

# Purification, Gene Cloning, Targeted Knockout, Overexpression, and Biochemical Characterization of the Major Pyrazinamidase from *Mycobacterium smegmatis*

HELENA I. M. BOSHOFF AND VALERIE MIZRAHI\*

Molecular Biology Unit, South African Institute for Medical Research, and Department of Haematology, University of the Witwatersrand Medical School, Johannesburg, South Africa

Received 9 July 1998/Accepted 9 September 1998

The pyrazinamidase from *Mycobacterium smegmatis* was purified to homogeneity to yield a product of approximately 50 kDa. The deduced amino-terminal amino acid sequence of this polypeptide was used to design an oligonucleotide probe for screening a DNA library of *M. smegmatis*. An open reading frame, designated *pzaA*, which encodes a polypeptide of 49.3 kDa containing motifs conserved in several amidases was identified. Targeted knockout of the *pzaA* gene by homologous recombination yielded a mutant, *pzaA::aph*, with a more-than-threelfold-reduced level of pyrazinamidase activity, suggesting that this gene encodes the major pyrazinamidase of *M. smegmatis*. Recombinant forms of the *M. smegmatis* PzaA and the *Mycobacterium tuberculosis* pyrazinamidase/nicotinamidase (PncA) were produced in *Escherichia coli* and were partially purified and compared in terms of their kinetics of nicotinamidase and pyrazinamidase activity. The comparable  $K_m$  values obtained from this study suggested that the unique specificity of pyrazinamide (PZA) for *M. tuberculosis* was not based on an unusually high PZA-specific activity of the PncA protein. Overexpression of *pzaA* conferred PZA susceptibility on *M. smegmatis* by reducing the MIC of this drug to 150  $\mu$ g/ml.

Pyrazinamide (PZA) is one of the most important drugs in tuberculosis chemotherapy, but in contrast to other first-line drugs, relatively little is known about its mechanism of action (12, 27). PZA is converted in the mycobacterium to pyrazinoic acid (POA) by the action of the enzyme pyrazinamidase. POA is thought to function as the toxic metabolite by virtue of its activity as a weak acid and/or by specifically inhibiting a metabolic process (12). In a recent seminal study, point mutations in the *M. tuberculosis pncA* gene that abrogated the pyrazinamidase/nicotinamidase activity of this organism were shown to confer resistance to PZA and the natural resistance of *M. bovis* to this drug was shown to be attributable to an inactivating point mutation in *pncA* (27). Subsequent studies have shown that 72 to 95% of PZA-resistant clinical isolates of *M. tuberculosis* are associated with *pncA* mutations (26, 29), suggesting that pyrazinamidase-catalyzed hydrolysis of PZA is essential for the activity of this drug in *M. tuberculosis*. This conclusion is consistent with the fact that POA esters show good antimycobacterial activity against PZA-susceptible and -resistant isolates of *M. tuberculosis* (4, 35). Paradoxically, other mycobacterial species such as *M. smegmatis* (5, 33) and *M. avium* (31) are known to possess pyrazinamidase activity yet are resistant to PZA. We therefore decided to investigate the PZA-hydrolyzing activity of *M. smegmatis*. We report the purification to homogeneity of the major pyrazinamidase from this organism and the subsequent cloning and targeted knockout of its encoding gene. We also show that overexpression of this enzyme conferred PZA susceptibility on *M. smegmatis*.

(Preliminary reports on the purification of this activity have been published elsewhere [2, 18]).

## MATERIALS AND METHODS

**Materials, bacterial strains, media, and growth conditions.** Enzymes were from Boehringer Mannheim or New England Biolabs; radiochemicals were from Amersham; the Sequenase 2.0 sequencing kit was from United States Biochemicals; and PZA, POA, nicotinamide, and other fine chemicals were from Sigma. The bacterial strains and plasmids used in this study are listed in Table 1, and the oligonucleotides are given in Table 2. *Escherichia coli* strains were grown in Luria-Bertani broth (LB) or agar (LA) for all plasmid isolations or in 2YT broth for expression of recombinant proteins (24). *E. coli* DH5 $\alpha$  was used for plasmid manipulations, JM101 was used for M13 cloning, and BL21 (DE3)pLysS was used for expression. Ampicillin, chloramphenicol, and kanamycin were used at 100, 34, and at 50  $\mu$ g/ml, respectively, for *E. coli*. *M. smegmatis* was maintained in MADC-Tw (Middlebrook 7H9 broth [Difco] supplemented with 0.085% NaCl, 0.2% glucose, and 0.05% Tween 80) and on Middlebrook 7H10 supplemented with 0.085% NaCl and 0.2% glucose as the solid medium.

**Purification of the *M. smegmatis* pyrazinamidase.** *M. smegmatis* ATCC 607 was grown and harvested as described by Scherman et al. (25). Cell pellets (35 g) were resuspended in 70 ml of buffer A (20 mM sodium phosphate [pH 6.3], 1 mM EDTA, 1 mM dithiothreitol [DTT]) containing 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ M leupeptin, and 0.2  $\mu$ M aprotinin and sonicated at 4°C for 15 cycles of a 15-s pulse followed by a 90-s cooling. The extract was clarified by centrifugation at 25,400  $\times$  g for 20 min at 4°C followed by 148,000  $\times$  g for 60 min at 4°C. Extracts (50 ml) were loaded on a 2.5- by 10-cm Q Sepharose Fast Flow column (Pharmacia LKB) equilibrated in buffer A and eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A (1,500 ml). Pyrazinamidase-containing fractions were pooled, concentrated to 8 ml by ultrafiltration through a 30-kDa exclusion limit membrane (Amicon), and fractionated on a HiLoad 26/60 Superdex (Pharmacia LKB) column in buffer A (0.8 ml/min). Peak fractions were loaded on a Mimetic Blue 1 A6XL column (0.5 by 3 cm; Affinity Chromatography Ltd.) equilibrated in buffer A, and the enzyme was eluted in 50 mM KCl in buffer A (15 ml). A second anion-exchange separation was performed on a MonoQ HR5/5 column (Pharmacia LKB) eluting with a 0 to 0.5 M NaCl linear gradient in buffer A (30 ml). Ammonium sulfate (1 M) was added to peak fractions, and the protein was fractionated on a phenyl-Superose HR5/5 column (Pharmacia LKB), eluting with a linear gradient of 1 to 0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in buffer A (30 ml). Protein concentrations were determined by a Bradford assay (Bio-Rad kit II), and proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Protein sequencing.** Cysteine residues were derivatized with 4-vinylpyridine, and the proteins were subsequently separated by electrophoresis on a 10% Tricine gel. After being blotted to a polyvinylidene difluoride membrane, the proteins were subjected to Edman degradation and analysis on an Applied Biosystems ABI476A protein sequencer.

**Cloning and sequencing of the *pzaA* gene.** The probe PZA-P1 (Table 2), designed from the amino-terminal sequence of the purified protein (MELYEL PLIEVAEKIRTKEVSPVEVTES), was used to screen a gridded plasmid library

\* Corresponding author. Mailing address: Molecular Biology Unit, SAIMR, P.O. Box 1038, Johannesburg 2000, South Africa. Phone: 2711-4899370. Fax: 2711-4899001. E-mail: 075val@chiron.wits.ac.za.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
<i>M. smegmatis</i> strains		
mc <sup>2</sup> 155	High-frequency transformation mutant of <i>M. smegmatis</i> ATCC 607	28
<i>pzaA::aph</i>	<i>pzaA</i> knockout mutant of <i>M. smegmatis</i> mc <sup>2</sup> 155; Km <sup>r</sup>	This work
[ <i>pzaA::aph</i> ](pOam)	<i>pzaA::aph</i> carrying <i>pzaA</i> on pOam; Km <sup>r</sup> , Hyg <sup>r</sup>	This work
mc <sup>2</sup> 155(pOam)	mc <sup>2</sup> 155 carrying <i>pzaA</i> on pOam; Hyg <sup>r</sup>	This work
Plasmids		
pGEM3Z(+)	<i>E. coli</i> cloning vector; Ap <sup>r</sup>	Promega
pET15b	<i>E. coli</i> expression vector; Ap <sup>r</sup>	Novagen
p45E5	pBluescript SK+ construct containing complete <i>M. smegmatis pzaA</i> with ca. 200 bp of upstream sequence	M. Everett
p38O20	pBluescript SK+ construct containing partial <i>M. smegmatis pzaA</i> with 380 bp of upstream sequence	M. Everett
pTam	pET15b derivative carrying <i>pzaA</i>	This work
pTncA	pET15b derivative carrying <i>pncA</i>	This work
pGam	pGEM3Zf(+) derivative carrying <i>M. smegmatis pzaA</i> with 382 bp of upstream sequence cloned as a <i>HindIII-XbaI</i> fragment	This work
pMam	M13mp19 construct containing the 1,824-bp <i>HindIII-XbaI</i> fragment from pGam	This work
pMamB	pMam derivative in which the internal <i>BamHI</i> site in <i>pzaA</i> was mutated to <i>BglII</i>	This work
pGamB	pGEM3Zf(+) construct carrying the <i>EcoRV-XbaI</i> fragment from pMamB	This work
pGamK	pGamB derivative carrying the <i>aph</i> gene in the <i>BglII</i> site engineered in <i>pzaA</i>	This work
pOLYG	Multicopy <i>E. coli-Mycobacterium</i> shuttle vector; Hyg <sup>r</sup>	P. O'Gaora
pOam	pOLYG derivative carrying the <i>HindIII-XbaI</i> fragment from pGam	This work
pHINT	<i>E. coli-Mycobacterium</i> integrating shuttle vector; Hyg <sup>r</sup>	P. O'Gaora
pHam	pHINT derivative carrying the <i>HindIII-XbaI</i> fragment from pGam	This work

of *M. smegmatis* provided by M. Everett (Glaxo Wellcome, Stevenage, United Kingdom). Two overlapping clones (p45E5 and p38O20) thus identified were subcloned in M13mp18/19 for DNA sequencing. The nucleotide sequence was analyzed with the Lasergene suite of programs (DNASTAR, Madison, Wis.).

**Expression of *M. smegmatis PzaA* and *M. tuberculosis PncA* in *E. coli*.** The *M. smegmatis pzaA* gene was amplified by PCR with Vent polymerase (Promega) and the AMD-F and AMD-R primer pair. The product was cloned in pET15b to create pTam, which directed the overexpression of a recombinant protein with the authentic PzaA sequence. Overexpression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to cultures at an optical density at 600 nm of 0.6. After 2 h at 37°C, the cells were harvested, washed, and resuspended at 0.2 g/ml in buffer B (20 mM sodium phosphate [pH 5.9], 1 mM EDTA, 1 mM DTT, 0.04% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>) containing protease inhibitors (Complete; Boehringer Mannheim), freeze-thawed once, and lysed by sonication. Clarified extracts were obtained by centrifugation at 20,000 × g for 60 min. Most of the recombinant PzaA was precipitated in inclusion bodies, but sufficient activity remained in the supernatant for further analysis. The protein was partially purified by loading a 50-ml extract on a 2.5- by 12-cm Q Sepharose Fast Flow column equilibrated in buffer B and eluting with a linear gradient of 0 to 0.5 M NaCl in buffer B (600 ml).

The *M. tuberculosis pncA* gene was amplified by PCR with the PncA-F and PncA-R primer pair (Table 2). The product was cloned in pET15b to form

pTncA. Since PncA-F introduced a Gly codon between the first and second codons of *pncA*, the recombinant form of PncA was designated PncA-G2+. Overexpression and lysis were carried out as described above, and the protein was partially purified by fractionating a 50-ml extract on a 2.5- by 12-cm Q Sepharose Fast Flow column equilibrated in buffer B and eluting with a linear gradient of 0 to 0.5 M NaCl in buffer B (600 ml). Peak fractions were treated with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (1.6 M) and loaded on a Toyopearl phenyl 650M 2- by 16-cm column [equilibrated in 1.6 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-buffer C] and eluted with a 300-ml linear gradient of 1.6 to 0 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in buffer C (10 mM Tris · HCl [pH 7.5], 1 mM DTT, 0.04% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>). Peak fractions were loaded on a 2- by 16-cm hydroxyapatite column (Fast Flow; Calbiochem) equilibrated in buffer D (1 mM potassium phosphate, 10 mM Tris · HCl [pH 8], 0.2 mM CaCl<sub>2</sub>, 0.04% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>), and the protein was eluted with a 300-ml linear gradient of 0 to 100% buffer E (0.3 M potassium phosphate [pH 7.4], 0.01 mM CaCl<sub>2</sub>, 0.04% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>).

**Enzyme assays.** The pyrazinamidase assay was a modification of the Wayne test (34). The activity was assayed by adding a 50-μl aliquot of test sample to 50 μl of a solution containing 40 mM PZA, 100 mM sodium phosphate (pH 6.5), and 2 mM DTT. After incubation at 37°C for 5 to 10 min, 10 μl of 20% FeNH<sub>4</sub>(SO<sub>4</sub>)<sub>2</sub> and 890 μl of 0.1 M Gly · HCl (pH 3.4) were added, precipitates were removed by centrifugation (13,000 × g for 10 min), and the OD<sub>480</sub> of the supernatant was determined. The POA concentration was determined from a standard curve. Since this modified Wayne test was limited in its sensitivity, a

TABLE 2. Oligonucleotides used in this study

Primer	Sequence (5'-3')	Strand	Position and description
<i>M. smegmatis pzaA</i> primers <sup>a</sup>			
PZA-P1	ATCGAGGTCGCGGAGAAGATCCGCACGAAG GAGGTCAGCCCGGTTCGAGGT	+	Guessmer used to clone the <i>pzaA</i> gene.
AMD-F	GGGGCCATGGAACTCTACGAA	+	Positions 382–396. G/C clamp and <i>NcoI</i> site are italic; start codon is underlined.
AMD-R	GGGGCATATGTTCAGCCACCTT	–	Positions 1777–1791. G/C clamp and <i>NdeI</i> site are italic; stop codon is underlined.
muAMD	GGATGCGGATAGATCTGCCCGTATCGCTGC	–	Positions 881–910. Used to mutate internal <i>BamHI</i> to <i>BglII</i> (underlined).
<i>M. tuberculosis pncA</i> primers <sup>b</sup>			
PncA-F	GGGGCCATGGGACGGGCGTTGATCATC	+	Positions 1–18. G/C clamp and <i>NcoI</i> site are italic; start codon is underlined; extra Gly2 codon is bold.
PncA-R	GGGGGGATCCTCAGGAGCTGCAAAC	–	Positions 548–561. G/C clamp and <i>BamHI</i> site are in italic; stop codon is underlined.

<sup>a</sup> Numbering according to GenBank accession no. AF058285.

<sup>b</sup> Numbering according to GenBank accession no. U59967.

TABLE 3. Summary of the purification of the *M. smegmatis* pyrazinamidase activity

Step	Vol (ml)	Concn of protein (mg/ml)	Total amt of protein (mg)	Sp act (U/mg) <sup>a</sup>	Total activity (U)	Yield (%)	Fold purification
Crude cell extract	70	12	840	0.45	378	100	1
Q Sepharose	190	0.39	75	3.53	265	71	8
Superdex 200	20	0.089	1.8	35.3	64	17	80
Blue 1 A6XL	3	0.083	0.25	201	50	13	448
Mono Q HR5/5	2.5	0.029	0.072	607	44	11	1,350
Phenyl-Superose	2	0.0085	0.017	1,471	25	6	3,270

<sup>a</sup> A unit (1 U) is defined as the number of micromoles of product formed per minute at 37°C.

coupled amidase enzyme assay based on ammonia release was developed to determine the  $K_m$  values for nicotinamide and PZA. The 750- $\mu$ l assay mixture consisted of 30 mM Tris · HCl (pH 7.5), 15 U of L-glutamate dehydrogenase per ml, 800  $\mu$ M  $\alpha$ -ketoglutarate, 160  $\mu$ M NADPH, and PZA (0; 70  $\mu$ M to 5 mM) or nicotinamide (0; 10  $\mu$ M to 5 mM) and the appropriate units of amidase, with the substrate being added last. The reaction mixtures were incubated at room temperature (21 to 25°C), and the optical density at 340 nm was monitored with a Shimadzu UV1601 spectrophotometer during the course of the reaction.

**Construction of a *pzaA* knockout mutant of *M. smegmatis*.** Plasmid pGam was constructed by cloning the 282-bp *HindIII*-*StuI* upstream fragment from p38O20 and the 1,542-bp *StuI*-*XbaI* fragment from p45E5 in pGEM-3Zf(+). The 1,824-bp *HindIII*-*XbaI* fragment from pGam was cloned in M13mp19 to produce pMam, which was used as the template for site-directed mutagenesis with a Muta-Gene kit (Bio-Rad) and the oligonucleotide muAMD (Table 2) to yield pMamB. The *EcoRV*-*XbaI* fragment from pMamB was cloned in pGEM3Zf(+) to yield pGamB. The *Tn903 aph* (kanamycin resistance) gene, carried on a 1.3-kb *BamHI* fragment (10), was cloned in the *BglII* site of pGamB to form pGamk. *M. smegmatis* was electroporated with 1  $\mu$ g of alkali-denatured pGamk (19) as described by Jacobs et al. (13), except that the cells were washed and prepared in 1% glycerol. Cells were plated on LA containing 10  $\mu$ g of kanamycin per ml, and colonies were grown in LB containing 10  $\mu$ g of kanamycin per ml. Genomic DNA was isolated as previously described (10), and Southern blots were probed with the *pzaA* PCR product described above. Cell extracts were prepared by resuspension of cell pellets from 10-ml cultures in 0.7 ml of 20 mM sodium phosphate (pH 6), disruption in a Bio 101/Savant FastPrep cell disruptor as previously described (7), and removal of cells and debris by centrifugation at 13,000  $\times$  g for 30 min.

**Construction of PzaA expression vectors.** The *M. smegmatis pzaA* gene was excised as a *HindIII*-*XbaI* fragment from pGam and cloned in the hygromycin resistance vectors pOLYG and pHINT (9) to form pOam and pHam, respectively (Table 1). The constructs were electroporated into *M. smegmatis*. DNA was extracted from the recombinants, and Southern blotting was performed as described above. The presence of pOLYG-based vectors was also confirmed by electrotransformation into *E. coli* (10). The pyrazinamidase activity of recombinant clones was determined as previously described (10).

**Determination of PZA susceptibility.** Dilutions of cultures were prepared in sterile water and plated on Middlebrook 7H10 adjusted to pH 5.2, supplemented with NaCl and glucose as described above, and containing a range of PZA concentrations (0, 50, 100, 150, 200, 300, 500, and 2,200  $\mu$ g/ml). Colonies were counted after the appropriate growth period, and the MIC was defined as the lowest PZA concentration that caused a 90% reduction in the colony count. Nicotinamide susceptibility studies were carried out in a similar manner.

**Nucleotide sequence accession number.** The *pzaA* gene sequence was deposited in GenBank under accession no. AF058285.

## RESULTS AND DISCUSSION

**Purification of the *M. smegmatis* pyrazinamidase and cloning of its encoding gene.** The pyrazinamidase activity from *M. smegmatis* was purified 3,270-fold by a combination of anion-exchange, gel filtration, dye affinity, and hydrophobic interaction chromatography (Table 3). SDS-PAGE of the purified protein revealed a major species at ca. 50 kDa (Fig. 1). The amino-terminal sequence was used to design a 50-mer oligonucleotide probe (PZA-P1 [Table 2]) for screening a gridded plasmid library of *M. smegmatis* DNA. An open reading frame (ORF) encoding 468 amino acids (49.3 kDa) was identified in positive clones and designated *pzaA*. Mycobacterial promoter elements (1) could not be identified in the region 382 nucleo-

tides upstream of the putative start codon, although a ribosome binding site (GAAAAGGAA) was found 7 nucleotides upstream of this site. The ORF had 52 and 64% amino acid identity and similarity, respectively, to an enantiomer-selective amidase from *Rhodococcus* (17) but showed little homology to the *M. tuberculosis* (27), *M. avium* (31), and *M. kansasii* PncA proteins. This observation is consistent with the fact that *M. avium* and *M. tuberculosis pncA* probes failed to hybridize to *M. smegmatis* genomic DNA (27, 31). PzaA belongs to a large family of enzymes which bear a conserved amidase signature (16, 17) and which act on a wide range of substrates. Although this family includes a putative amidase from *M. tuberculosis*, it is unlikely to hydrolyze nicotinamide or PZA, since loss of the *pncA*-encoded amidase function is sufficient to abrogate the pyrazinamidase/nicotinamidase activity of this organism (26, 27).

**Biochemical characterization of *M. smegmatis* PzaA and comparison with *M. tuberculosis* PncA.** The pyrazinamidase specific activity of a cell extract of *M. tuberculosis* H37Rv (0.02 U/mg) was found to be considerably lower than that of *M. smegmatis* (0.45 U/mg). To determine whether the difference in susceptibilities of *M. tuberculosis* and *M. smegmatis* to PZA could be ascribed to different specificities of the amidases for their substrates, PncA and PzaA were expressed as nonfusion recombinant proteins in *E. coli* and were partially purified for use in steady-state kinetic studies (Fig. 2). The  $K_m$  values for nicotinamide and PZA were not significantly different for the two enzymes (33 and 300  $\mu$ M for PncA-G2+ and 25 and 400  $\mu$ M for PzaA). The  $K_m$  values for nicotinamide were within the ranges reported for other bacterial and yeast nicotinamidases (3, 20, 32, 36), suggesting that these mycobacterial amidases are theoretically competent for the incorporation of nicotinamide as a precursor of NAD synthesis via the Preiss-Handler pathway (22). The high  $K_m$  values for PZA suggested that PzaA and PncA have an equally low specificity for this drug. The biochemical data thus ruled out the possibility that the 20-fold higher apparent pyrazinamidase activity observed in crude extracts of *M. smegmatis* was an artifact of the assay conditions

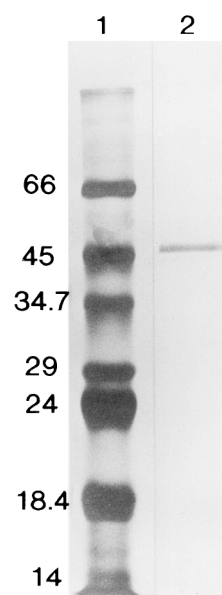


FIG. 1. Purification of the pyrazinamidase from *M. smegmatis*. Lanes: 1, molecular weight markers (sizes shown in thousands); 2, phenyl-Superose fraction that was subjected to N-terminal amino acid sequencing. Samples were fractionated by SDS-PAGE in a 10% gel, and bands were visualized by silver staining.

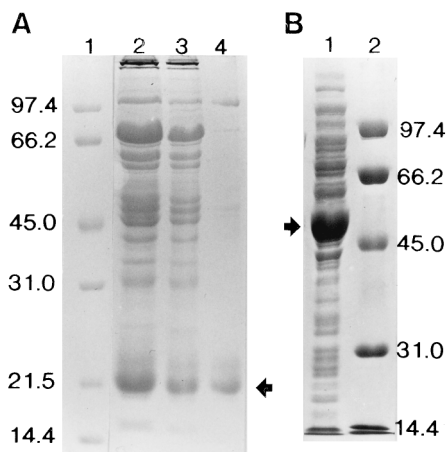


FIG. 2. Partial purification of recombinant forms of *M. smegmatis* PzaA and *M. tuberculosis* PncA (PncA-G2+) expressed in *E. coli*. (A) PncA-G2+. Lanes: 1, molecular weight markers; 2, Q Sepharose fraction; 3, Toyopearl phenyl fraction; 3, hydroxyapatite fraction. (B) PzaA. Lanes: 1, Q Sepharose fraction; 2, molecular weight markers. Marker sizes are as indicated (in thousands), and the positions of the recombinant amidases are shown by arrows. Samples were fractionated by SDS-PAGE in a 10% gel which was stained with Coomassie brilliant blue.

and confirmed that *M. smegmatis* is at least as proficient as *M. tuberculosis* in producing POA. On the basis of these observations, we therefore concluded that the relative resistance of *M. smegmatis* to PZA must be due to other factors such as the nature of the downstream target(s) of POA.

**Construction and phenotypic characterization of a *pzaA* knockout mutant of *M. smegmatis*.** Allelic exchange mutagenesis was used to confirm that *pzaA* encodes the major pyrazinamidase of *M. smegmatis*. The gene was disrupted by insertion of a drug resistance marker at a locus corresponding to the proposed active site of the encoded amidase (Fig. 3). Allelic exchange was detected in 4 of the 40 recombinants, with the remainder corresponding to products of site-specific single crossover (Fig. 3). The *M. smegmatis pzaA::aph* mutant displayed a 3-fold reduction in pyrazinamidase specific activity (Table 4). The residual pyrazinamidase activity of the mutant could be inactivated by heat (data not shown), suggesting that in addition to PzaA, *M. smegmatis* contains other minor, PZA-hydrolyzing enzymes. Comparison of the growth rate of cultures of the *pzaA::aph* mutant grown in MADC-Tw to that of wild-type controls suggested that loss of the amidase activity conveyed no obvious growth disadvantage (data not shown). Since disruption of a gene required in the recycling pathway of an essential cofactor might cause a decreased survival rate under conditions of nutrient limitation, the viability of stationary-phase cultures of the *pzaA::aph* mutant was also compared to the wild-type control, but no differences were detected (data not shown). This observation argues against the salvage of nicotinamide in the NAD biosynthetic pool by the sequential action of nicotinamidase and nicotinic acid phosphoribosyltransferase (PncB [8]) during stationary phase in *M. smegmatis* and is consistent with the inability to demonstrate the presence of PncB in *M. tuberculosis* by using nicotinic acid as a precursor for the synthesis of intermediates of the pyridine nucleotide cycle (6, 14). Therefore, continued de novo synthesis and/or bypass of this arm of the recycling pathway by the action of nicotinamide mononucleotide deamidase would be the preferred routes for the formation of NAD during the stationary phase of *M. smegmatis*.

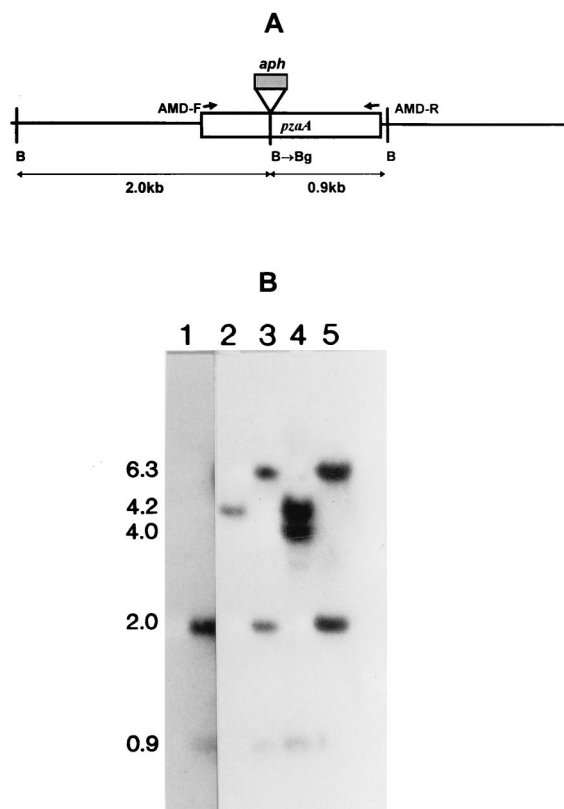


FIG. 3. Targeted knockout of the *M. smegmatis pzaA* gene. (A) Restriction map of the *pzaA* locus showing the site of insertion of the *aph* marker (hatched box) in the *pzaA* gene (open box). The positions of the primers used to amplify the PzaA ORF, and the *Bam*HI (B) and *Bgl*II (Bg) sites are indicated. (B) Southern blot of recombination products. Lanes: 1, wild-type *M. smegmatis* mc<sup>2</sup>155; 2, representative double-crossover product, *pzaA::aph*; 3 and 5, single crossover (downstream); 4, single crossover (upstream). The gel was probed with the *pzaA* PCR product, and the sizes of the hybridizing bands are as indicated (in kilobases).

**PZA susceptibility testing in *M. smegmatis* and in recombinants with altered pyrazinamidase activity.** The fact that the pyrazinamidase activity of the *pzaA::aph* knockout mutant was restored by complementation with a single copy of the *pzaA* gene carried on pHam confirmed that *pzaA* encodes the major pyrazinamidase of *M. smegmatis* (Table 4). However, the level of pyrazinamidase activity was unexpectedly high (threefold higher than in the wild type). Overexpression of PzaA in

TABLE 4. Pyrazinamidase specific activities and MICs for *M. smegmatis* strains<sup>a</sup>

Parental strain of <i>M. smegmatis</i>	Sp act (U/mg) <sup>b</sup>	MIC of PZA (μg/ml)	MIC of Nam (μg/ml)
Wild type			
mc <sup>2</sup> 155	0.45 ± 0.04	≥2,200	>2,200
mc <sup>2</sup> 155(pOam)	10 ± 1	150	150
mc <sup>2</sup> 155:pHam	1.12 ± 0.05	≥2,000	500
Mutant			
<i>pzaA::aph</i>	0.15 ± 0.03	≥2,000	>2,200
[ <i>pzaA::aph</i> ](pOam)	9 ± 1	150	200
[ <i>pzaA::aph</i> ]:pHam	1.04 ± 0.03	ND <sup>c</sup>	ND

<sup>a</sup> MIC determinations were performed at pH 5.2.

<sup>b</sup> Mean and standard deviation from three independent experiments.

<sup>c</sup> ND, not determined.

*M. smegmatis* from the multicopy plasmid, pOam, resulted in a 25-fold increase in the specific activity of the pyrazinamidase enzyme in cell lysates, which was also considerably higher than expected from the estimated copy number of pAL5000-based plasmids (30). Therefore, the *pzaA* expression cassette contained in pHam and pOam appeared to direct a higher level of expression of *pzaA* than the corresponding, chromosomally encoded gene. One possible explanation is that the chromosomal copy of *pzaA* is negatively regulated by flanking sequences which were not present in the *pzaA* cassette in pOam and pHam (382 nucleotides of upstream sequence). Consistent with this notion is the fact that regulation of amidase gene expression is a commonly observed phenomenon (11, 15, 17, 21). However, on the basis of the available data, we cannot exclude the possibility that the high-level expression directed by pOam and pHam was instead the result of a cloning artifact.

Overexpression of *pzaA* resulted in a 10-fold increase in the susceptibility of *M. smegmatis* to PZA (Table 4), suggesting that when produced at sufficiently high levels, POA can overwhelm relatively insensitive downstream target(s) and render the organism susceptible to this drug. The fact that nicotinamide and PZA inhibited the PzaA overexpressor with similar MICs suggests that nicotinic acid and POA have similar inhibitory effects, although it is unclear whether the toxicity is attributable to general, intracytoplasmic acidification or to inhibition of a specific cellular target(s). We note that the site of production of POA might also play an important role in determining the susceptibility of a mycobacterium to PZA. Indeed, Raynaud et al. (23) showed that 27% of the nicotinamidase activity of *M. tuberculosis* is secreted into the extracellular medium and that the cell-associated activity could be detected in its outermost capsule whereas *M. smegmatis* nicotinamidase was not detected in the extracellular medium but was buried much deeper within the outer capsule.

PZA-resistant revertants of mc<sup>2</sup>155(pOam) that were able to grow at 2 mg of PZA per ml were isolated at a frequency of  $3 \times 10^{-3}$  to  $4 \times 10^{-3}$ . Of the 16 clones initially isolated, 4 were subsequently shown to be genuinely resistant to this concentration of PZA. In all four of the revertants, the pyrazinamidase activity was reduced to wild-type levels as a result of gross rearrangements within the *pzaA* region of pOam, as detected by restriction analysis of the recovered plasmid (data not shown). We note that such a mechanism for PZA resistance caused by abrogation of pyrazinamidase overproduction in *M. smegmatis* precisely mirrors the dominant role of PncA inactivation in PZA resistance in *M. tuberculosis* (27, 29).

#### ACKNOWLEDGMENTS

We gratefully acknowledge the financial support of the Glaxo Wellcome Action TB Initiative, the South African Medical Research Council, and the South African Institute for Medical Research Foundation.

We are deeply indebted to Neil Freeman, Glaxo Wellcome, for carrying out the protein sequencing, to Steven Martin and Martin Everett for providing the gridded library and for recovering clones, and to Ken Duncan for advice, encouragement, and constructive criticisms. We thank F. Kiepela and W. Sturm for advice during the early part of this project, P. O'Gaora for providing pOLYG and pHINT, and Bill Jacobs, Oren Zimhony, and Jeffery Cox for providing mc<sup>2</sup>155 and for critically reviewing the manuscript.

#### REFERENCES

- Bashyam, M. D., D. Kaushal, S. K. Dasgupta, and A. K. Tyagi. 1996. A study of mycobacterial transcriptional apparatus: identification of novel features in promoter elements. *J. Bacteriol.* **178**:4847-4853.
- Boshoff, H. I., and V. Mizrahi. 1997. Characterization of the pyrazinamidase activity of *Mycobacterium smegmatis*, abstr. B-72, p. 42. In Abstracts of the ASM Conference on Tuberculosis: Past, Present and Future 1997. American Society for Microbiology, Washington, D.C.
- Calbreath, D. F., and J. G. Joshi. 1971. Inhibition of nicotinamidase by nicotinamide adenine dinucleotide. *J. Biol. Chem.* **246**:4334-4339.
- Cynamon, M. H., S. P. Klemens, T. S. Chou, R. H. Gimi, and J. T. Welch. 1992. Antimycobacterial activity of a series of pyrazinoic acid esters. *J. Med. Chem.* **35**:1212-1215.
- Damato, J. J., M. T. Collins, and J. K. McClatchy. 1984. Niacin, nitrate and pyrazinamide studies using Middlebrook 7H10 broth. *Eur. J. Clin. Microbiol.* **3**:546-549.
- Dudley, M. A., and H. P. Willett. 1965. Nicotinamide adenine dinucleotide synthesis by *Mycobacterium tuberculosis* var. *bovis*. *Proc. Soc. Exp. Biol. Med.* **119**:807-812.
- Durbach, S. I., S. J. Andersen, and V. Mizrahi. 1997. SOS induction in mycobacteria. Analysis of the DNA binding activity of a LexA-like repressor and its role in the DNA damage induction of the *recA* gene from *Mycobacterium smegmatis*. *Mol. Microbiol.* **26**:643-653.
- Foster, J. W., and A. G. Moat. 1980. Nicotinamide adenine dinucleotide biosynthesis and pyridine nucleotide cycle metabolism in microbial systems. *Microbiol. Rev.* **44**:83-105.
- Garbe, T. R., J. Barathi, S. Barnini, Y. Zhang, C. Abou-Zeid, D. Tang, R. Mukherjee, and D. B. Young. 1994. Transformation of mycobacterial species using hygromycin resistance as a selectable marker. *Microbiology* **140**:133-138.
- Gordhan, B. G., S. J. Andersen, A. R. De Meyer, and V. Mizrahi. 1996. Construction by homologous recombination and phenotypic characterization of a *polA* mutant of *Mycobacterium smegmatis*. *Gene* **178**:125-130.
- Hashimoto, Y., M. Nishiyama, S. Honirouchi, and T. Beppu. 1994. Nitrile hydratase gene from *Rhodococcus* sp. N-774 requirement for its downstream region for efficient expression. *Biosci. Biotechnol. Biochem.* **58**:1859-1865.
- Heifets, L. B., M. A. Flory, and P. J. Lindholm-Levy. 1989. Does pyrazinoic acid as an active moiety of pyrazinamide have specific activity against *Mycobacterium tuberculosis*? *Antimicrob. Agents Chemother.* **33**:1252-1254.
- Jacobs, W. R., G. V. Kalpana, J. D. Cirillo, L. Pascopella, S. B. Snapper, R. A. Udani, W. Jones, R. G. Barletta, and B. R. Bloom. 1991. Genetic systems for mycobacteria. *Methods Enzymol.* **381**:537-555.
- Kasárov, L. B., and A. G. Moat. 1972. Metabolism of nicotinamide adenine dinucleotide in human and bovine strains of *Mycobacterium tuberculosis*. *J. Bacteriol.* **110**:600-603.
- Komeda, H., M. Kobayashi, and S. Shimizu. 1996. A novel gene cluster including the *Rhodococcus rhodochrous* J1 *nhlBA* genes encoding low molecular mass nitrile hydratase (L-Nhase) induced by its reaction product. *J. Biol. Chem.* **271**:15796-15802.
- Mayaux, J. F., E. Cerebelaud, F. Soubrier, D. Faucher, and D. Petre. 1990. Purification, cloning and primary structure of an enantiomer-selective amidase from *Brevibacterium* sp. strain R312: structural evidence for genetic mapping with nitrile reductase. *J. Bacteriol.* **172**:6764-6773.
- Mayaux, J. F., E. Cerebelaud, F. Soubrier, P. Yeh, F. Blanche, and D. Petre. 1991. Purification, cloning, and primary structure of a new enantiomer-selective amidase from a *Rhodococcus* strain: structural evidence for a conserved genetic coupling with nitrile reductase. *J. Bacteriol.* **173**:6694-6704.
- Miller, L. P., J. A. Marcinkiewicz, J. S. Blanche, and W. R. Jacobs, Jr. 1996. Purification and characterization of the *M. smegmatis* pyrazinamidase, abstr. U-79, p. 115. In Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
- Oh, S.-H., and K. Chater. 1997. Denaturation of circular or linear DNA facilitates targeted integrative transformation of *Streptomyces coelicolor* A3(2): possible relevance to other organisms. *J. Bacteriol.* **179**:122-127.
- Pardee, A. B., E. J. Benz, Jr., D. A. St. Peter, J. N. Krieger, M. Meuth, and H. W. Trieshmann, Jr. 1971. Hyperproduction and purification of nicotinamide deamidase, a microconstitutive enzyme of *Escherichia coli*. *J. Biol. Chem.* **246**:6792-6796.
- Parish, T., E. Mahenthalingam, P. Draper, E. Dabis, and M. J. Colston. 1997. Regulation of the inducible acetamidase gene of *Mycobacterium smegmatis*. *Microbiology* **143**:2267-2276.
- Preiss, J., and P. Handler. 1958. Biosynthesis of diphosphopyridine nucleotides. I. Identification of the intermediates. *J. Biol. Chem.* **233**:483-492.
- Raynaud, C., G. Etienne, P. Peyron, M. A. Laneelle, and M. Daffe. 1998. Extracellular enzyme activities potentially involved in the pathogenicity of *Mycobacterium tuberculosis*. *Microbiology* **144**:577-587.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Scherman, M., A. Weston, K. Duncan, A. Whittington, R. Upton, L. Deng, R. Comber, J. D. Friedrich, and M. McNeil. 1995. Biosynthetic origin of mycobacterial cell wall arabinosyl residues. *J. Bacteriol.* **177**:7125-7130.
- Scorpio, A., P. Lindholm-Levy, L. Heifets, R. Gilman, S. Siddiqi, M. Cynamon, and Y. Zhang. 1997. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **41**:540-543.
- Scorpio, A., and Y. Zhang. 1996. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat. Med.* **2**:662-667.

28. **Snapper, S. B., R. E. Melton, T. Kieser, S. Mustafa, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of high efficiency plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol. Microbiol.* **4**:1911–1919.
29. **Sreevatsan, S., X. Pan, Y. Zhang, B. N. Kreiswirth, and J. M. Musser.** 1997. Mutations associated with pyrazinamide resistance in *pncA* of *Mycobacterium tuberculosis* complex organisms. *Antimicrob. Agents Chemother.* **41**: 636–640.
30. **Stolt, P., and N. G. Stoker.** 1996. Functional definition of regions necessary for replication and incompatibility in the *Mycobacterium fortuitum* plasmid pAL5000. *Microbiology* **142**:2795–2802.
31. **Sun, Z., A. Scorpio, and Y. Zhang.** 1997. The *pncA* gene from naturally pyrazinamide-resistant *Mycobacterium avium* encodes pyrazinamidase and confers pyrazinamide susceptibility to resistant *M. tuberculosis* complex organisms. *Microbiology* **143**:3367–3373.
32. **Tanigawa, Y., M. Shimoyama, K. Dohi, and I. Ueda.** 1972. Purification and properties of nicotinamide deamidase from *Flavobacterium peregrinum*. *J. Biol. Chem.* **247**:8036–8042.
33. **Tarnok, L., and E. Rohrscheidt.** 1976. Biochemical background of some enzymatic tests used for the differentiation of mycobacteria. *Tubercle* **57**: 145–150.
34. **Wayne, L. G.** 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. *Am. Rev. Respir. Dis.* **109**:147–151.
35. **Yamamoto, S., I. Toida, N. Watanabe, and T. Ura.** 1995. In vitro antimycobacterial activities of pyrazinamide analogs. *Antimicrob. Agents Chemother.* **39**:2088–2091.
36. **Yan, C., and D. L. Sloan.** 1987. Purification and characterization of nicotinamide deamidase from yeast. *J. Biol. Chem.* **262**:9082–9087.