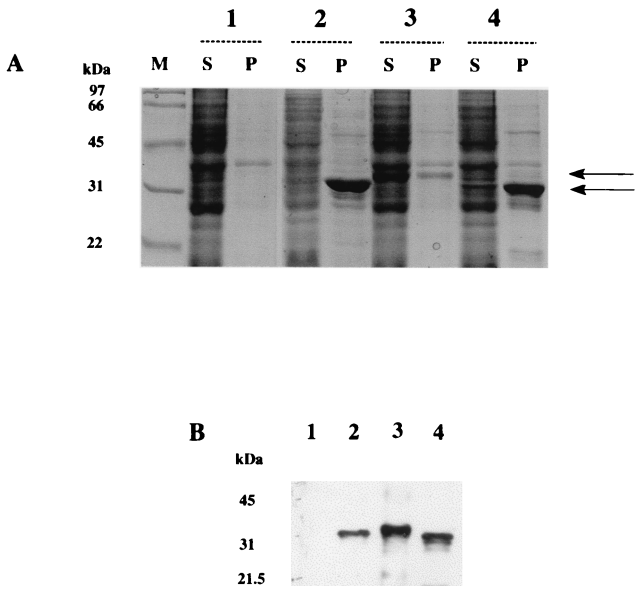

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of recombinant NAT proteins in *E. coli*. (A) Gels were stained with Coomassie blue. *E. coli* cells were transformed with pET28b alone (lanes 1) or with pET28b constructs containing the coding region of *nat* from *M. tuberculosis* (lanes 2), *S. typhimurium* (lanes 3), or *M. smegmatis* (lanes 4). Lysates were used to generate supernatants (S) and pellets resuspended in the original volume of lysate (P). Individual lanes were loaded with 15 μl of each fraction. The upper and lower arrows indicate the migration of recombinant *S. typhimurium* and mycobacterial NATs, respectively. Lane M, molecular mass markers. (B) Western blot analysis using an antiserum (1:100,000) against recombinant *S. typhimurium* NAT developed by using an enhanced chemiluminescence detection system (27). Lane 1, supernatant from *E. coli* transformed with pET28b alone; lane 2, pellet from *E. coli* transformed with *M. tuberculosis* NAT; lane 3, supernatant from *E. coli* transformed with *S. typhimurium* NAT; lane 4, supernatant from *E. coli* transformed with *M. smegmatis* NAT.

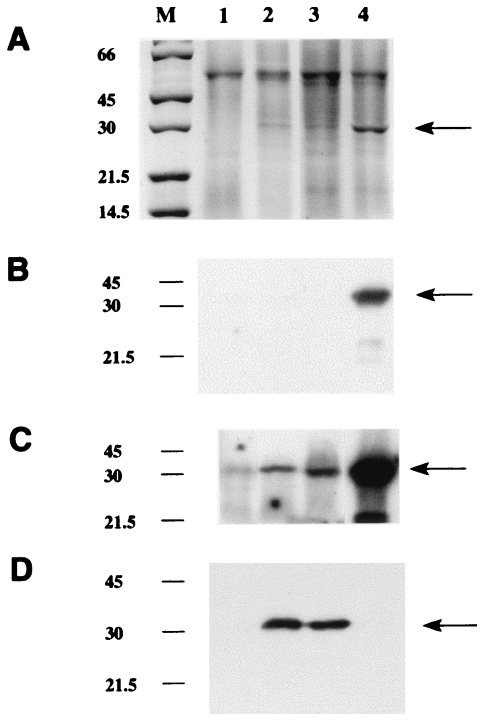

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of recombinant NAT proteins in *M. smegmatis*. Gels were loaded with 15 μl of supernatants from lysates of *M. smegmatis* transformed with pACE-1 alone (lane 1), with *M. tuberculosis nat* (lanes 2 and 3), or with *M. smegmatis nat* (lane 4) (M, molecular mass markers [in kilodaltons]). (A) Staining with Coomassie blue. The arrow indicates the additional band in the supernatants transformed with a *nat* gene. (B to D) Western blots developed by using an enhanced chemiluminescence detection system as described previously (27), where exposure time for detection of the second antibody is either 1 min (B and D) or 20 min (C). In each panel, lane 1 has been loaded with 10 μg of total protein and lanes 2 to 4 have been loaded with 600 ng of total protein. An antiserum raised against recombinant *M. tuberculosis* NAT protein synthesized in *E. coli* is shown in panel D. The antiserum used in panels B and C is the antiserum against pure *S. typhimurium* NAT (24). Both antisera were used at a dilution of 1:100,000.

cross-reacts less with the *M. tuberculosis* protein than with the *M. smegmatis* protein (Fig. 2A, lane 4S, and Fig. 2B, lane 4).

The detection of NAT activity using the arylamines anisidine, 4-aminoveratrole, and *p*-aminobenzoic acid was carried out by a colorimetric assay (24), and activity with the hydrazine isoniazid was detected by the method of Olson et al. (19). Bacterial lysates and supernatants were diluted up to 50-fold with 20 mM Tris-HCl (pH 7.5) and 2 mM dithiothreitol before being used. Controls were carried out with identically diluted samples of *E. coli* cell lysate fractions that had been transformed with pET28b alone and demonstrated no activity under the conditions used. Likewise, insoluble pellets from *E. coli* cells expressing the *M. tuberculosis nat* gene or the *M. smegmatis nat* gene had no NAT enzymic activity. It was concluded that the NAT protein in these pellets was in inclusion bodies. Recombinant NAT from *M. smegmatis* in the supernatant fraction of *E. coli* is active in acetylating a series of substrates, including isoniazid, anisidine, and 4-aminoveratrole, which are also substrates of the *S. typhimurium* enzyme. The arylamine *p*-aminobenzoic acid is poorly acetylated by NAT from both *M. smegmatis* and *S. typhimurium* (24). Isoniazid is a better substrate (K_m, 25 μM; V_{max}/K_m 2,520 × 10^{-6} liter · min^{-1} · mg of $protein^{-1}$) than are the arylamines anisidine (K_m, 300 μM; V_{max}/K_m, 16 × 10^{-6} liter · min^{-1} · mg of $protein^{-1}$) and 4-aminoveratrole (K_m, 650 μM; V_{max}/K_m, 25 × 10^{-6} liter · min^{-1} · mg of $protein^{-1}$) for the NAT enzyme from *M. smegmatis*. These results suggest the enzyme has a substrate specificity similar to that of the *S. typhimurium* enzyme (24, 32).

Expression of mycobacterial *nat* genes in *M. smegmatis*. *M. smegmatis* and *M. tuberculosis nat* genes were also expressed by using the mycobacterial expression vector pACE-1. Competent *M. smegmatis* cells (Mc^2155 [26]) were electroporated with 1 μg of plasmid DNA, either alone or containing the *nat* insert, at 700 Ω, 2.5 kV, and 25 μF (26). *M. smegmatis* cells were grown on the selective medium 7H9 supplemented with albumin-dextrose-catalase (Difco) or 7H11 agar supplemented with oleic acid-albumin-dextrose-catalase (Difco) containing hygromycin (50 μg/ml). Minimal medium was used for gene expression using the pACE-1 constructs (21). These constructs yield recombinant protein when acetamide (2 mg/ml) is supplied as the sole carbon source in the growth medium. *M. smegmatis* cell pellets were sonicated on ice 10 times, each for 1 min, with 30-s intervals between sonications. The resulting cell lysates were centrifuged at 100,000 × *g* for 60 min at 4°C. Supernatants were kept, and the pellets were resuspended to the original volume in resuspension buffer. With this expression vector, recombinant mycobacterial NAT protein was detected predominantly in the supernatant fraction (Fig. 3A). Cells expressing the *M. smegmatis nat* (Fig. 3A, lane 4) routinely had more recombinant protein than the cells expressing the *M. tuberculosis nat* gene (Fig. 3A, lanes 2 and 3). The sizes of the induced protein bands were around 30 kDa, which corresponds to the expected sizes for NAT proteins without a

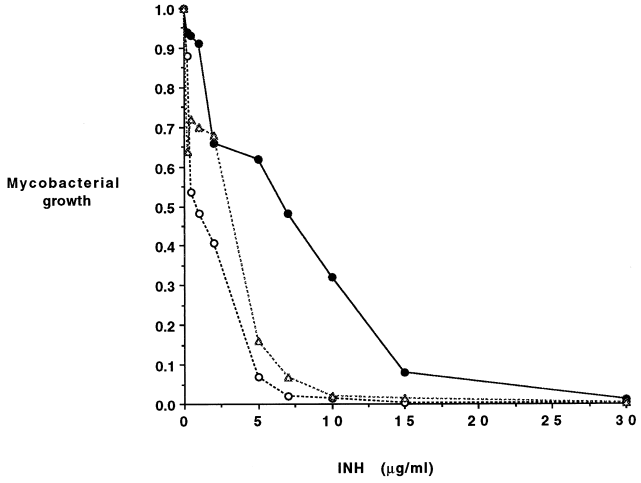

FIG. 4. Effect of expression of *M. tuberculosis nat* on the growth of *M. smegmatis* in the presence of isoniazid (INH). Results for cultures of *M. smegmatis* either transformed with pACE-1 alone (open circles) or with pACE-1 containing *M. tuberculosis nat* (solid circles) grown in minimal medium containing acetamide and cultures of pACE-1 containing *M. tuberculosis nat* grown in minimal medium containing glucose (triangles) are shown.

hexahistidine tag. The antiserum raised against the pure *S. typhimurium* NAT identified a band in the supernatants of *M. smegmatis* induced to express the *M. smegmatis nat* gene (Fig. 3B and C, lane 4) and, with longer exposure of the autoradiograph, the presence of *M. tuberculosis* NAT (Fig. 3C, lanes 2 and 3). There was also an indication of endogenous NAT (Fig. 3C, lane 1). The sizes of the endogenous and induced proteins (which have no hexahistidine tags) illustrated in Fig. 3 are indistinguishable, as expected. An antiserum raised against recombinant *M. tuberculosis* NAT, prepared by immunizing rabbits with insoluble recombinant protein excised from a polyacrylamide gel slice, was used at a dilution of 1:100,000 and is highly specific for *M. tuberculosis* NAT (Fig. 3D, lanes 2 and 3). It does not recognize *M. smegmatis* NAT under the conditions shown in Fig. 3D.

Overproduction of mycobacterial *nat* genes in *M. smegmatis* results in increased resistance to isoniazid. We have investigated whether the expression of the *nat* gene from *M. tuberculosis* alters the sensitivity of *M. smegmatis* to isoniazid in vivo. *M. smegmatis* transformants of pACE-1 either alone or containing the *nat* gene from *M. tuberculosis* were grown in minimal medium containing either acetamide to induce synthesis of protein or an equivalent carbon concentration of glucose which does not induce protein synthesis from the pACE-1 vector. Cultures were grown for up to 48 h in the presence of different amounts of isoniazid, and cell growth was determined at 600 nm with a plate reader (Titertek Multiskan). It was observed that when the expression of the *nat* gene was induced, there was an increase in the concentration of isoniazid in the growth medium that could be tolerated (Fig. 4). There was no change in the response to isoniazid in *M. smegmatis* cultures containing the *nat* gene construct when the growth conditions did not induce synthesis of recombinant NAT protein. In contrast, *M. smegmatis* cells, which contained only vector, were equally sensitive to isoniazid, irrespective of whether acetamide or glucose was the carbon source.

Discussion. The existence of highly conserved arylamine *N*-acetyltransferase sequences in bacteria other than *S. typhimurium* suggests that the enzyme is conserved in evolution. The role of NAT in endogenous metabolism is unclear, although it has been suggested that in eukaryotes, NAT (in particular, the human NAT1 isoenzyme) plays a role in folate catabolism (16, 31). The very poor activity of the NAT from *M. smegmatis* or *S. typhimurium* with *p*-aminobenzoic acid can be rationalized on the basis that a supply of *p*-aminobenzoic acid is essential for folate synthesis in prokaryotes. The activity profile is more like that of the human isoenzyme NAT2 which is responsible for the inactivation, by acetylation, of isoniazid (18). NATs are present in several bacterial species, and a sequence similar to NAT has been identified in the completed genome sequence of *E. coli* (1). It has been demonstrated in the present study that the activity in *E. coli* with all substrates tested is less than 1% of the activity of the other recombinant NATs overproduced in *E. coli*. In addition to the overall homology, alignment of NAT sequences (Fig. 1) necessitates the introduction, in the bacterial sequences, of a gap of around 20 amino acids on the C-terminal region flanking the N- and C-terminal regions of the molecule (12). It has been suggested that the N-terminal region is predominantly alpha helix, while the C-terminal portion is predominantly beta sheet. The gap corresponds to the region linking these two secondary structure domains of the molecule. The bacterial NAT enzymes appear to be particularly stable to proteolysis, in contrast to the mammalian NAT enzymes (25), which may be due to the lack of a randomly structured loop in the bacterial NATS.

The expression of NAT in mycobacteria has important implications. Mycobacteria are exquisitely sensitive to isoniazid (14), although there are differences among the mycobacteria in their levels of sensitivity to the drug. *M. tuberculosis* will not grow in 0.2 μg of isoniazid per ml (21), and *M. smegmatis* will not grow in 5 μg of isoniazid per ml (7). When we induced the synthesis of more NAT in *M. smegmatis*, growth of the mycobacteria was not arrested until the concentration of isoniazid was 15 μg/ml. Isoniazid is inactivated in humans through acetylation (8, 9). The results presented here demonstrate that NAT is expressed endogenously in *M. smegmatis* and that the NAT proteins from mycobacteria can acetylate isoniazid and thus appear able to inactivate isoniazid in vivo. There is a body of evidence to demonstrate that the antimycobacterial activity of isoniazid in mycobacteria relies on the drug first becoming activated (11, 17, 23). It is already known that the acetylation of isoniazid inactivates the drug. The results presented here support the concept that the acetylation of isoniazid in mycobacteria acts in competition with the activation through oxidation. It is therefore important to investigate *nat* expression in other mycobacteria, including particularly *M. tuberculosis*, in which development of isoniazid resistance cannot be accounted for completely by currently identified loci (29).

We are extremely grateful to the Wellcome Trust for financial support.

We thank S. Martin for the preparation of the gridded libraries and D. Young, K. Duncan, and J. Sinclair for very helpful discussions. We also thank James Sandy for excellent technical assistance.

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