Estimation of Pyrazinamidase Activity Using a Cell-free In vitro Synthesis of PncA and its Association with Pyrazinamide Susceptibility in Mycobacterium tuberculosis

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Abstract

Background: The main mechanism of resistance to PZA in Mycobacterium tuberculosis relies on mutations on its pyrazinamidase/nicotinamidase. Recently, a rapid colorimetric test relying on the PCR-based in vitro-synthesized-PZase assay has been reported for PZase activity determination from clinical M. tuberculosis isolates but the assay has not been compared with other tests to evaluate PZA susceptibility in M. tuberculosis isolates. Methods: In this study, we have used the PCR-based in vitro-synthesized-PZase assay to analyze the specific pyrazinamidase (PZase) activity of PncA mutants and have correlated the results to the PZA susceptibility phenotype determined by culture in acidic agar medium at pH 6.0. A set of 23 clinical isolates displaying mutated pncA genes (11 PZA-resistant and 12 PZA-susceptible) and 55 PZA-susceptible clinical strains displaying a wild-type pncA gene were tested. Results: Among the 23 mutants tested, 4 corresponded to mutations not reported before (I5T, Y99S, T142R and P77L+V131G). Of the 11 PncA mutants expressed from PZA-resistant clinical isolates, 9 were expressed in vitro at yields > 50% relative to the wild type enzyme. Among them, 6 enzymes (T47P, H51P, H51R, H57D, L85R and T142R) showed no detectable activity, while the relative activities for the 3 others, V9A (27%), G97D (10%) and A146V (28%) were low compared to the wild-type PZase. The remaining two mutants, I5T and V9G, presented very low relative expression (5%) and relative activities values of 12 and 1%, respectively. Twelve mutants were expressed from PZA-susceptible isolates. Their expression was similar to the wild type enzyme and behaved as active pyrazinamidase with specific relative activities ranging from 34 to 314%. Finally, discrepant results were observed for two mutants, V7A and P62T. Conclusion: Thus, this study provides the proof of concept that the PCR-based in vitro-synthesized-PZase assay represents a promising rapid approach for the evaluation of PZA susceptibility based on the estimation of the relative PZase activity from clinical isolates.

Keywords: Cell-free expression, Mycobacterium tuberculosis, PncA, pyrazinamidase, pyrazinamide, resistance, tuberculosis

Introduction

The emergence of multidrug-resistant (MDR) Mycobacterium tuberculosis impacts the control of tuberculosis (TB). In 2014, the World Health Organization estimated that there were 9.6 million cases of TB and that 1.5 million people died from TB worldwide. Among the extant cases of TB, 480,000 were MDR-TB.1 Pyrazinamide (PZA) is a first-line anti-TB drug which plays a very important role in shortening the duration of TB treatment.2,3 PZA is a prodrug that is converted to its active form, pyrazinoic acid (POA), by a pyrazinamidase (PZase) encoded by the pncA gene. PZA has a bactericidal effect on M. tuberculosis, especially under acidic conditions. Three hypotheses have been formulated regarding the mode of action of POA.4 First, POA would be pumped out of the cell by efflux pumps to be protonated POA (HPOA).
in the acidic environment of the bacilli. HPOA could then re-enter the cell and exert its lethal effect by releasing protons inside the cell, acidifying the cytoplasm and disrupting the mycobacterial membrane properties. In a second hypothesis, POA would bind to the 30S ribosomal protein S1 (RpsA), thereby preventing the binding of RpsA to tmRNA and blocking trans-translation. This mechanism would protect against defective mRNA being stalled in a ribosome, otherwise resulting in transcription errors, reading frame alterations or mRNA damage. Finally, PZA could inhibit pantothenate and coenzyme A synthesis by targeting PanD, an aspartate decarboxylase involved in the synthesis of beta-alanine. The main mechanism of resistance to PZA in *M. tuberculosis* relies on the inactivation of the PZase PncA by mutations in the *pncA* gene which introduce amino acid substitutions, insertions, or deletions in the protein.

PZA phenotypic determination can be assessed by several approaches based on culture methods including automated culture systems such as Bactec 460, mycobacteria growth indicator tube (MGIT), and BacT/ALERT MB. One important problem with these techniques is the lack of reproducibility of the growth of *M. tuberculosis* at the acidic pH (5.5) used to test PZA susceptibility on automated systems, leading to difficulties in interpreting phenotypic results. An agar medium with an acidic pH of 6.0 ensuring a better growth of *M. tuberculosis* comparatively to the poor growth generally observed on the usual media at pH 5.5 has been previously proposed to perform PZA susceptibility testing with *M. tuberculosis* by an agar proportion method.

The Wayne test is the main biochemical approach to measure the PZase activity of PncA. The test is based on the addition of PZA to a buffer solution containing the PZase, followed by the quantification of POA using ferrous ammonium sulfate which reacts with POA to yield a red compound. Recently, a rapid colorimetric test relying on the polymerase chain reaction (PCR)-based in vitro-synthesized PZase assay has been reported for PZase activity determination from *M. tuberculosis* clinical isolates. Briefly, the test is based on the amplification from clinical isolates of a long PCR fragment containing the full-length *pncA* gene, including its putative promoter and additional expression sequences, allowing the synthesis of PZase in a cell-free wheat germ protein expression system. The activity of the in vitro-synthesized PZase is then assessed by a colorimetric method similar to the quantitative Wayne assay. Even though there have been some attempts to use this assay to evaluate PZase susceptibility from *M. tuberculosis* isolates, the results were not always conclusive.

Molecular methods based on DNA amplification and sequencing techniques can detect all the mutations that can occur in the *pncA* gene. The approach, which is now widely used in routine laboratories, is affordable and reliable, but the interpretation of the effects of the mutations remains somewhat difficult. There is a large number of studies in the literature reporting the evaluation of the PZase activity of PncA mutants, either directly from *M. tuberculosis* cultures using the Wayne test, or indirectly from *Escherichia coli*-expressed recombinant proteins by measuring PZA hydrolysis and correlating the PZase activities to PZA susceptibility of the corresponding *M. tuberculosis* isolates. However, most of the studies are based on (i) sets of nonisogenic clinical isolates and (ii) various techniques for PZA susceptibility testing, hence there is a lack of concordance when the results from different studies are compared. Notably, several of these reports have described mutations found in susceptible clinical isolates, suggesting that some of the amino acid modifications occurring in PncA could be phenotypically silent mutations which do not impair significantly the PZase activity. Therefore, it is of critical importance not only to detect mutations in PncA, but also to measure directly the PZase activity associated with the detected mutations.

In this report, we have investigated a collection of *M. tuberculosis* clinical isolates having various mutations in *pncA*. The phenotype of PZA susceptibility associated with these mutants was determined using a commercial test based on the pH 6.0 agar medium described by Heifets and Sanchez. In parallel, the impact of the corresponding *pncA* mutations on the PZase activity was assessed by a PCR-based in vitro-synthesized cell-free PZase assay. Using these two approaches, we provide evidence that the PCR-based in vitro-synthesized PZase assay could be used to determine PZA susceptibility from clinical isolates in a relatively short time.

### Materials and Methods

#### Strains and culture conditions

A set of 23 clinical isolates displaying mutated *pncA* genes, including 11 PZA-resistant and 12 PZA-susceptible strains, were selected among the clinical samples received at the National Reference Center of Tuberculosis of France. Phenotypic resistance to PZA was determined using the acidic agar medium previously described by Heifets and Sanchez, which is made of 7H11 agar base containing 300 μg/ml of PZA, with a pH adjusted to 6.0 by the addition of monosodium phosphate. The corresponding HSTB PZA agar plates were obtained from Biocentric (France). PZA susceptibility was defined by <1% of growth on the PZA-containing medium compared to the PZA-free control plate. In addition to the 23 PncA mutants, 55 PZA-susceptible clinical strains displaying a wild-type (*WT*) *pncA* gene were tested.

#### Cloning, expression, and purification of the PncA control enzymes

Three PncA proteins were cloned, expressed, and purified as His-tag recombinant proteins to be used as reference enzymes in the enzymatic studies: the WT PZase of *M. tuberculosis* H37Rv (active enzyme), the PZase of *Mycobacterium bovis* (naturally harboring a H57D substitution, inactive enzyme), and a *M. tuberculosis* mutant PZase harboring the T87M substitution (active enzyme). The three proteins

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were produced as previously reported with the following modifications. The H37Rv pncA gene was amplified and introduced in the vector pET29a between the restriction sites HindIII and NdeI. The T87M and H57D mutations were introduced in pncA WT using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The recombinant plasmids were introduced into E. coli TOP10 to be purified and transformed into E. coli BL21 (DE3) (Stratagene). In order to express the proteins, one colony was inoculated in 4 mL of Luria-Bertani (LB) broth and cultured overnight to inoculate a volume of 500 mL of LB medium containing 30 µg/mL of kanamycin that was incubated at 37°C under agitation. When the culture reached an absorbance of 0.6 at 600 nm, isopropyl-β-D-thiogalactoside was added at a final concentration of 0.4 mM and the culture was shaken at 18°C overnight (16 h). After centrifugation, the pellet was resuspended in 20 mL of Bis-Tris 20 mM pH 6.0-DTT 1 mM, sonicated, and centrifuged at 4°C during 30 min at 10,000 rpm. The supernatant was recovered and mixed to 1 mL of spermidine at 90 mg/mL before 2 h of centrifugation at 10,000 rpm (4°C). The new supernatant was filtered using a 0.20 µm filter Miniartis (Sartorius Stedim biotech) and was loaded onto a 5 mL HiTrap™ Q HP column equilibrated in a Bis-Tris 20 mM pH 6.0-DTT 1 mM buffer. The protein was eluted on an Akta chromatography system using a linear gradient of NaCl (0-1 M) in buffer Bis-Tris 20 mM pH 6.0-DTT 1 mM. The eluted fractions were concentrated using Amicon ultra 15-mL concentrators (Millipore). The protein concentration was estimated using the Bradford assay (Biorad).

**Enzymatic activity measurement of control enzymes by the colorimetric Wayne assay**

The enzymatic activity of the three control enzymes (PncA WT of *M. tuberculosis*, PncA-H57D of *M. bovis*, and PncA-T87 of *M. tuberculosis*) was determined by measuring the reaction of PZA hydrolysis in a colorimetric assay, as previously described. 1.5 µM of PZase was incubated in 0.5 mL of reaction medium containing 2 mM of PZA in a sodium phosphate buffer 50 mM (pH 6.6). At different times of the reaction, 45 µL of the reaction mix was mixed with 10 µL of ferrous ammonium sulfate 20% and then with 1 mL of cold glycine-HCl 100 mM buffer at pH 3.4. The mixture was centrifuged at 4°C, 14,000 rpm during 5 min and its absorbance was measured at 450 nm.

**In vitro expression of selected PncA proteins**

The *in vitro* expression of the PncA mutants identified in our collection of *M. tuberculosis* clinical isolates and of the H37Rv WT PZase was carried out using the approach described by Li et al. The PCR-based *in vitro* expression system, the RTS™ 100 Wheat Germ CECF kit, was used to express the PncA proteins, with the following modifications. Two sets of primers were used in DNA amplifications: Primers RT-F (5'-CGGACGGATTTGTGCTCACC-3') and RT-R (5'-GGGGATGAGGTTGTCGTTGAAAG-3') for the *Long* PCR reaction and primers F-5 (5'-TAATACGACTCACTATAAGGATCTACCTCCCGACCAAGTTAACATACTCACCACAGCTTACAATACTCCCCACA CAGCTTTACAAATCCTCCCCACGCTGCCCCAGGATGAGGACT-3') and R-1 (5'-CCGGCCGCAACGTAAGGAGGACGT-3').

The Long PCR reaction mix (50 µL) consisted of 0.2 µL of AmpliTaq Gold DNA Polymerase (Applied Biosystems), 25 µL of water, 5 µL of 10× PCR Gold buffer, 5 µL of MgCl₂, 5 µL of dNTP 10 mM, 5 µL of primer RT-F (4 µM), 5 µL of primer RT-R (4 µM), and 5 µL of DNA template obtained by heat-shock protein from a suspension of *M. tuberculosis* cells. DNA amplification was performed under the following conditions: denaturation step at 98°C for 120 s, followed by 35 cycles of denaturation (98°C for 10 s), annealing (64°C for 65 s), and extension (72°C for 60 s), with a final elongation step of 10 min at 72°C. The expected size of the DNA amplicons was checked by electrophoresis on 1% agarose gel (918 bp for the Long PCR reaction).

The second PCR reaction solution (50 µL) consisted of 0.2 µL of AmpliTaq Gold DNA Polymerase (Applied Biosystems), 25 µL of water, 5 µL of 10× PCR Gold buffer, 5 µL of MgCl₂, 5 µL of dNTP 10 mM, 5 µL of primer F-5 (4 µM), 5 µL of primer R-1 (4 µM), and 5 µL DNA template which corresponded to a 1:10 dilution of the first PCR amplification. This reaction was performed under the following conditions: denaturation step at 98°C for 30 s, followed by 35 cycles of annealing (98°C for 10 s), annealing (68°C for 60 s), and extension (72°C for 30 s), with a final elongation at 72°C for 10 min. After having checked the sizes of the DNA amplicons (698 bp), the presence of the mutations was verified by sequencing using an ABI 310 sequencer with the BigDye terminator method (Applied Biosystems).

PncA mutants were expressed *in vitro* using the product of the second PCR reaction. The protocol described by Li et al. was used, following the instructions of the manufacturer of the RTS 100 Wheat Germ CECF Kit (5 PRIME) with the following modifications. For each *in vitro* expression assay, 6 µg of DNA template was used, and the expression reaction was performed at 24°C during 20 h. Each series of four mutants tested also included the expression of the PZases of *M. tuberculosis* (WT enzyme) and *M. bovis* (natural H57D variant) as internal positive and negative controls, respectively.

**Pyrazinamidase Western blot assay**

The *in vitro*-expressed PncA proteins were resolved on 14% SDS-PAGE gels and were reveled in a Western blot format using a rabbit sera anti-PncA wt enzyme. To permit comparisons between gels, we adjusted the variability of the total amount of proteins corresponding to the mutated PZAses.
by including in each gel a sample of H37Rv and M. bovis WT PncA proteins for later normalization and estimation of relative expression yields. The fractions were heated at 95°C for 5 min in sample buffer (0.1% SDS, 0.025% [w/v] bromophenol blue, 1% glycerol, 0.0025 M Tris-glycine-HCl, pH 8.0, and 1% β-mercaptoethanol). Electrophoresis was performed at 180 V. Gels were washed three times in sodium phosphate 0.1 M pH 7.4 during 15 min while polyvinylidene difluoride (PVDF) membranes were incubated in ethanol for 20 min. Proteins were electrically transferred onto a PVDF membrane at 0.25 A for 25 min in a Trans Blot SD Semi-dry Transfer cell (Bio-Rad, Hercules, CA, USA) with filter papers wetted in the buffer solution. After the transfer, membranes were washed three times in sodium phosphate pH 7.4, 0.3% Tween 20. A blocking solution of sodium phosphate pH 7.4, 0.3% Tween-20, 3% BSA was prepared as a diluent for the sera sample. Rabbit sera anti-PncA wt enzymes were provided by Dr. Mirko Zimic as lyophilized powder. The final serum dilutions of 1:375, 1:150, and 1:75 were used. The membranes were incubated at room temperature for 150 min, washed four times with sodium phosphate pH 7.4, 0.3% Tween 20, and incubated for 90 min in a solution containing rabbit IgG horseradish peroxidase-conjugated antibody (RD Systems) diluted at 1:20,000 in sodium phosphate pH 7.4, 0.3% Tween 20. The membranes were washed three times in sodium phosphate pH 7.4, 0.3% Tween 20 and three times in sodium phosphate pH 7.4. Finally, 2 mL of the final mix (containing substrates A and B) of Pierce ECL Plus Western Blotting Substrate (Thermo Scientific) was added to each membrane which was incubated for 5 min. The reacting bands were visualized using the ImageQuant LAS 4000 system (GE Healthcare Life Sciences), and the intensity of the PZase bands was determined to estimate the relative protein production rates with H37Rv as reference [arbitrarily set to 1, Table 1].

### Specific relative enzymatic activity

The enzymatic activities of the in vitro-expressed PncA proteins were determined using the conditions of the reaction of PZA hydrolysis described above for the three control enzymes, with the following modifications. For each mutant, 30 µL of in vitro expression reaction was incubated in 0.5 mL of reaction medium containing PZA 2 mM and sodium phosphate buffer 50 mM pH 6.6. The fractions for the colorimetric reaction were taken and processed exactly as described above for the control enzymes.

The relative enzymatic activity for a given mutant was obtained by dividing its enzymatic activity at the beginning of the kinetic reaction in the colorimetric Wayne assay by the WT PZase activity measured in the same batch with H37Rv. The

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### Table 1: Phenotypic and enzymatic characteristics of PncA mutants from M. tuberculosis clinical strains

| Mutation in PncA | Phenotype of resistance to PZAa | Relative PZase activity (%) | Relative PncA expression in vitro | Number of PZA-R isolates reported with mutation | Number of PZA-S isolates reported with mutation | References |
|------------------|---------------------------------|----------------------------|----------------------------------|-----------------------------------------------|-----------------------------------------------|------------|
| WT+              | S (55)                          | 100                        | 1                               |                                               |                                               | [This report] |
| I5T              | R 10% (1)                       | 12                         | 0.05                            |                                               |                                               | [22] |
| V9A              | R 2% (1)                        | 27                         | 0.5                             |                                               |                                               | [12, 20] |
| V9G              | R 100% (1)                      | 1                          | 0.05                            |                                               |                                               | [20, 23, 24] |
| T47P             | R 100% (1)                      | 0                          | 0.5                             |                                               |                                               | [20, 25-32] |
| H51P             | R 10% (1)                       | 0                          | 3                               | 10                                            | 1                                             | [20, 31-35] |
| H51R             | R 2% (1)                        | 0                          | 0.5                             | 18                                            | 2                                             | [20] |
| H57D+            | R 100% (2)                      | 1.5                        | 1.5                             |                                               |                                               | [This report] |
| L85R             | R 10% (1)                       | 0                          | 1.5                             | 11                                            |                                               | [19, 20, 22, 36-39] |
| G97D             | R 100% (1)                      | 10                         | 3                               | 10                                            |                                               | [12, 20, 26, 27, 40-42] |
| T142R            | R 100% (1)                      | 0                          | 1.5                             |                                               |                                               | [This report] |
| A146V            | R 2% (1)                        | 28                         | 1.5                             | 4                                             |                                               | [20, 26, 31] |
| I6L              | S (1)                           | 117                        | 2                               | 8                                             | 124                                           | [20, 24, 39, 43] |
| T47A             | S (2)                           | 34                         | 0.5                             | 24                                            | 52                                            | [20, 22, 27, 32, 35, 39, 43-49] |
| D63A             | S (1)                           | 83                         | 1                               | 6                                             | 1                                             | [20, 39, 47, 50] |
| Y64D             | S (1)                           | 200                        | 4                               | 3                                             | 1                                             | [22, 36, 51] |
| T87M             | S (1)                           | 147                        | 1.5                             | 3                                             |                                               | [24, 50, 52] |
| Y99S             | S (1)                           | 82                         | 2                               |                                               |                                               | [This report] |
| R140S            | S (2)                           | 120                        | 3                               | 21                                            | 1                                             | [19, 32, 53] |
| T167I            | S (5)                           | 314                        | 3                               |                                               |                                               | [14] |
| M175V            | S (1)                           | 62                         | 1                               | 14                                            |                                               | [20, 32, 49, 50, 54-56] |
| P77L + V131G     | S (1)                           | 53                         | 1.5                             | 1d                                            |                                               | [This report] and [20] |
| V7A              | S (1)                           | 5                          | 1                               | 1                                             |                                               | [33] |
| P62T             | S (1)                           | 4                          | 0.1                             | 2                                             |                                               | [28, 37] |

a: S: Susceptible, R: Resistant, wt: Wild-type, b: Amino acid substitution found in M. bovis which is naturally resistant to PZA, c: mutant reported with only one (P77L) of the 2 mutations found in the clinical isolate
specific relative PZase activities of the different PncA mutants expressed in the different batches were defined as the relative activities of the mutants divided by their relative rates of production assessed from the Western blot assay.

Results
Phenotypic and enzymatic characterization of PncA control enzymes

Three PncA proteins were used as references in the present study. The reference for a fully active PZase was the WT PncA protein of *M. tuberculosis* H37Rv. The PZase from *M. bovis*, which is naturally resistant to PZA, was used as negative control for the PZase activity because this enzyme naturally harbors a H57D substitution that suppresses the catalytic activity (His-57 is one of the three essential His-residues coordinating the metal ion in PncA).[17] Finally, we also included the T87M mutant as reference enzyme which was isolated from a PZA-susceptible strain and was shown on preliminary experiments conducted by our group to retain a PZase activity comparable to that of the WT enzyme. The three control enzymes were cloned in pET29a, and then expressed and purified successfully as soluble His-tag proteins (data not shown). The results of the kinetic measurements of these control proteins by the colorimetric assay are shown in Figure 1a, indicating relative activities of 6% and 100% for the purified *M. bovis* PZase (H57D) and the T87M mutant, respectively.

Phenotypic and enzymatic characterization of selected PncA mutants

Fifty-five clinical strains displaying a WT pncA gene were assayed on the agar pH 6.0 medium containing PZA at a concentration of 300 µg/ml. All were found to be phenotypically susceptible to PZA [Table 1], and the WT PncA proteins tested by the PCR-based in vitro-synthesized PZase assay displayed PZase activities comparable to that of the WT PncA control [shown on Figure 1b and c for two in vitro-expressed WT enzymes]. Twenty-three pncA mutants were investigated [Table 1]. It is notable that mutations I5T, Y99S, T142R, and P77L+V131G were not reported before. All the mutants presented higher than 50% of relative expression, except I5T, V9G, and P62T which displayed 5%, 5%, and 10% of relative expression, respectively [Table 1]. Examples of results of the PCR-based in vitro-synthesized PZase assay are shown in Figure 1b and c for the control enzymes (*M. tuberculosis* H37Rv PncA WT, the mutant T87M, and *M. bovis* H57D) and seven of the PncA mutants (I6L, G97D, R140S, M175V, I5T, T47A, and H51R). The results obtained for the 23 mutants are summarized in Table 1 which shows the relative enzymatic activities and relative expression levels along with the phenotypic results. Even though each experiment set was evaluated only once, the reliability of the kinetics in different batches is indicated by the reproducibility of the kinetics of PZA hydrolysis by the two reference enzymes PncA WT and PncA H57D that were systematically included in the six sets of independent experiments [Figure 1].

Eleven clinical isolates having mutations in the pncA gene were found to be phenotypically resistant to PZA with a percentage of colonies growing on the PZA-containing medium compared to the control medium without antibiotic varying from 2% to 100% [Table 1]. Analysis of these 11 mutants by the PCR-based

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**Figure 1:** Time course colorimetric assays of wild-type and mutant PncA proteins. (a) Kinetics of pyrazinamide hydrolysis by the three purified control enzymes (PncA wild-type from *Mycobacterium tuberculosis* H37Rv and the two mutants from *Mycobacterium tuberculosis* T87M and *Mycobacterium bovis* H57D). (b and c) Kinetics of pyrazinamide hydrolysis by in vitro-expressed pyrazinamidase. Each series includes the wild-type PncA protein of *Mycobacterium tuberculosis*, the variant H57D of *Mycobacterium Bovis*, and four *Mycobacterium tuberculosis* PncA mutants.
in vitro-synthesized cell-free PZase assay indicated that their relative activities were drastically reduced compared to the WT PncA enzyme [from 0% to 28%, Table 1]. The amount of enzyme produced for each variant was assessed by Western blot analysis [Figure 2 and Table 1], and the PZase activities were reported as specific relative activities in Figure 3. Seven PZases expressed in vitro (G9V, T47P, H51P, H51R, H57D, L85R, and T142R) showed no significant activity. Three PncA mutants, V9A, G97D, and A146V, displayed specific activities of 54%, 3%, and 19%, respectively, when compared to the WT PZase [Figure 4]. Notably, the I5T mutant presented a specific relative activity of 240% [Figure 4], a value due to the very low level of expression (5%) of the mutant protein, which had a weak relative activity (12%) compared to PncA WT [Table 1].

On the other hand, ten clinical isolates harboring pncA genes with missense mutations were found on phenotypic testing to be susceptible to PZA and to produce active PZases on the in vitro assay [relative activity values ranging from 34% to 314%, Table 1], with specific relative activities between 35% and 105% [Figure 4]. Three mutants, T87M, D63A, and A146V, presented specific activities similar to the WT enzyme (98%, 83%, and 105%, respectively). Mutants L61L, T47A, Y64D, and M175V showed intermediate specific relative activities (59, 68, 50–62%). Finally, P77L-V131G, Y99S, and R140S presented lower specific relative activities at 35%, 41%, and 40%, respectively [Figure 4].

We observed two discrepant results. The V7A mutant was obtained from a PZA-susceptible isolate but was unexpectedly associated with a reduced specific relative activity of 5% (one can note here that based on the Western blot results, V7A was expressed in vitro with a yield comparable to that of PncA WT). Similarly, P62T, which displayed a very low relative PZase activity value of 4% [Table 1] was isolated from a susceptible clinical isolate. Compared to V7A, P62T was expressed in vitro at a very low yield (10%), explaining the fairly high specific activity worked out for this mutant (40%) [Figure 4].

**DISCUSSION**

The analytical approach used in this study was based on the in vitro synthesis of PncA in a cell-free wheat germ protein expression system coupled with a rapid colorimetric test allowing evaluation of PZase activities. We first validated this approach using three purified control enzymes, the H37Rv WT enzyme from *M. tuberculosis*, the naturally inactive variant H57D from *M. bovis*, and the pncA mutant T87M obtained from a *M. tuberculosis* PZA-susceptible clinical isolate. When compared to the purified enzymes, the in vitro-synthesized PZases showed a very similar enzymatic behavior. The WT pncA protein and the mutant pncA-T87M synthesized in vitro were active and showed the same level of PZase activity, while H57D was found, as expected, to be nearly devoid of catalytic activity (specific relative activity of 0.29% ± 0.13%). The in vitro-synthesized PZase activity testing of the 23 pncA mutants was then conducted by a series of six enzymes, each batch including four mutants and the two control enzymes PncA WT and H57D in order to take into account the possible variations of the expression yields in the different assays. Among the 23 tested PncA mutants, 11 consisted of enzymes expressed from clinical isolates classified as PZA resistant by the phenotypic method. Seven of them (V9G, T47P, H51P, H51R, H57D, L85R, and T142R) were characterized by a lack of PZase activity while they displayed in vitro expression levels comparable to those of the control enzymes (apart for V9G which showed a low expression level). Based on the systematic review of mutations in PncA associated with PZA resistance published by Ramirez-Busby and Valafar, the seven amino acid modifications found in the inactive PncA variants can be all considered as very high confident resistance mutations. A search for these mutations in the literature confirmed that they were virtually all reported in PZA-resistant isolates [Table 1]. Analysis of the three-dimensional (3D) structure of the *M. tuberculosis* PZase indicated that the amino acid residues at positions 9 (Val), 47 (Thr), 51 (His), and 57 (His) are located in the immediate vicinity of the active site [Figure 3a]. Val-9 is found one residue next to the catalytic aspartate at position 8 and likely contributes to the positioning of the Asp-8 side chain in the active site. On the other side, the side chain of Thr-47 also stabilizes Asp-8, while those of His-51 and His-57 both contribute to the coordination of a metal ion (Mn2+, Fe2+, Mg2+, or Zn2+) essential for the binding of PZA [Figure 3a] (Sheen). The two remaining residues, Leu-85 and Thr-142, are more distant to the catalytic center, but one can note that Thr-142 is located on the helix bearing...
the catalytic Cys-138 holding the PZA molecule, while the side chain of Leu-85 is at only 3.92 Å from the side chain of Val-9 mentioned above [Figure 3a].

The last four mutants identified in the PZA-R group, IST, V9A, G97D, and A146V, displayed different levels of relative PZase activity in the in vitro assay [from 10% to 28%, Table 1]. Two mutations V9A and G97D occur at critical positions localized one residue next to catalytic residues, Asp-8 and Lys-96, respectively. The A146V mutation, which was previously ranked among the very high confidence resistance mutations,[15] is located far from the active site (average distance of 15 Å) but, like T142R, may affect the structural properties of the helix bearing the catalytic Cys-138 [Figure 3a]. The last mutation, IST, which is also located far from the active site [at 11 Å from Asp-8, Figure 3a], occurs in the middle of a β strand contributing to the PncA β sheet [Figure 3a]. It has been previously suggested that this kind of substitution entails significant alteration of the folding and/or stability of the pncA protein,[17] a hypothesis which is here substantiated by the very low expression level observed on Western blot analysis for the IST mutant [Figure 2]. Due to this low expression level, the specific relative activity of the IST mutant is unexpectedly high [240%, Figure 4] despite its low relative activity of 12%. Considering that it was found in a PZA-susceptible strain, it is very likely that the IST PZase is not stable enough and/or not properly folded to confer PZA resistance in vivo.

It is worth to highlight that the extent of the impairment of PZase activity linked to a mutation at a given position in pncA greatly depends on the nature of the amino acid replacement occurring at this position. In our study, the substitution of Thr-47 by Pro entailed a complete loss of PZase activity and was associated with phenotypic resistance, while its replacement by Ala resulted in an active PncA mutant identified in a PZA-susceptible isolate [Table 1]. Similarly, the two mutants V9A and V9G exhibited distinct enzymatic behaviors, the former being 27 folds more active than the latter. These observations reinforce the importance of determining the PZase activity before proposing a phenotypic interpretation for different mutations occurring at a given position in PncA.

The specific relative PZase activity of the ten remaining mutants estimated by the cell-free in vitro expression PZase assays and the Western blot assays found all mutants to be active PZase. These enzymes displayed different levels of specific relative activity: 35%–41% for P77L-V131G, Y99S, and R140S; 50%–68% for I16L, T47A, Y64D, and M175V; and 83%–105% for D63A, T87M, and T167I. These results agree well with the results of the PZA susceptibility phenotypic assay that classified the corresponding clinical isolates as PZA susceptible. In the literature, several of these amino acid substitutions have been previously associated with PZA-S isolates [Table 1]. For instance, I6L has been reported by Ramirez-Busby and Valafar[15] to be a very high confidence susceptible mutation (in the literature, it was reported in 124 PZA-S strains versus 8 PZA-R only) [Table 1]. The T47A mutation was reported in 68% of the published studies in PZA-S isolates and in 32% in PZA-R isolates [Table 1].[15] Other mutations such as D63A and T87M were not previously linked to PZA-S isolates in the literature, but they are clearly associated with PZA susceptibility and an unaltered PZase activity in our study (specific relative PZase activities of 83% and 98%, respectively) [Table 1]. Regarding R140S, this mutation was previously reported in 21 PZA-resistant strains from Quebec (Canada), but these strains had the same PncA mutation profile consisting of an 8-nucleotide deletion plus the amino acid substitution Arg140→ Ser.[19] It is therefore very likely that resistance to PZA in these isolates stemmed from the deletion in pncA rather than from the R140S substitution that did not impair the PZase activity in our study [Table 1]. Finally, the double mutant P77L+V131G is reported here...
for the first time, with a resulting specific PZase activity of 35% from a strain susceptible to PZA, contrasting with a previous study reporting a PZA-R mutant with P77L alone.[20] Further investigations are required to clarify the impact of both mutations on PZase activity. At the structural level, the PZA-S-associated mutations are more scattered and more distant from the active site than the PZA-R-associated mutations [Figure 3b]. In particular, eight mutations (D63A, Y64D, P77L, T87M, Y99S, R140S, T167I, and M175V) affect residues located at the surface of the PncA protein, their side chains being oriented toward the solvent [e.g. D63, Y64, Y99, T167, and M175 as shown in Figure 3b]. These structural features agree well with the phenotypic and the enzymatic results reported here for these mutations (susceptibility to PZA and active PncA proteins). On the other hand, mutations such as I6L and T47A occur at the level of buried residues, but the replacements of Ile-6 by Leu and Thr-47 by Ala can be considered as conservative enough to preserve the PZase activity of the mutants (specific relative PZase activity of 59% and 68%, respectively).

As shown in Figure 4, two mutants, V7A and P62T, constituted an ambiguous category of mutants yielding discrepant results as they were identified in PZA-S strains but showed very low relative PZase activities (5% and 4%, respectively) [Table 1]. In the 3D structure of pncA, V7A occurs one residue before the catalytic Asp-8 and is considered in the systematic review of Ramirez-Busby and Valafar and the recent publication of Yadon et al. to be a high-confidence resistance mutation associated with high minimum inhibitory concentration (MIC) values.[19] The other mutation, P62T, which affects a proline residue located in the middle of a β strand contributing to the PncA β sheet [Figure 3a], gives rise to a very low level of expression in vitro (10 folds lower compared to pncA-wt). Like V7A, different amino acid substitutions at the level of residue P62 (P62Q/L/R) have been reported as high-confidence resistance mutations associated with high MIC values.[15,21] Therefore, the kinetic results and the structural analysis made in our study by the in vitro approach are rather concordant with the previously published phenotypic results (PZA resistance), and it is likely that the phenotypic analysis made in the present study misclassified the two mutants in the PZA-S category. This unexpected result could be either due to unexplained technical difficulties when performing PZA dug susceptibility testing or to the PZA MICs for the two mutants being lower than the PZA concentration of 300 μg/ml used on the pH 6.0 solid medium.

**Conclusion**

The approach presented here for evaluating PZase activity from clinical isolates using the PCR-based in vitro-synthesized PZase assay can provide results in <3 days. It compares favorably with the results given by a phenotypic method based on the use of an acidic agar medium at pH 6.0 since almost no activity is associated with PZA-R isolates while specific relative activities of 35%–105% are associated with PZA-S isolates. This study establishes the proof of concept that the PCR-based in vitro-synthesized PZase assay represents a promising approach for rapid evaluation of PZA susceptibility directly from tuberculous patients’ samples. However, it has to be highlighted here that this approach should be correlated with other standard phenotypic methods such as the MGIT 960 assay and should be further evaluated on large collections of PncA mutants.

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**Conflicts of interest**

There are no conflicts of interest.

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