Published in final edited form as:
Tuberculosis (Edinb). 2009 March ; 89(2): 109–113. doi:10.1016/j.tube.2009.01.004.

EFFECT OF PYRAZINAMIDASE ACTIVITY ON PYRAZINAMIDE RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

Patricia Sheen[a], **Patricia Ferrer**[a], **Robert H. Gilman**[a,b,*], **Jon López-Llano**[a], **Patricia Fuentes**[a], **Eddy Valencia**[c], and **Mirko J. Zimic**[d]

a *Laboratorio de Enfermedades Infecciosas. Laboratorios de Investigación y Desarrollo. Facultad de Ciencias, Universidad Peruana Cayetano Heredia. Av. Honorio Delgado 430 San Martín de Porres, Lima-Peru*

b *Department of International Health, School of Public Health, Johns Hopkins University, Baltimore, USA*

c *Laboratorio de Tuberculosis. Instituto Nacional de Salud, Lima, Peru Capac Yupanqui 1400 Jesús María-Lima11*

d *Laboratorio de Bioinformática. Laboratorios de Investigación y Desarrollo. Facultad de Ciencias, Universidad Peruana Cayetano Heredia Av. Honorio Delgado 430 San Martín de Porres, Lima-Peru*

Abstract

Resistance of *Mycobacterium tuberculosis* to pyrazinamide is associated with mutations in the *pncA* gene, which codes for pyrazinamidase. The association between the enzymatic activity of mutated pyrazinamidases and the level of pyrazinamide resistance remains poorly understood. Twelve *M. tuberculosis* clinical isolates resistant to pyrazinamide were selected based on Wayne activity and localization of pyrazinamidase mutation. Recombinant pyrazinamidases were expressed and tested for their kinetic parameters (activity, $k_{cat,}$ $K_{m,}$ and efficiency). Pyrazinamide resistance level was measured by Bactec-460TB and 7H9 culture. The linear correlation between the resistance level and the kinetic parameters of the corresponding mutated pyrazinamidase was calculated.

The enzymatic activity and efficiency of the mutated pyrazinamidases varied with the site of mutation and ranged widely from low to high levels close to the corresponding of the wild-type enzyme. The level of resistance was significantly associated with pyrazinamidase activity and efficiency, but only 27.3% of its statistical variability was explained.

Although pyrazinamidase mutations are indeed associated with resistance, the loss of pyrazinamidase activity and efficiency as assessed in the recombinant mutated enzymes is not sufficient to explain a high variability of the level of pyrazinamide resistance, suggesting that complementary mechanisms for pyrazinamide resistance in *M. tuberculosis* with mutations in *pncA* are more important than currently thought.

Keywords

Tuberculosis; pyrazinamidase; *pncA*; enzymatic activity; resistance

*Corresponding author: E-mail: rgilman@jhsph.edu. Johns Hopkins School of Public Health. 615, N. Wolfe St. Room W5515. Baltimore, MD. 21205., Phone: (410) 614-3639, Fax: (410) 614-6060.

INTRODUCTION

Pyrazinamide (PZA) is an important first-line drug for tuberculosis (TB) and appears to be the most important drug in killing latent *Mycobacterium tuberculosis* [1–3]. The emergence of strains resistant to PZA represents an important public health problem, as both primary and secondary line treatment schemes include PZA. Multi-drug resistant (MDR) tuberculosis, defined as isoniazid and rifampin resistant, is increasing globally [4, 5]; and more than 30% of Peruvian MDR TB strains are also resistant to PZA [6].

PZA-susceptible *M. tuberculosis* isolates possess a pyrazinamidase (PZAse) that is constitutively expressed [7, 8] and hydrolyzes PZA to pyrazinoic acid (POA) which is the lethal molecule [9–11]. A defective POA efflux pump is required to accumulate intracellular POA [7].

Mutations in the PZAse coding gene (*pncA*) are scattered throughout its sequence with some degree of clustering in the regions that contain the catalytic residues of PZAse [12–14]. The catalytic residues comprise the active site (D8, K96, A134 and C138) and the metal-binding site (D49, H51 and H71) [12]. According to previous studies, the ion likely to bind the metal coordination site would be zinc or iron [12, 15]. The specific activity of recombinant mutated PZAses varies as much as one thousand-fold depending on the site of the mutation [15, 16]. It is suggested that mutations causing significant loss of PZAse activity are those that produce a physical-chemical alteration of the active site or the metal-binding site. Mutations located farther away are thought to have less effect on PZAse activity [12, 16].

Only one study has addressed the correlation of PZAse activity with the 'yes/no' microbiological resistance [17]. The study showed that low levels of PZAse activity are found in resistant isolates with *pncA* mutations, however it did not examine other kinetic parameters or its association with the quantitative level of resistance.

In this study we will examine the correlation between the kinetic parameters of recombinant mutated PZAses cloned from PZA resistant *M. tuberculosis* clinical strains and the microbiological PZA resistance level.

MATERIAL AND METHODS

Selection of *M. tuberculosis* sputum isolates

In a recent study, we identified 26 *pncA* unique sequences with single missense mutation in *M. tuberculosis* clinical isolates [18]. We selected 12 of these strains based upon the Wayne activity and type of mutations. We also included the PZA-susceptible wild-type reference strain H37Rv. The selected strains displayed negative, weak, and positive Wayne activities. Mutations were of three types: mutations of the metal-binding residues (D49N, H51R), mutations close to the metal-binding or active-site (D12A, D12G, K48T, T76P, F94L, and T135P), and mutations distant from these sites (G24D, Y34D, G78C and L116P).

Cloning of the *M. tuberculosis pncA* gene

The DNA was extracted from 7H11 cultures as described previously [19]. The entire *pncA* gene was amplified by PCR using oligonucleotides with restriction sites for NcoI and XhoI (5′ CCC CCA TGG GCC GGG CGT TGA TCA TC and 5′ CCC CTC GAG GGA GCT GCA AAC CAA CTC). The purified *pncA* gene amplification (575bp) was double digested and inserted in the pET28a plasmid using T4 DNA ligase (NEBioLabs,Ipswich, MA) for 16 h at 16°C. The digested expression vector was dephosphorylated with Shrimp Alkaline Phosphatase (Fermentas) before ligation. For purification, a six-histidine tag was added to the carboxy-terminal end. *Escherichia coli* Novablue cells (Novagen, San Diego, CA) were transformed directly from the ligation reaction according to the heat shock protocol [20]. Plasmid DNA was

extracted with the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and sequenced in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA) in both directions.

Expression and purification of the recombinant PZAses

The PZAses were transformed into *E. coli* BL21(DE3)pLysS cells (Novagen) as described above for protein expression. Isopropyl β-D-thiogalactoside was added to fresh overnight cultures to achieve a final concentration of 1 mM and incubated for 4 h at 37°C. The cells were harvested by centrifugation at 4,830g and 4°C for 10 min. The pellet was resuspended in 20 ml of binding buffer (20 mM imidazole, 0.5 M NaCl and 20 mM phosphate buffer pH 7.4) and lysed by repeated freeze-thaw cycles followed by sonication using a S3000 sonicator (Misonix, Farmingdale, NY). After centrifugation at 17,572 g and 4ºC for 30 min the supernatant was loaded on a 5 ml His-bind column (Pharmacia, Piscataway, NJ) and washed with 40 mM imidazole, 0.5 M NaCl and 20 mM phosphate buffer, pH 7.4.

The bound protein was eluted with 60 mM imidazole, 0.5 M NaCl and 20 mM phosphate buffer, pH 7.4. Aliquots of the fractions obtained were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and those containing pure PZAse (molecular weight: 20.7 kDa) were combined. The purified protein was concentrated 10 times and then washed 3 times with 20 mM Tris-HCl, pH 7.9 by filtration using cellulose membranes with 10 kDa pore size in an Ultracel Amicon Ultrafiltration system (Millipore, Billerica, MA) at 4ºC. The protein concentration was determined according to the method of Bradford [20].

PZA resistance level of *M. tuberculosis*

Bactec 460TB (Bactec)—Mycobacterial growth was estimated by measuring the release of $^{14}CO_2$ under PZA inhibition at pH 6.0 according to the manufacturer's instructions (Becton Dickinson, Sparks, MD). A concentration of 100 μg/ml was used as the breakpoint to determine PZA resistance. The Bactec PZA resistance level (BZRL) was estimated by the ratio of the radioactive growth index (GI) of the culture media containing the bacteria and PZA, and the radioactive GI of the control (i.e. bacteria in culture media without PZA), expressed as a percentage. The GI was read at the moment in which the control was at a GI of at least 200. The Bactec test was repeated twice for each strain.

Culture in 7H9 media—*M. tuberculosis* was cultured in 7H9 culture broth enriched with ADC (albumin, dextrose and catalase) at pH 6.0 with different concentrations of PZA [21]. The PZA minimum inhibitory concentration (MIC) was estimated.

Wayne test—The Wayne test is a qualitative colorimetric method that detects POA, which is released by the bacilli into the culture media [22]. The presence of a red color detected by visual inspection indicates PZAse activity [23–25]. Based on the color intensity, we classified the selected strains as having a positive, weak or negative Wayne activity. The Wayne test was repeated four times for each strain, and when discrepancies occurred, the most frequent result was considered.

Kinetic parameters of the recombinant enzyme

PZAse kinetic parameters were calculated using the hydrolysis reaction of PZA with some modifications [12]. Briefly, PZA was used from 0 to 4 mM and incubated with 1 μM PZAse in 50 mM sodium phosphate pH 6.5. To prevent the hydrolysis of more than 10% of the initial PZA, 1 min was used. The incubation period was increased to 2 h for mutants with very low activity. 10 μL of 20% $FeNH_4(SO_4)_2$ was added, followed immediately by adding 890 μL of 100 mM glycine-HCl (pH 3.4) to stop the reaction. OD was measured at 450 nm in a 96-well plate using 200 μL of reaction.

The amount of POA produced was estimated by interpolation in a standard curve of known concentrations. Each recombinant PZAse was tested at least 3 times, and at least 2 different batches of recombinant protein were analyzed for each strain. To minimize any loss of activity due to storage, recombinant PZAses were analyzed within one week after produced.

To measure the affinity of PZAse with PZA, we estimated the Michaelis constant (K_m). To determine the number of times each enzyme-substrate complex converts substrate to its product per unit time, we estimated the catalytic constant (k_{cat}). To determine how efficiently PZA is converted into POA, we calculated the enzymatic efficiency (*Eff*) as k_{cat}/K_m [26, 27]. The enzymatic activity was estimated as the amount of POA produced in a 1-min reaction divided by the total amount of enzyme.

Statistical analysis

The pooled data of all the repetitions were fitted to the Lineweaver–Burk plot using the software Microcal Origin [28]. The kinetic parameters, k_{cat}, K_m, and *Eff* were calculated by fitting the data in a [total PZAse]/[reaction velocity] versus 1/[PZA], and each parameter was estimated with its 95% confidence interval. The correlations between the kinetic parameters and the PZA resistance were estimated. The Pearson correlation was used for the BZRL and the Spearman correlation for the 7H9 MIC and the Wayne activity. The correlation between the PZA resistance and the site of mutations was also calculated by the Spearman test. The variability of the BZRL explained by the kinetic parameters was estimated by the R^2 coefficient of a linear regression. The *Eff* of each mutated PZAse was compared to the wild type in a multiple linear regression by modeling [total PZAse]/[reaction velocity] and testing for the interaction between 1/[PZA] with an indicator variable referring to the strains. All the statistical analysis was performed using the statistical software Stata 10.

RESULTS

Cloning, expression and purification of *M. tuberculosis* PZAse

The PZAses were cloned, expressed and purified at a final concentration of 2 mg/ml. The purified protein was stored at −70ºC for further analysis. The purity of the recombinant PZAse was confirmed as a single band in a Coomassie blue stained SDS-PAGE (Figure 1).

PZA susceptibility parameters in the *M. tuberculosis* sputum isolates

The BZRL, the 7H9 PZA MIC, and the Wayne activity of the 13 strains are shown in Table 1. All the mutant isolates were resistant according to Bactec except for the strain K48T, which was susceptible according to the 7H9 culture with a PZA MIC of less than 50 μg/ml. In addition K48T showed a positive Wayne activity and the lowest BZRL (20%). The linear correlation between the PZA MIC and the BZRL was 71.1% (P=0.004). The correlation between the PZA MIC and the Wayne activity was 61.4% (P= 0.02). The correlation between the BZRL and the Wayne activity was 68.7% (P= 0.01).

Kinetic parameters of recombinant PZAses

The kinetic parameters k_{cat}, K_m, *Eff*, and the enzymatic activities are shown in Table 2. The k_{cat} and K_m varied among each other as much as six thousand and ten fold respectively. The efficiency of the *pncA* mutants was diverse. Three PZAses (D49N, H51R, and T135P), had almost no efficiency ($\leq$0.55 $mM^{-1}\cdot min^{-1}$); five (D12A, G24D, Y34D, G78C, and F94L), retained a middle level of efficiency (97.65 – 460.19 $mM^{-1}\cdot min^{-1}$) and four (T76P, L116P, D12G, and K48T), exhibited efficiencies similar to that of the wild type (551.28 – 847.66 $mM^{-1}\cdot min^{-1}$). The enzymatic efficiency was correlated with k_{cat} (71.8%, P=0.004), but was not significantly correlated with K_m (P=0.13).

Mutations in the metal-binding amino acids (D49N and H51R) did not change the K_m significantly compared to the wild type, but k_{cat} for both was extremely low resulting in almost undetectable enzymatic efficiency. However, the mutation K48T close to the metal-binding site residue D49, retained a relatively high efficiency (551.28 $mM^{-1} \cdot min^{-1}$). The lowest enzymatic activity, k_{cat}, and *Eff* corresponded to the mutation T135P that is close to the active site residue A134. Other mutations close to the active site also had greatly diminished the *Eff*. Mutations distant to the catalytic site caused varied enzymatic *Eff*; for example the mutation L116P exhibited the highest efficiency, but not significantly higher than that of H37Rv (P=0.82).

Association between PZA levels of resistance with kinetic parameters

The linear correlations between the PZA resistance measurements and the kinetic parameters are shown in Table 3. The PZA MIC was negatively correlated with the enzymatic activity (P=0.028), the k_{cat} (P=0.047), and the *Eff* (P=0.018). The BZRL was also negatively correlated with the enzymatic activity, k_{cat} and *Eff*, but with a borderline significance. With a significance level of 0.067, the variability of the BZRL explained by the PZAse activity was only 27.3%. The k_{cat} and *Eff* explained similar amounts of variability of the BZRL (24.3% and 21.4% respectively) with borderline significance. The *Eff* across the categories of Wayne and the Bactec "yes/no" resistance did not vary notably, but a trend starting from the value of the wild type susceptible strain to slightly lower values for the resistant strains was observed. The Wayne activity and K_m were not significantly associated with any of the susceptibility parameters. The highest association was observed between the *Eff* and the PZA MIC with a correlation coefficient of −0.64 (P = 0.018). The location of the mutations within the enzyme did not show a statistically significant association with the enzymatic kinetic parameters, although was a trend that mutations closer to the catalytic site were associated with a lower activity and *Eff*.

DISCUSSION

In this study, we have investigated the kinetic parameters of mutated PZAses from *M. tuberculosis* clinical isolates resistant to PZA. We demonstrated that only 27.3% of the statistical variability of resistance is explained by the PZAse activity and there is a wide variation in the enzymatic activity in recombinant proteins associated with mutations in *pncA*. In some cases these proteins had enzymatic levels similar to the wild-type PZAse of the susceptible strain (H37Rv). Confirming previous findings, we have demonstrated that mutations affecting the metal-binding residues cause the lowest enzymatic activity and are associated with the highest PZA resistance. We also confirmed that the resistance level is negatively and significantly correlated with the enzymatic activity and that mutations close to catalytic site cause a major reduction of the enzymatic activity and appear to be associated with high PZA resistance.

The fact that the *Eff* showed a high association with the level of PZA resistance is explained because the modulation of the rate of product formation depends upon both the ability of the enzyme to bind the substrate ($1/K_m$) and the rate at which the complex enzyme-substrate produces the product (k_{cat}). Therefore, the PZAse *Eff* (k_{cat}/K_m) is the parameter that best resembles the overall rate of production of POA. The higher variability of k_{cat} compared to K_m is consistent with the fact that none of the selected mutations directly affected the residues of the active site, thus the affinity for PZA was not expected to be highly altered.

The use of recombinant enzymes expressed in *E. coli* is not a limitation. The activity of a recombinant PZAse should not be different than a native protein in *M. tuberculosis* because this is a strict cytosolic enzyme, thus its folding in *E. coli* is expected to be similar in *M.*

tuberculosis. This is probably why the recombinant PZAses have always been produced in *E. coli* and not in *M. tuberculosis*[12, 15, 16, 29].

Negative Wayne activity is usually associated with PZA resistance [24, 25] and indicates the inability of the bacteria to release POA to the extracellular environment. This condition can be caused by the low expression of PZAse or enzymatic malfunction due to *pncA* mutations [7, 11, 13, 30, 31]. However, some PZA-resistant isolates with *pncA* mutations retain PZAse activity [14, 17, 30, 32], suggesting an inappropriate gene expression or an altered POA efflux pump.

In another study, the *Pyrococcus horikoshii* PZAse showed an enzymatic activity of 17.3 μmol POA·min^{-1}·mg^{-1} protein [12], which is similar to what we found in *M. tuberculosis* H37Rv. This is probably explained by the similarity of the amino acid sequences (37% identity). In contrast, a study in *M. tuberculosis* that characterized a partially purified H37Rv PZAse yielded a lower estimate of enzymatic activity of 0.022 μmol POA·min^{-1}·mg^{-1} protein (1.33 U·mg^{-1} protein, 1 U = amount of PZAse required to produce 1 μmol of POA/h) [16]. This discrepancy maybe due to the use of partially purified enzymes as well as of a longer enzymatic incubation period (4 hours), compared to the 1 min incubation period used in our study. We verified that the linearity of PZA hydrolysis is satisfied within the first 3 min of reaction when using 3 μM of pure enzyme. Given that the rate of product formation decays with time, an incubation time longer than the linearity period underestimates the enzymatic activity. In a recent study [15] the enzymatic activity of a recombinant H37Rv PZAse was estimated as 89.6 U·mg^{-1} protein (1 U = amount of PZAse required to produce 1 μmol of POA/min), which is close to the value estimated by us. The 95% confidence interval of K_M estimated in our study for the recombinant H37Rv PZAse (1.24 +/− 0.44 mM) is close to that calculated in one other study using partially purified *M. tuberculosis* PZAse that found a lower K_m of 0.3 mM [29].

Drug resistance is not a binary “yes/no” condition, but a continuous phenotype. The level of resistance is the natural parameter being measured. A biologically meaningful causal pathway to explain the PZA-resistant phenotype is: “*pncA* mutation” → “PZAse activity” → “level of PZA resistance” → “yes/no PZA-resistant phenotype”, therefore the PZAse activity is a proximal predictor of the level of PZA resistance. Several studies have demonstrated that *pncA* mutations are highly associated with a “yes/no” PZA-resistant phenotype [8, 33–36] and that mutations in the active site have a deleterious effect on the recombinant enzymes [15, 16]. However, these studies have not explored the relationship between enzymatic activity and the level of PZA resistance. Despite the high association between the presence of *pncA* mutations and PZA resistance, our study shows that even though the PZAse activity is significantly correlated with the level of PZA resistance, it does not explain an important amount of the variability of this parameter. This is clearly not fully consistent with the proposed causal pathway. Under the assumption that the expression level of the PZAse is similar in all the strains studied, our data suggests that mechanisms other than low PZAse activity, and thus a loss of PZA hydrolysis capacity, should occur frequently and are probably linked to *pncA* mutations. It is also possible that PZAse activity participates through an unstable non-linear mechanism of PZA resistance; therefore a slight variation of the normal PZAse activity causes a large change in the resistance level. Further studies to measure the *pncA* expression level or the release rate of POA are required to understand the combined effect of the PZAse function on the level of PZA resistance. Conclusion: We have found that the enzymatic efficiency of mutated PZAses accounts for some but not all the variability of PZA resistance level; therefore, an alternate mechanism other than the alteration of the PZAse function must occur more frequently and be more important than previously believed.

Acknowledgments

We acknowledge Drs. Mark A. Swancutt, Carlton Evans, David Moore, Alberto Mendoza, Laurie Baumann and Marjory Bravard for their advice and review of the manuscript. The National Institute of Allergy and Infectious Diseases, National Institutes of Health US, funded this research under the terms of Award No # 1 R03 AI067608-0. P. Sheen and MJ Zimic received some support from TMRC New Tools to Understand and Control Endemic Parasites # 1 P01 AI51976. J. López-Llano received an award from the MAE-AECI fellowship (Spain).

References

1. Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. Tubercle 1985;66:219–25. [PubMed: 3931319]
2. Steele MA, Des Prez RM. The role of pyrazinamide in tuberculosis chemotherapy. Chest 1988;94:845–50. [PubMed: 3048929]
3. Hu Y, Coates AR, Mitchison DA. Sterilising action of pyrazinamide in models of dormant and rifampicin-tolerant *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 2006;10:317–22. [PubMed: 16562713]
4. Ministerio de Salud. Magnitud del problema de TB MDR y cobertura de los tratamientos para TB MDR. In: Bonilla, C.; Jave, H.; Cortez, Y.; Bayona, JCM.; Mukherjee, J., editors. Construyendo alianzas estratégicas para detener la Tuberculosis: La experiencia peruana. Lima, Perú: Ministerio de Salud; 2006. p. 67-73.
5. Zignol M, Hosseini MS, Wright A, Weezenbeek CL, Nunn P, Watt CJ, Williams BG, Dye C. Global incidence of multidrug-resistant tuberculosis. J Infect Dis 2006;194:479–85. [PubMed: 16845631]
6. Vasquez-Campos L, Asencios-Solis L, Leo-Hurtado E, Quispe-Torres N, Salazar-Lindo E, Bayona J, Becerra MC. Drug resistance trends among previously treated tuberculosis patients in a national registry in Peru, 1994–2001. Int J Tuberc Lung Dis 2004;8:465–72. [PubMed: 15141740]
7. Zhang Y, Mitchison D. The curious characteristics of pyrazinamide: a review. Int J Tuberc Lung Dis 2003;7:6–21. [PubMed: 12701830]
8. Cheng SJ, Thibert L, Sanchez T, Heifets L, Zhang Y. pncA mutations as a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis*: spread of a monoresistant strain in Quebec, Canada. Antimicrob Agents Chemother 2000;44:528–32. [PubMed: 10681313]
9. Konno K, Feldmann FM, McDermott W. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am Rev Respir Dis 1967;95:461–9. [PubMed: 4225184]
10. Zhang Y, Wade MM, Scorpio A, Zhang H, Sun Z. Mode of action of pyrazinamide: disruption of Mycobacterium tuberculosis membrane transport and energetics by pyrazinoic acid. J Antimicrob Chemother 2003;52:790–5. [PubMed: 14563891]
11. Wade MM, Zhang Y. Mechanisms of drug resistance in Mycobacterium tuberculosis. Front Biosci 2004;9:975–94. [PubMed: 14766424]
12. Du X, Wang W, Kim R, Yakota H, Nguyen H, Kim SH. Crystal structure and mechanism of catalysis of a pyrazinamidase from *Pyrococcus horikoshii*. Biochemistry 2001;40:14166–72. [PubMed: 11714269]
13. Scorpio A, Lindholm–Levy P, Heifets L, Gilman R, Siddiqi S, Cynamon M, Zhang Y. Characterization of pncA mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 1997;41:540–3. [PubMed: 9055989]
14. Lemaitre N, Sougakoff W, Truffot-Pernot C, Jarlier V. 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. Antimicrob Agents Chemother 1999;43:1761–3. [PubMed: 10390238]
15. Zhang H, Deng JY, Bi LJ, Zhou YF, Zhang ZP, Zhang CG, Zhang Y, Zhang XE. Characterization of *Mycobacterium tuberculosis* nicotinamidase/pyrazinamidase. Febs J 2008;275:753–62. [PubMed: 18201201]
16. Lemaitre N, Callebaut I, Frenois F, Jarlier V, Sougakoff W. Study of the structure-activity relationships for the pyrazinamidase (PncA) from *Mycobacterium tuberculosis*. Biochem J 2001;353:453–8. [PubMed: 11171040]

17. Suzuki Y, Suzuki A, Tamaru A, Katsukawa C, Oda H. Rapid detection of pyrazinamide-resistant *Mycobacterium tuberculosis* by a PCR-based in vitro system. J Clin Microbiol 2002;40:501–7. [PubMed: 11825963]
18. Sheen, P. Mycobacterium tuberculosis. Baltimore: School of Public Health, Johns Hopkins University; 2008. Molecular diagnosis of pyrazinamide resistance and molecular understanding of the pyrazinamidase functionality.
19. van Soolingen D, Hermans PW, de Haas PE, Soll DR, van Embden JD. Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. J Clin Microbiol 1991;29:2578–86. [PubMed: 1685494]
20. Simonian, M.; Smith, J. Short Protocols in Molecular Biology. New York: John Wiley & Sons; 1999.
21. Scorpio A, Zhang Y. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat Med 1996;2:662–7. [PubMed: 8640557]
22. Wayne LG. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am Rev Respir Dis 1974;109:147–51. [PubMed: 4203284]
23. Kent, PTKB. Public health mycobacteriology: a guide for the level III laboratory. Atlanta: USDHHS: Centers for Disease Control; 1985.
24. McClatchy JK, Tsang AY, Cernich MS. Use of pyrazinamidase activity on Mycobacterium tuberculosis as a rapid method for determination of pyrazinamide susceptibility. Antimicrob Agents Chemother 1981;20:556–7. [PubMed: 6805419]
25. Trivedi SS, Desai SG. Pyrazinamidase activity of Mycobacterium tuberculosis--a test of sensitivity to pyrazinamide. Tubercle 1987;68:221–4. [PubMed: 3129848]
26. Voet, D.; Voet, J. Biochemistry. Jonh Wiley & Sons, Inc; 1995.
27. Fersht, A. Structure and mechanis in protein science. United States of America: W.H. Freeman and Company; 1999.
28. Fersht, A. Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding. New York: W.H. Freeman and Company; 1999. The basic equations of enzyme kinetics; p. 103-31.
29. Boshoff HI, Mizrahi V. Purification, gene cloning, targeted knockout, overexpression, and biochemical characterization of the major pyrazinamidase from *Mycobacterium smegmatis*. J Bacteriol 1998;180:5809–14. [PubMed: 9811635]
30. Mestdagh M, Fonteyne PA, Realini L, Rossau R, Jannes G, Mijs W, De Smet KA, Portaels F, Van den Eeckhout E. Relationship between pyrazinamide resistance, loss of pyrazinamidase activity, and mutations in the *pncA* locus in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 1999;43:2317–9. [PubMed: 10471589]
31. Butler WR, Kilburn JO. Susceptibility of *Mycobacterium tuberculosis* to pyrazinamide and its relationship to pyrazinamidase activity. Antimicrob Agents Chemother 1983;24:600–1. [PubMed: 6418066]
32. Brown TJ, Tansel O, French GL. Simultaneous identification and typing of multi-drug-resistant Mycobacterium tuberculosis isolates by analysis of pncA and rpoB. J Med Microbiol 2000;49:651–6. [PubMed: 10882091]
33. Endoh T, Yagihashi A, Uehara N, Kobayashi D, Tsuji N, Nakamura M, Hayashi S, Fujii N, Watanabe N. Pyrazinamide resistance associated with pncA gene mutation in Mycobacterium tuberculosis in Japan. Epidemiol Infect 2002;128:337–42. [PubMed: 12002553]
34. Marttila HJ, Marjamaki M, Vyshnevskaya E, Vyshnevskiy BI, Otten TF, Vasilyef AV, Viljanen MK. pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis isolates from northwestern Russia. Antimicrob Agents Chemother 1999;43:1764–6. [PubMed: 10390239]
35. Lee KW, Lee JM, Jung KS. Characterization of pncA mutations of pyrazinamide-resistant *Mycobacterium tuberculosis* in Korea. J Korean Med Sci 2001;16:537–43. [PubMed: 11641519]
36. Portugal I, Barreiro L, Moniz-Pereira J, Brum L. pncA mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* isolates in Portugal. Antimicrob Agents Chemother 2004;48:2736–8. [PubMed: 15215139]

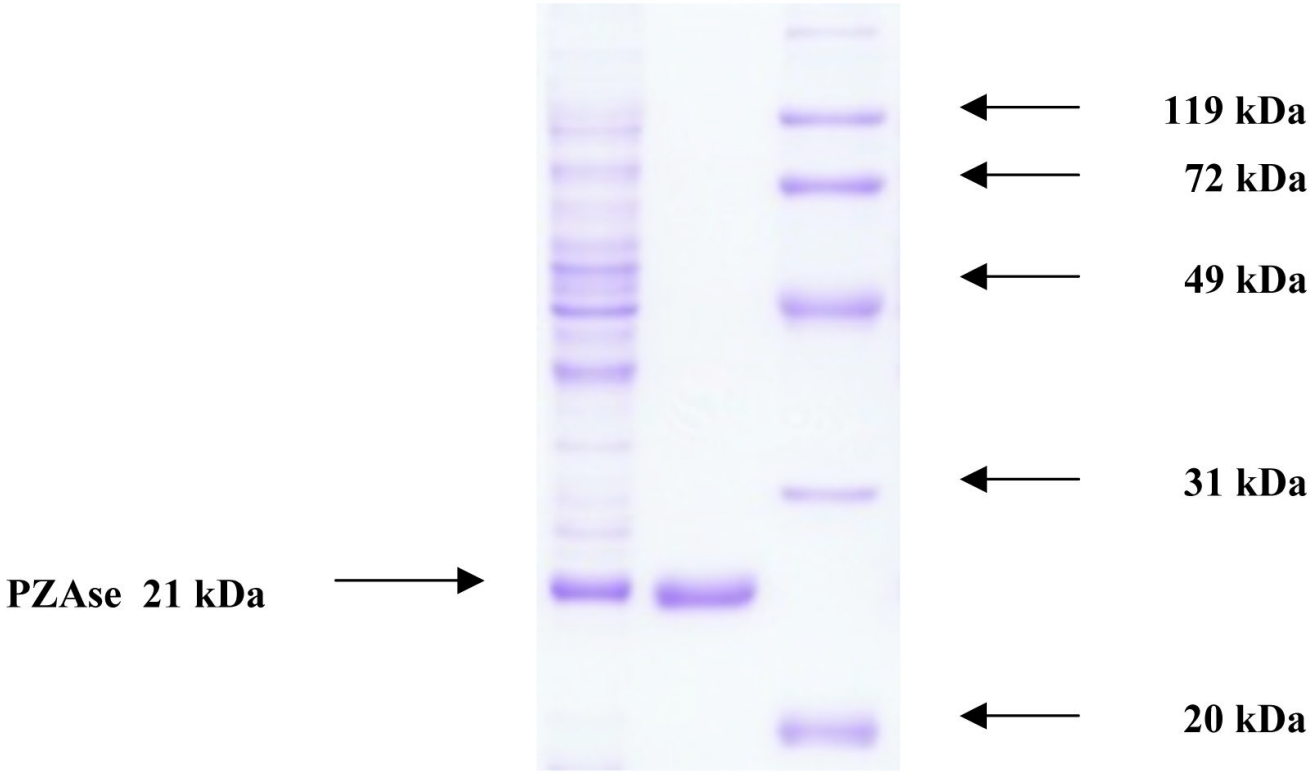

Figure 1. Coomasie blue SDS-PAGE of recombinant pyrazinamidase from *Mycobacterium tuberculosis* wild-type H37Rv strain

Lane 1: crude lysate of *E. coli* BL21(DE3)pLysS transformed with plasmid pET28a::His_6-PncA after induction with 1mM IPTG; lane 2: affinity-chromatography-purified recombinant pyrazinamidase, 0.5 mg/ml; lane 3: Standard Molecular Weight (Gibco BRL).

Table 1

Pyrazinamide susceptibility parameters and localization of *pncA* mutations

Mutation	Localization of mutation in PZAse	PZA MIC	Wayne activity	Bactec 460TB	
				% Growth index	Susceptibility
T135P	Close AS	>800	Negative	98	R
H51R	MBS	>800	Negative	90	R
D49N	MBS	400	Negative	74	R
T76P	Close MBS	200	Negative	89	R
L116P	Distant	100	Negative	46	R
D12A	Close AS	400	Weak	77	R
D12G	Close AS	400	Weak	70	R
F94L	Close AS	400	Weak	70	R
G24D	Distant	200	Weak	68	R
Y34D	Distant	100	Weak	78	R
G78C	Distant	100	Weak	27	R
K48T	Close MBS	≤50	Positive	20	R
Wild type	No mutation	≤50	Positive	1	S

% Growth Index, Percentage of growth on 100 μg/ml PZA-broth compared to a PZA-free broth.

%Growth Index more than 11% is considered PZA resistant. AS, active site; MBS, metal-binding site; R, resistance, S, susceptible; NS, not significant.

Table 2

Kinetic parameters of *Mycobacterium tuberculosis* pyrazinamidases

Mutation	PZAse activity (μmol POA·min^{-1}·mg^{-1} PZAse)		K_m (mM)		k_{cat} (min^{-1})		PZAse efficiency (mM^{-1}·min^{-1})		
	Median	IQR	Mean	95% CI (+/−)	Mean	95% CI (+/−)	Mean	95% CI (+/−)	P-value
T135P	0.02	0.02	0.93	0.45	0.45	0.25	NS		*0.000*
H51R	0.006	0.001	1.41	0.18	0.17	0.01	0.12	0.70	*0.000*
D49N	0.045	0.014	3.09	1.19	1.53	0.45	0.55	0.50	*0.000*
T76P	8.99	0.95	0.31	0.05	202.98	7.8	650.33	89.71	0.34
L116P	50.15	5.68	1.56	0.15	1324.5	85.19	847.66	37.11	0.820
D12A	9.24	3.19	0.99	0.16	245.17	19.20	248.79	20.04	*0.000*
D12G	14.00	2.52	0.55	0.06	368.46	14.96	716.83	64.78	*0.042*
F94L	21.19	8.89	2.00	0.47	712.92	111.46	348.6	30.48	*0.010*
G24D	4.28	3.25	0.42	0.25	100.13	16.30	236.2	107.40	*0.046*
Y34D	20.58	8.35	0.83	1.05	386.4	210.06	460.19	362.01	0.568
G78C	6.96	0.83	1.07	0.14	105.16	7.02	97.65	6.10	*0.000*
K48T	10.45	1.06	0.44	0.05	241.82	8.34	551.28	40.03	*0.036*
Wild type	38.40	18.36	1.24	0.44	1005.41	199.91	806.6	144.60	

IQR, interquartile range; 95% CI, 95% confidence interval; P-value for the comparison of the enzymatic efficiency between each mutated PZAse and the wild-type; NS, not significant. The enzymatic activity was estimated as the amount of POA produced in a 1 min reaction divided by the total amount of enzyme.

Table 3

Analysis of correlation between the pyrazinamidase kinetic parameters and pyrazinamide resistance levels

	Correlation coefficient	Linear regression R^2	P-value
MIC – Activity	−0.61		0.028
MIC – k_{cat}	−0.56		0.047
MIC – K_m	0.22		0.477
MIC – Efficiency	−0.64		*0.018*
Bactec – Activity	−0.52	0.273	0.067
Bactec – k_{cat}	−0.49	0.243	0.087
Bactec – K_m	0.04	0.002	0.899
Bactec – Efficiency	−0.46	0.214	0.111
Wayne – Activity	−0.47		0.103
Wayne – k_{cat}	−0.45		0.124
Wayne – K_m	−0.24		0.420
Wayne – Efficiency	−0.36		0.221