Characterization of \textit{pncA} Mutations in Pyrazinamide-Resistant \textit{Mycobacterium tuberculosis} in Brazil

Vivian de F. Sumnieski Rodrigues, 1,2 Maria Alice Telles, 3 Marta Osório Ribeiro, 2 Patrícia Izquierdo Cafrune, 2 Maria Lucia Rosa Rossetti, 2 and Arnaldo Zaha 1*  
Centro de Biotecnologia do Estado do Rio Grande do Sul, Universidade Federal do Rio Grande do Sul (UFRGS), 1 and Centro de Desenvolvimento Científico e Tecnológico (CDCT), Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS), 2 Porto Alegre, and Laboratório de Bacteriologia da Tuberculose, Instituto Adolfo Lutz, São Paulo, 3 Brazil

Received 11 August 2004; Returned for modification 1 September 2004; Accepted 27 September 2004

In this study the nucleotide sequence of the \textit{pncA} gene from 59 \textit{Mycobacterium tuberculosis} clinical isolates was analyzed. Mutations in the \textit{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 \textit{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 \textit{M. tuberculosis} (15, 17, 29), since PZase activity is lost in PZA-resistant isolates (11, 33). The identification of the \textit{pncA} gene of \textit{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 \textit{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 \textit{pncA} gene of \textit{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 antitubercular 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 \textit{pncA} gene (730 bp) was amplified with primers (\textit{pncA1-f}, 5'-TCGGTCATGTTCGCGATCG-3'; \textit{pncA1-r}, 5'-GA TTGCCGACGTGTCAGAC-3'; \textit{pncA2-f}, 5'-GTCTGCGACACGGCGGCAATC-3'; and \textit{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 using GenBank.

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

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Nucleotide</th>
<th>Codon (no.)</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G&gt;C at 7</td>
<td>GCG&gt;C CG (3)</td>
<td>Ala&gt;Pro</td>
</tr>
<tr>
<td>2*</td>
<td>GC insertion at 76</td>
<td>Frameshift(^b)</td>
<td>NA(^d)</td>
</tr>
<tr>
<td>3</td>
<td>5-bp deletion at 74</td>
<td>Frameshift(^b)</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>G deletion at 136</td>
<td>Frameshift(^b)</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>C&gt;A at 151</td>
<td>CAC&gt;AAC (51) (^b)</td>
<td>His&gt;Asn</td>
</tr>
<tr>
<td>3</td>
<td>5-bp insertion at 182</td>
<td>Frameshift(^b)</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>T&gt;C at 202</td>
<td>TGG&gt;GCG (68) (^b)</td>
<td>Trp&gt;Arg</td>
</tr>
<tr>
<td>2</td>
<td>T deletion at 213</td>
<td>Frameshift(^b)</td>
<td>NA</td>
</tr>
<tr>
<td>1</td>
<td>A&gt;C at 226</td>
<td>ACT&gt;CCT (76)</td>
<td>Thr&gt;Pro</td>
</tr>
<tr>
<td>2</td>
<td>A&gt;G at 245</td>
<td>CAT&gt;CGT (82)</td>
<td>His&gt;Arg</td>
</tr>
<tr>
<td>1</td>
<td>A&gt;T at 246</td>
<td>CAT&gt;CTT (82) (^b)</td>
<td>His&gt;Leu</td>
</tr>
<tr>
<td>3</td>
<td>C&gt;G at 297</td>
<td>TAC&gt;TAG (99)</td>
<td>Tyr&gt;Stop</td>
</tr>
<tr>
<td>1</td>
<td>C&gt;G at 309</td>
<td>TAC&gt;TAG (103)</td>
<td>Tyr&gt;Stop</td>
</tr>
<tr>
<td>2</td>
<td>G insertion at 315</td>
<td>Frameshift(^b)</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>T&gt;G at 386</td>
<td>GTC&gt;GCG (128)</td>
<td>Val&gt;Gly</td>
</tr>
<tr>
<td>2</td>
<td>T&gt;C at 414</td>
<td>TGT&gt;TGC (138)</td>
<td>Cys&gt;Cys</td>
</tr>
<tr>
<td>1</td>
<td>C deletion at 422</td>
<td>Frameshift(^b)</td>
<td>NA</td>
</tr>
<tr>
<td>3*</td>
<td>T&gt;G at 464</td>
<td>GTG&gt;GGG (155)</td>
<td>Val&gt;Gly</td>
</tr>
<tr>
<td>1</td>
<td>G&gt;A at 485</td>
<td>GGT&gt;GAT (162) (^b)</td>
<td>Gly&gt;Asp</td>
</tr>
<tr>
<td>3</td>
<td>C&gt;A at 512</td>
<td>GCG&gt;GAG (171) (^b)</td>
<td>Ala&gt;Glu</td>
</tr>
</tbody>
</table>

\(^a\) Number of codon position was counted from the start codon (ATG) of the \textit{pncA} gene.

\(^b\) New mutation not reported in previous studies.

\(^*\) 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.
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 2 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 suggested 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.

We thank Tatiana Gregianini and Terimar Ruoso Moresco for helping in sequencing analysis and Lia Possuelo for assistance with genotyping analysis.

This work was supported by CAPES and by Centro de Desenvolvimento Científico e Tecnológico da Fundação Estadual de Produção e Pesquisa em Saúde (CDCT/FEPPS).
REFERENCES
ERRATUM

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

Vívian de F. Sumnienski Rodrigues, Maria Alice Telles, Marta Osório Ribeiro, Patricia Izquierdo Cafrune, Maria Lucia Rosa Rossetti, and Arnaldo Zaha

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

Volume 49, no. 1, p. 444–446, 2005. Page 444, left column, last line: The pncA2-r primer sequence should read “5’-GCTTTGCG CGAGCGCTCCA-3’.”