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Characterization of New Mutations in Pyrazinamide-Resistant Strains of *Mycobacterium tuberculosis* and Identification of Conserved Regions Important for the Catalytic Activity of the Pyrazinamidase PncA

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A new set of mutations, including transposition of the insertion sequence IS*6110*, was identified in the *pncA* gene from 19 pyrazinamide-resistant *Mycobacterium tuberculosis* strains. Alignment of the PncA protein from *M. tuberculosis* with homologous proteins from different bacterial species revealed three highly conserved regions in PncA which may play an important role in the processing of pyrazinamide.

Recently, the gene *pncA*, encoding the pyrazinamidase (PZase) of *Mycobacterium tuberculosis*, was identified (8); mutations in *pncA* have been shown to be associated with pyrazinamide (PZA) resistance (1, 5, 9, 10). However, the mutations found in the amino acid sequence of the PZase from *M. tuberculosis* have not been investigated with respect to their locations in conserved regions which may have an important catalytic function in the enzyme. In this study, we present an analysis of the sequence of the *pncA* gene from 35 epidemiologically unrelated (i.e., with distinct restriction fragment length polymorphism patterns) strains of *M. tuberculosis* isolated during the last decade in France, and we assess whether

TABLE 1. Characteristics of PZA-R clinical isolates of *M. tuberculosis*

Strain (year of isolation)	Main associated drug resistance[a]	PZA MIC (μg/ml)[b]		PZase activity	Variation(s) in:	
		pH 5.6	pH 5.8		Nucleotide sequence	Amino acid sequence
2701 (1997)	H R E S	1,500	2,000	Negative	None	None
8017 (1988)	H R S	500	1,000	Negative	None	None
7283 (1986)	H R S	500	1,500	Negative	None	None
7337 (1986)	H R	NG	1,000	Negative	G7→C	A3→P
7759 (1987)	H R E S	>2,000	>2,000	Negative	T38→C	F13→S
9420 (1992)	H R E S	NG	500	Positive	A181→C	T61→P
3303 (1994)	H R	>2,000	>2,000	Negative	C206→T	P69→L
2759 (1994)	H R S	NG	1,000	Negative	A287→C	K96→T
480 (1994)	H R E	NG	1,500	Negative	A308→G	Y103→C
7911 (1987)	H R	>2,000	>2,000	Negative	G395→A	G132→D
7508 (1987)	H R E	1,500	1,500	Negative	T464→G	V155→G
9701 (1997)	H R S	500	2,000	Negative	T545→C	L182→S
7501 (1987)	H R E	1,000	1,500	Negative	C260→T and nucleotide G deletion at position 436	T87→M
2134 (1996)	S	1,000	1,000	Negative	Nucleotide A insertion at position 192	
8220 (1989)	H R E S	500	1,500	Negative	Nucleotide C insertion at position 306	
7582 (1988)	H R E S	500	1,000	Negative	Two nucleotide G insertions at position 391	
9638 (1992)	H R E	NG	1,000	Negative	68-bp deletion at position 419	
7169 (1986)	H R S	>2,000	>2,000	Negative	29-bp insertion at position 215	
8250 (1989)	H R E	NG	1,000	Negative	1,355-bp insertion at position 341	

[a] H, isoniazid; R, rifampin; S, streptomycin; E, ethambutol.
[b] NG, no growth.

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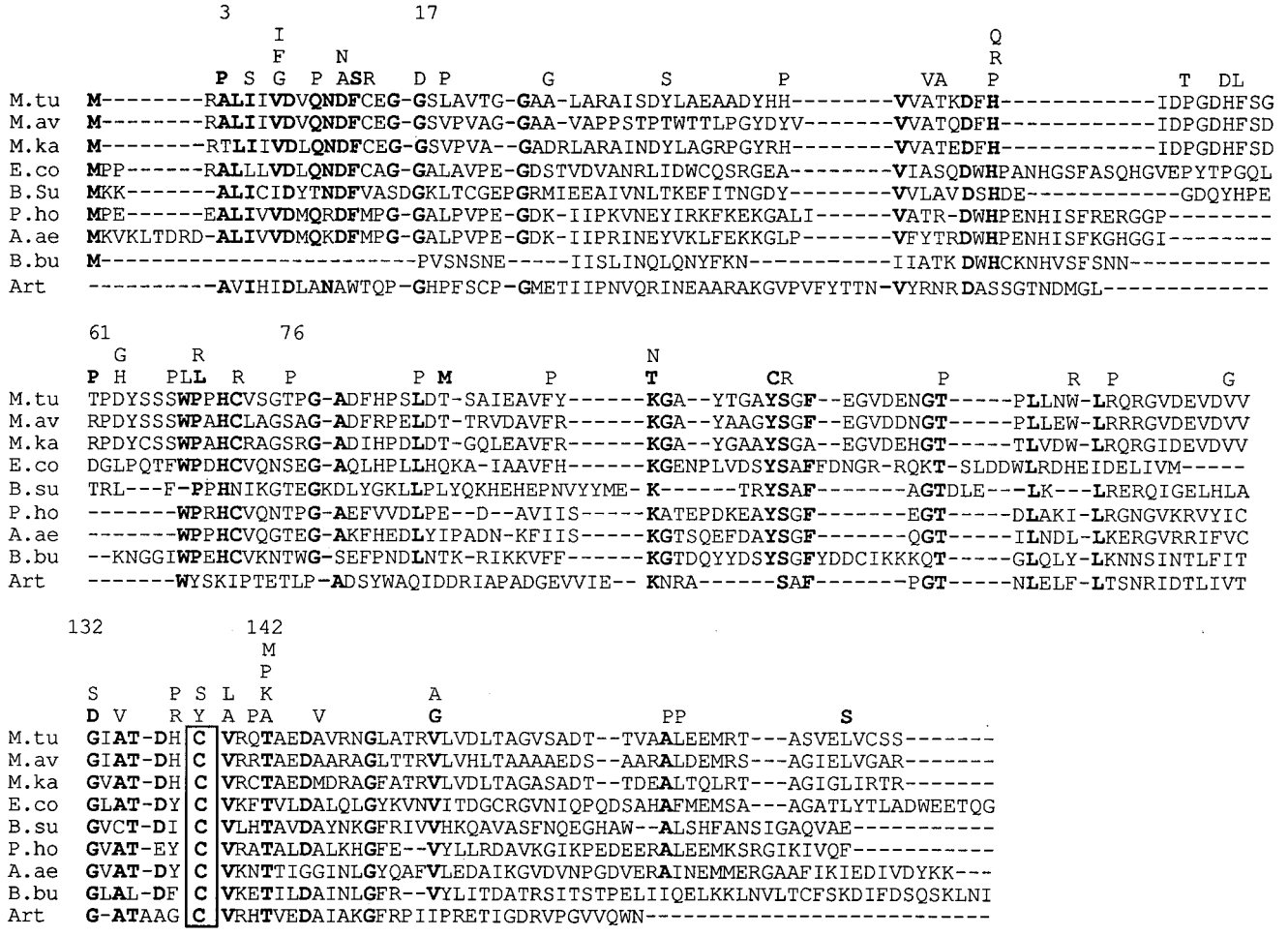

FIG. 1. Alignment of the deduced amino acid sequences of the *pncA* genes from *M. tuberculosis* (M.tu), *Mycobacterium avium* (M.av), *Mycobacterium kansasii* (M.ka), *Escherichia coli* (E.co), *Bacillus subtilis* (B.su), *Pyrococcus horikoshii* (P.ho), *Aquifex aeolicus* (A.ae), and *Borrelia burgdorferi* (B.bu) (accession no. U59967, U80820, AF002663, M26934, Z99120, AP000004, AE000717 and AE000785, respectively) and of the sequence encompassing residues 46 to 210 of the *N*-carbamoylsarcosine amidohydrolase from *Arthrobacter* sp. (Art) (accession no. P32400). Conserved amino acid residues are shown in boldface type, and the putative active cysteine is boxed. All distinct substitutions leading to PZA resistance are shown above the alignment, with boldface letters for those identified in the present study and lightface letters for those previously reported (1, 5, 8–10).

the mutations occurred randomly or in particular regions of the PZase.

MICs of PZA were determined at two different pH values (5.6 and 5.8), as previously described (8). Among the 35 clinical isolates, 16 were susceptible (MIC $\leq$50 μg/ml) and 19 were highly resistant (MIC $\geq$ 500 μg/ml) to PZA. PZase activity tested by the Wayne method (11) showed that all PZA-susceptible (PZA-S) isolates were PZase positive and all PZA-resistant (PZA-R) isolates lacked PZase activity except isolate 9420 (Table 1). Finally, the *pncA* gene as well as the 105-bp upstream and 60-bp downstream regions were amplified from each strain by using the conditions and the set of primers, P1 and P6, previously described by Scorpio et al. (9). The nucleotide sequences of the PCR products were generated with an Applied Biosystems, Inc., automatic DNA sequencer (model 377).

As expected, the 16 PZA-S strains had no detectable mutations and 16 (84%) of the 19 PZA-R isolates harbored mutations in *pncA*, thus confirming that *pncA* mutations are the major mechanism of resistance to PZA in *M. tuberculosis*. The remaining three PZA-R isolates with no mutations in *pncA* had also no mutations in the putative 105-bp upstream regulatory region. All mutations were new except the G132→D substitution (5). One isolate (8250) harbored a 1,355-bp insertion at position 341 in *pncA* which generated a duplication of 3 bp, suggesting that this event resulted from a transposition process. This insertion was sequenced by using a second set of primers, Y1 (5′-CTACACCGGAGCGTACAGCG-3′) and Y2 (5′-GGCGCACACAATGATCGGTG-3′), corresponding to nucleotides 294 to 313 and 402 to 421 in *pncA*, respectively. Comparison of the sequence obtained with those contained in the GenBank database revealed that it corresponded to the nucleotide sequence of IS*6110*. To our knowledge, this is the first report of transposition of a complete IS*6110* copy in a gene involved in susceptibility to antituberculous drugs. Recent reports have shown that IS*6110* transposes preferentially to hot-spot regions such as the direct repeat, *ipl*, and DK1 loci (2–4). Interestingly, we found that insertion of IS*6110* in strain 8250 occurred in a region of 10 bp encompassing nucleotides 337 to 346 in *pncA* (GGCAC ↓ GCCAC) which is identical to the region located between nucleotides 382 and 391 in the *ipl* locus (2).

It is generally considered that the mutations leading to PZA resistance are scattered along the *pncA* gene (6). However,

Scorpio et al. (9) mentioned some degree of clustering of mutations in three regions (positions 5 to 12, 69 to 85, and 132 to 142) of the PncA protein among the 26 strains harboring distinct mutations. We analyzed the data presented here together with those previously obtained from strains recovered in North America (8–10), South America (1), and Asia (5) to enhance the number and the geographic diversity of strains. We found that 34 (54%) of the 63 distinct amino acid substitutions reported in the literature occurred preferentially in three distinct regions (positions 3 to 17, 61 to 76, and 132 to 142) that are close to those described by Scorpio et al. (9). These regions represent only 22% of the full length of the PncA protein (Fig. 1). Alignment of the amino acid sequences of PZases from various species reveals that the three regions contain highly conserved residues, thus supporting the idea that these regions could be structurally and/or catalytically important for the activity of the enzyme against PZA. To assess such a hypothesis, the amino acid sequences of the PZases aligned in Fig. 1 were compared with that of the *N*-carbamoylsarcosine amidohydrolase (CSHase) from *Arthrobacter* sp., an enzyme for which the three-dimensional structure has been determined and which exhibits an amidohydrolytic function similar to that of the PZase (7). Strikingly, the alignment (Fig. 1) indicates that the region encompassing residues 132 to 142 in the *M. tuberculosis* PZase includes a perfectly conserved cysteine at position 138 corresponding to the catalytic cysteine found at position 177 in the CSHase (7). In addition, residues Ala-172 and Thr-173 in the CSHase, which may facilitate the addition of the nucleophilic thiol group of Cys-177 to the substrate, are also conserved in most PZases and correspond to residues Ala-134 and Thr-135 in *M. tuberculosis*. The region from positions 61 to 76 in *M. tuberculosis* also contains an aromatic residue, Trp-68, which is shared by most PZases and is aligned with the Trp at position 111 in the *Arthrobacter* CSHase. Interestingly, in the crystal structure of this enzyme, the aromatic residue Trp-111 may define, with other aromatic residues, a hydrophobic pocket in which the substrate could bind. Finally, the region from position 3 to 17 in *M. tuberculosis* harbors the residue Asp-8, which is also present in the CSHase at position 51. The structural data on the CSHase indicate that the acidic residue Asp-51 may increase the nucleophilicity of Cys-177 (7). Taken altogether, these data suggest that Cys-138, Ala-134, Thr-135, Trp-68, and Asp-8 in the *M. tuberculosis* PZase could be key residues for hydrolysis of PZA. Consequently, the mutations occurring within or close to the regions containing these residues could result in conformational modifications of the active site of the PZase and, consequently, in a loss of activity, as observed in most of our PZA-R strains. It should be pointed out, however, that some of the mutations leading to PZA resistance are not located within the conserved regions. One can hypothesize that the loss of PZase activity observed in such mutants is likely due to improper folding or instability of the PZase protein.

In conclusion, although the mutations found in *pncA* are scattered along the gene, the present data and analysis suggest that they occur preferentially in conserved regions of *pncA* which might be very important for the binding and processing of PZA. Further biochemical and structural studies will be necessary to clarify the structure-function relationships of the corresponding residues in the PZase.

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