

## Characterization of *pncA* Mutations in Pyrazinamide-Resistant *Mycobacterium tuberculosis* in Brazil

Vívian de F. Sumnienski Rodrigues,<sup>1,2</sup> Maria Alice Telles,<sup>3</sup> Marta Osório Ribeiro,<sup>2</sup>  
Patrícia Izquierdo Cafrune,<sup>2</sup> Maria Lucia Rosa Rossetti,<sup>2</sup> and Arnaldo Zaha<sup>1\*</sup>

Centro de Biotecnologia do Estado do Rio Grande do Sul, Universidade Federal do Rio Grande do Sul (UFRGS),<sup>1</sup> and Centro de Desenvolvimento Científico e Tecnológico (CDCT), Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS),<sup>2</sup> Porto Alegre, and Laboratório de Bacteriologia da Tuberculose, Instituto Adolfo Lutz, São Paulo,<sup>3</sup> Brazil

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**In this study the nucleotide sequence of the *pncA* gene from 59 *Mycobacterium tuberculosis* clinical isolates was analyzed. Mutations in the *pncA* gene were identified in 29 of 40 pyrazinamide-resistant isolates, and no pyrazinamidase activity was detected in 39 of them. Twelve mutations found in this work have not been described previously.**

Pyrazinamide (PZA) is an important first-line drug used for the short-course treatment of tuberculosis in combination with isoniazid and rifampin (18, 26). PZA appears to kill semidormant bacilli that persist in acidic-pH environments inside macrophages (6, 9, 18). PZA susceptibility is usually determined by growth of *Mycobacterium tuberculosis* on a medium containing PZA and measurement of the MIC (23). However, these conventional culture-based methods require up to 3 months and exhibit high discordance rates among laboratories (7). Alternatively, PZA susceptibility has also been determined by detecting pyrazinamidase (PZase) activity of cultured *M. tuberculosis* (15, 17, 29), since PZase activity is lost in PZA-resistant isolates (11, 33). The identification of the *pncA* gene of *M. tuberculosis* (24), which encodes PZase, has given new clues in the study of PZA resistance mechanisms. Some PZA-resistant isolates retain PZase activity, suggesting that there are other mechanisms of resistance (9).

We have characterized the type and frequency of mutations in the *pncA* gene of PZA-resistant clinical isolates from Brazil. The isolates were selected by standardized testing for susceptibility to PZA (2) and correlated with PZase activity. In order to define the characteristics of the *pncA* gene of *M. tuberculosis* PZA-resistant strains from Brazil, a total of 40 resistant strains isolated from 1998 to 2003 were selected according to culture availability in health centers. All 40 strains presented resistance to other drugs (variable profiles), and 36 (90%) were multidrug resistant. We also analyzed 19 strains susceptible to first-line antimycobacterial drugs. The PZase activity was measured using the Wayne method (32), which was performed twice, as well as the susceptibility testing and all sequencing reactions. DNA was extracted using the cetyltrimethylammonium bromide method (30). The complete *pncA* gene (730 bp) was amplified with primers (*pncA1-f*, 5'-TCGGTCATGTTTCGCGATCG-3'; *pncA1-r*, 5'-GATTGCCGACGTGTCCAGAC-3'; *pncA2-f*, 5'-GTCTGGACAGTCGGCAATC-3'; and *pncA2-r*, 5'-GCTTTGCGGCGAGCG

CCTCCA-3'), resulting in two fragments. The first is 380 bp long and contains the putative promoter region, starting 80 bp upstream of the start codon and ending at codon 90. The second (350 bp) starts at codon 84 and ends 70 bp downstream of the stop codon of the gene. PCR products were purified with MicroSpin S-300 HR columns (Amersham Biosciences, Piscataway, N.J.) and sequenced using the Big Dye Terminator Cycle sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, Calif.) in the ABI Prism 3100 DNA sequencer (Applied Biosystems). Nucleotide sequences were analyzed us-

TABLE 1. *pncA* nucleotide and amino acid changes in PZA-resistant *M. tuberculosis* clinical isolates from Brazil

No. of isolates	Change in:		
	Nucleotide	Codon (no.) <sup>a</sup>	Amino acid
1	G>C at 7	GCG>CCG (3)	Ala>Pro
2 <sup>c</sup>	GC insertion at 76	Frameshift <sup>b</sup>	NA <sup>d</sup>
1	5-bp deletion at 74	Frameshift <sup>b</sup>	NA
1	G deletion at 136	Frameshift <sup>b</sup>	NA
1	C>A at 151	CAC>AAC (51) <sup>b</sup>	His>Asn
3	5-bp insertion at 182	Frameshift <sup>b</sup>	NA
1	T>C at 202	TGG>CGG (68) <sup>b</sup>	Trp>Arg
1	T deletion at 213	Frameshift <sup>b</sup>	NA
1	A>C at 226	ACT>CCT (76)	Thr>Pro
1	A>G at 245	CAT>CGT (82)	His>Arg
1	A>T at 246	CAT>CTT (82) <sup>b</sup>	His>Leu
1	C>G at 297	TAC>TAG (99)	Tyr>stop
1	C>G at 309	TAC>TAG (103)	Tyr>stop
2	G insertion at 315	Frameshift <sup>b</sup>	NA
1	T>G at 386	GTC>GGC (128)	Val>Gly
2	T>C at 414	TGT>TGC (138)	Cys>Cys
1	C deletion at 422	Frameshift <sup>b</sup>	NA
3 <sup>c</sup>	T>G at 464	GTG>GGG (155)	Val>Gly
1	G>A at 485	GGT>GAT (162) <sup>b</sup>	Gly>Asp
3	C>A at 512	GCG>GAG (171) <sup>b</sup>	Ala>Glu

<sup>a</sup> Number of codon position was counted from the start codon (ATG) of the *pncA* gene.

<sup>b</sup> New mutation not reported in previous studies.

<sup>c</sup> Groups of isolates that showed related spoligotypes and identical double-repetitive-element PCR profiles.

<sup>d</sup> NA, not applicable.

\* Corresponding author. Mailing address: Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Rua Bento Gonçalves 9500, Prédio 43421, CEP 91501-970 Porto Alegre, RS, Brazil. Phone: 55 51 3316-6054. Fax: 55 51 3316-7309. E-mail: zaha@cbiot.ufrgs.br.

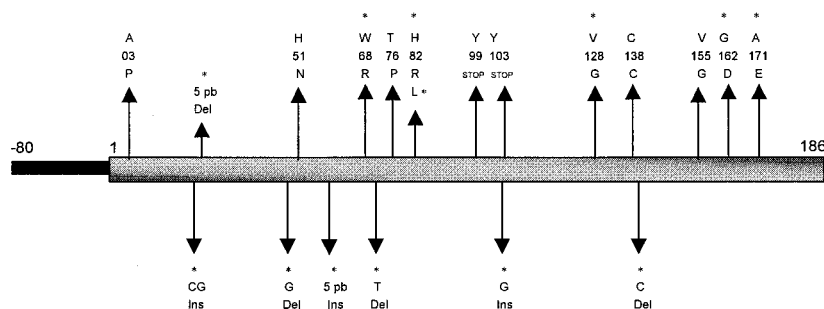


FIG. 1. Schematic representation of mutations in *pncA* gene of *M. tuberculosis*, found in this work. Asterisk indicates a new mutation not reported in previous studies. Ins, insertion; Del, deletion.

ing CROMAS version 1.45 and BLAST (Basic Local Alignment Search Tool).

Twenty-nine of 40 PZA-resistant *M. tuberculosis* clinical isolates (72.5%) exhibited mutations in the *pncA* gene compared to the wild-type (H37Rv) sequence (Table 1). Similar results were reported previously (24, 27), but 12 of the mutations found have not been described previously (1, 3, 5, 8, 12, 14, 16, 19, 20, 21, 22, 24, 27, 28, 31, 33) (Fig. 1). The majority of the mutations identified were single-nucleotide substitutions (18 of 29), two isolates of which yielded the stop codon by single-nucleotide substitutions at positions 297 and 309, presumably leading to premature termination of protein synthesis (12, 13) (Table 1). Of the remaining 16 isolates, 14 exhibited nucleotide substitutions resulting in amino acid replacements and 2 were synonymous (silent), although their PZase test was negative. The remainder presented either insertions or deletions. Nucleotide deletions were observed in four isolates, and in one of them, 5 bp was deleted. Nucleotide insertions were found in seven isolates, and two of them presented insertions of 2 nucleotides and three presented insertions of 5 nucleotides. The remaining two presented insertions of 1 nucleotide (Table 1). We still found three pairs of three isolates and another three pairs of two isolates showing the same mutation (Table 1). We analyzed those isolates using spoligotyping (10) and observed a group of three isolates and a group of two isolates showing related spoligotypes (only one spacer of difference). In order to confirm their relatedness, we performed double-repetitive-element PCR (4), and isolates had identical profiles. Once those isolates also presented the same susceptibility profile and were shown to belong to patients from the same city, it suggested transmission of an already resistant strain among the patients in each group. The remaining isolates presented unrelated spoligotypes (more than 3 spacers of difference), indicating that they are actually different strains which happened to acquire by chance the same type of mutation.

We found that 11 (61.1%) of the 18 isolates with nucleotide substitution occurred preferentially in one region (codons 128 to 171). Scorpio et al. (25) described some degree of clustering of mutations in three regions (codons 5 to 12, 69 to 85, and 132 to 142) of the *pncA* gene. Lemaitre et al. (13) reported that the alignment of the amino acid sequences of PZases from various species reveals that this region contains highly conserved residues, supporting the idea that this region should be structurally and/or catalytically important for the PZase activity. Possibly, these mutations could result in conformational mod-

ification of the PZase active site and, consequently, in loss of activity, as observed in virtually all PZA-resistant strains. In this work, PZase activity was measured in all isolates. Thirty-nine (97.5%) of the 40 PZA-resistant isolates were PZase negative, confirming the susceptibility tests, and one isolate showed PZase activity but no mutations in the *pncA* gene. The identification of a wild-type *pncA* gene in 11 PZA-resistant isolates suggests the existence of additional drug resistance mechanisms. Sequencing of the *pncA* gene regulatory region revealed no mutations in the isolates analyzed. The possible mechanisms for PZA resistance in these isolates remain to be explained. Recently two alternatives have been proposed, the active efflux of bactericidal pyrazinoic acid from the organism (34) and defects in PZA uptake by the organism (33).

Several authors have described the diversity of mutations in the *pncA* gene of PZA-resistant strains, and the frequency of mutation varied among studies (72 to 97%) (9, 33). In Brazil, the conventional screening of PZA resistance is based on the test described by Canetti et al. (2), which is a time-consuming procedure and exhibits high discordance rates among different laboratories. The use of automated systems such as BACTEC MGIT 460 is expensive and technically demanding. Automatic sequencing is an auxiliary method, which allows the characterization of mutations in the *pncA* gene and provides the possibility of developing genotypic methods to rapidly detect PZA resistance, such as single-strand conformation polymorphism analysis and PCR-restriction fragment length polymorphism, according to the features of Brazilian strains.

In this study, we described the high diversity of the *pncA* gene in strains of PZA-resistant *M. tuberculosis* from Brazil. We also found strains that did not present a *pncA* gene mutation but were PZase negative, suggesting the participation of other *M. tuberculosis* genomic regions in resistance to PZA. Furthermore, our study reveals 12 new mutations in the *pncA* gene of *M. tuberculosis* that possibly are involved in resistance to PZA.

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