Mutations in \textit{panD} encoding aspartate decarboxylase are associated with pyrazinamide resistance in \textit{Mycobacterium tuberculosis}

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Pyrazinamide (PZA) is a frontline anti-tuberculosis drug that plays a crucial role in the treatment of both drug susceptible and multidrug-resistant tuberculosis (MDR-TB). PZA is most commonly associated with mutations in the \textit{pncA} gene encoding nicotinamidase/pyrazinamidase which converts the prodrug PZA to the active form pyrazinoic acid (POA). RpsA (ribosomal protein S1) involved in trans-translation was recently shown to be a target of PZA and mutations in RpsA are found in some PZA-resistant TB strains. However, some other PZA-resistant strains lack mutations in either \textit{pncA} or \textit{rpsA}. To identify potential new mechanisms of PZA resistance, we isolated 174 \textit{in vitro} mutants of \textit{M. tuberculosis} H37Rv resistant to PZA to search for resistant isolates that do not have \textit{pncA} or \textit{rpsA} mutations. Whole genome sequencing analyses revealed that the 5 PZA-resistant mutants had different mutations all occurring in the same gene \textit{panD} encoding aspartate decarboxylase, which is involved in synthesis of \textit{l}-alanine that is a precursor for pantothenate and co-enzyme A biosynthesis. \textit{panD} mutations were identified in naturally PZA-resistant \textit{Mycobacterium canetti} strain and a PZA-resistant MDR-TB clinical isolate. Future studies are needed to address the role of \textit{panD} mutations in PZA resistance and confirm PanD as a new target of PZA.

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\textbf{INTRODUCTION}

Pyrazinamide (PZA) is an important first-line tuberculosis (TB) drug used in combination with other TB drugs for the treatment of both drug susceptible TB and multidrug-resistant tuberculosis (MDR-TB).\textsuperscript{1} PZA plays a critical role in modern TB chemotherapy\textsuperscript{2} by shortening the treatment from previously 9-12 months to 6 months. The unique sterilizing activity of PZA in shortening the treatment is due to its high activity against persisting bacteria that are not killed by other TB drugs.\textsuperscript{3} Because of its indispensable sterilizing activity, all new TB drug candidates in clinical trials are used together with PZA.\textsuperscript{4-7}

PZA is an unconventional and paradoxical TB drug, characterized by lack of activity against growing bacteria under normal culture conditions,\textsuperscript{8} but high activity against non-replicating persisters at acid pH (e.g. pH 5.5).\textsuperscript{9} Even under acid pH conditions \textit{in vitro}, PZA has a high minimal inhibitory concentration (MIC) of 50–100 \textmu g/mL at pH 5.5–6.0. PZA is a peculiar drug whose activity is influenced by various factors such as acid pH, culture age, starvation, weak acids, energy inhibitors and microaerophilic/anaerobic conditions. Despite the importance of PZA in shortening the treatment of TB, its mechanism of action is the least understood of all TB drugs. Structurally, PZA is an analog of nicotinamide. Mutation in \textit{pncA} encoding nicotinamidase/pyrazinamidase (PZase)\textsuperscript{10} is the major mechanism for PZA resistance in \textit{M. tuberculosis}.\textsuperscript{10-12} Like isoniazid,\textsuperscript{13} PZA is a prodrug which requires activation to its active form pyrazinoic acid (POA) by \textit{M. tuberculosis} PZase enzyme.\textsuperscript{10} Recently, we identified a new target of PZA as ribosomal protein S1 (RpsA, Rv1630), a vital ribosomal protein involved in trans-translation.\textsuperscript{14} Trans-translation is involved in degradation of potentially toxic protein products formed in stressed bacteria required for persister survival. Mutations in \textit{rpsA} have been found in some PZA-resistant strains without \textit{pncA} mutations.\textsuperscript{14-16} However, some PZA-resistant strains, which are typically low level PZA resistant (MIC=200–300 \textmu g/mL, PH 6.0) and PZase positive do not have mutations in either \textit{pncA} or the \textit{rpsA} gene.\textsuperscript{12,15,17} To identify new mechanisms of PZA resistance, in this study, we isolated a large number of \textit{in vitro} generated mutants resistant to PZA and characterized these strains for novel mutations in their genomes by whole genome sequencing. Sequence analyses of 5 low level PZA-resistant isolates without \textit{pncA} or \textit{rpsA} mutations indicate mutations in the \textit{panD} gene encoding aspartate alpha-decarboxylase as a potential new mechanism of PZA resistance.

\textbf{MATERIALS AND METHODS}

Isolation of \textit{M. tuberculosis} mutants resistant to PZA and PZA susceptibility testing

\textit{Mycobacterium tuberculosis} H37R\textit{v} was grown in 7H9 liquid medium (Difco) supplemented with 0.05% Tween 80 and 10% bovine serum albumin-dextrose-catalase (ADC) enrichment at 37 °C for approximately
10–14 days (mid- to late-exponential phase) with occasional agitation as described. Pyrazinamide (Sigma-Aldrich Co.) was dissolved in deionized water at a stock concentration of 10 mg/mL and filter-sterilized and incorporated into 7H11 agar plates containing ADC at concentrations of 200 μg/mL, pH 6.0. Mutants that grew on the PZA-containing plates after 3–4 weeks incubation at 37 °C were picked and grown in 7H9 liquid medium for confirming PZA resistance phenotype by repeated PZA susceptibility testing. The PZA susceptibility testing of the PZA-resistant mutants was performed on 7H11 agar plates containing 100 μg/mL, 200 μg/mL, 300 μg/mL PZA (pH6.0) as described. Briefly, genomic DNA from 175 M. tuberculosis H37Rv to identify potential mutations in the pncA gene. The 650 bp PCR products were then sequenced as above to identify possible mutations in the pncA gene. The 650 bp pncA PCR products were sequenced as above to identify possible mutations in pncA.

Polymerase chain reaction (PCR) and DNA sequencing

The pncA PCR was performed using P1 primer (5’TCTCGTCA-TGGTTCCGATCC-3’; from -105 base pair (bp) upstream of pncA) and P6 primer (5’-GTTTGGCAGGCTC-3’; from 60 base pair downstream of the stop codon) as described. Briefly, genomic DNA from 175 in vitro isolated PZA-resistant mutants was isolated (see below) and used as templates for PCR as follows: heat denaturation at 94 °C 15 min followed by 30 cycles of 94 °C 0.5 min, 55 °C 0.5 min, 72 °C 1 min followed by extension at 72 °C for 7 min. The PCR reaction was then cooled to 4 °C. The pncA PCR products were then sequenced by ABI 377 DNA sequencer at Johns Hopkins Genetic Resources Core Facility, and the pncA sequences from different mutant isolates were compared against the wild type pncA sequence of M. tuberculosis H37Rv to identify potential mutations in the pncA gene. The rpsA gene was also PCR amplified, and the PCR products were sequenced for 5 mutants without pncA mutations using primers and conditions as previously described. Primers (panD_F: 5’TCTC-ACGGTTCCGAGGCTG-3’ and panD_R: 5’TATCGGCC-ACGGGTACACCTT-3’) were used to amplify a 650 bp PCR product that contains the whole panD gene from PZA-resistant M. tuberculosis strains using the same condition as above for amplifying the pncA gene. The 650 bp panD PCR products were sequenced as above to identify possible mutations in panD.

PZase activity determination

The PZase enzyme test (the Wayne PZase test) was performed as described, with the following modifications. Briefly, PZA was added to 100 μg/mL final concentration to 1 mL M. tuberculosis log phase cultures in Eppendorf tubes and incubated at 37 °C overnight, and then 2% ferrous sulfate was added for color development. PZA in the presence of positive PZase enzyme from the M. tuberculosis will be converted to POA, which then reacts with ferrous ion to produce a brown colored compound, which can be detected as an indication of positive PZase activity.

Whole genome sequencing

The genomic DNA for whole genome sequencing was isolated as previously described. The genomic DNA samples from the 5 PZA-resistant mutants that were positive for PZase and did not have pncA mutations were subjected to whole genome sequencing using Illumina HiSeq 2000 machine. Paired-end sequencing libraries for genomic DNA of each strain were barcoded and constructed with insert sizes of approximately 300 bp using TruSeq DNA Sample Preparation kits (Illumina, USA) according to manufacturer’s instruction. For each strain, 1.0 G–1.5 G bases (230-fold to 350-fold genome coverage) were generated after barcodes were trimmed. High-quality data were aligned with the reference sequence of M. tuberculosis H37Ra (NC_000925) using SOAPaligner. We used M. tuberculosis H37Ra genome sequence as a reference strain for sequence comparison with the PZA-resistant mutants derived from M. tuberculosis H37Rv because of the significant number of sequencing errors in the original H37Rv genome sequence in the database. Only reads where both ends aligned to the reference sequence were used for single nucleotide variant (SNV) and insertion and deletion (InDels) analysis. SNVs and InDels ranging from 1 to 5 bp were sorted and called at minimum reads of 10. In order to eliminate the genomic differences of H37Ra and H37Rv in our analysis, SNVs and InDels shared between H37Ra and H37Rv were further filtered and annotated for gene locus and mutation types with the nearest coding sequences. Synonymous mutations and PE/PPE mutations within coding sequence were not included in the final analysis to focus on mutations that are most likely involved in PZA resistance.

RESULTS

Isolation of PZA-resistant mutants

The flow chart of isolation and characterization of in vitro mutants resistant to PZA is shown in Figure 1. The wild type M. tuberculosis strain H37Rv was susceptible to 100 μg/mL PZA (pH 5.9). To isolate PZA-resistant spontaneous mutants, early stationary phase cultures of M. tuberculosis H37Rv were plated on 7H11 agar plates containing 200 μg/mL PZA (pH 5.9). Through several rounds of isolation, about 300 mutants were obtained. After repeated PZA susceptibility testing to rule out false resistance, a total of 174 mutants were obtained that were consistently resistant to PZA. These 174 PZA-resistant mutants were subjected to further analysis by pncA sequencing as below.

pncA sequencing revealed new PZA-resistant mutants without pncA mutations

To identify desired mutants that do not have pncA mutations which would indicate possible new mechanisms of PZA resistance, we isolated genomic DNA from the 174 PZA-resistant mutants and performed PCR to amplify the pncA gene. DNA sequencing analysis of the pncA PCR products revealed that 169 of the 174 (97.1%) PZA-resistant mutants had various pncA mutations while 5 mutants, S6, S9, S10, S11, S13, did not have any pncA mutations. Sequencing analysis of rpsA, another gene involved in PZA resistance, did not show any rpsA mutations in the 5 mutants without pncA mutations. PZase assay showed that the 5 mutants were positive for the enzyme activity, which is consistent with the above pncA sequencing results and also ruled out a pncA promoter or regulatory mutation that could result in lack of PZase enzyme activity as a possible cause of the PZA resistance in the 5 mutants. The above findings suggest that the 5 PZA-resistant mutants harbor possible new mechanisms of PZA resistance independent of pncA or rpsA mutations.

Whole genome sequencing identified a new gene panD closely associated with PZA resistance

To identify possible new mechanisms of PZA resistance, we subjected the 5 PZA-resistant mutants without pncA or rpsA mutations to whole genome sequencing using Illumina Hi-Seq2000. After filtering out PE/PPE family genes and the genomic differences between H37Ra and H37Rv, only 3, 2, 4, 5 and 3 SNVs were identified respectively for
mutants S6, S9, S10, S11 and S13, and only 1 InDel was identified for mutants S6, S9 and S10, respectively (Supplementary information Table 1). Comparative genome sequence analyses of the 5 PZA-resistant strains revealed that they all had mutations in a single gene, panD, encoding aspartate alpha-decarboxylase (Table 1). It is interesting to note that the 5 mutants had 5 different mutations in the panD gene. Mutant S6 had an A128S mutation (Ala to Ser change at amino acid position 128), S9 and S10 had identical panD mutation V138A, S11 had two mutations causing H21R and I49V substitutions, S13 had an E130G substitution in the panD gene. These 5 mutations revealed by whole genome sequencing were confirmed to be genuine by PCR sequencing of the panD gene from each of the 5 individual mutants.

panD is located in an operon lysS-Rv3603c with panC (pantothenate synthetase), Rv3603c (conserved hypothetical alanine and leucine rich protein), Rv3600c (hypothetical protein), Rv3559c (hypothetical protein), and lysS (lysyl-tRNA synthetase 1) (Figure 2). panD encodes a 139 amino acid (15 kD) protein involved in synthesis of β-alanine from decarboxylation of L-aspartate required for pantothenate (vitamin B5) and co-enzyme A (CoA) biosynthesis (Figure 3).

Identification of panD mutations in clinical isolates
Mycobacterium canetti, a member of the M. tuberculosis complex that causes human TB in some regions of Africa, is naturally resistant to PZA but lacks pncA mutations. It is interesting to note that it contained a non-synonymous mutation of T to C change at nucleotide position 350 causing M117T change and a silent mutation (C39G) in panD (Table 1). In addition, an MDR-TB clinical isolate resistant to PZA was found to harbor a mutation of T to C change at nucleotide position 400 causing amino acid substitution of P134S (Table 1).

DISCUSSION
In this study, we found that mutations in panD are closely associated with PZA resistance.

Table 1 panD mutations identified in PZA-resistant mutants or clinical isolates

| M. tuberculosis strains | Nucleotide change | Amino acid change |
|------------------------|------------------|------------------|
| M. tuberculosis S6      | G382T            | A128S            |
| M. tuberculosis S9      | T413C            | V138A            |
| M. tuberculosis S10     | T413C            | V138A            |
| M. tuberculosis S11     | A62G, A145G      | H21R, I49V       |
| M. tuberculosis S13     | A389G            | E130G            |
| M. canetti K116         | C39G, T350C      | M117T            |
| M. tuberculosis HT158   | T400C            | P134S            |

*M. tuberculosis S6, S9, S10, S11, S13 refer to the 5 PZA-resistant mutants derived from M. tuberculosis strain H37Rv that do not have pncA or rpsA mutations. M. tuberculosis HT158 is an MDR-TB clinical isolate resistant to PZA.
background of the clinical strains that differ from each other and from the sequenced type strains. By using whole genome sequencing of isogenic mutants from the same strain H37Rv, we were able to identify mutation of the panD gene as a possible new mechanism of PZA resistance. Besides the in vitro isolated mutants that have panD mutations, we also found panD mutations in clinical isolates such as M. canettii and a clinical strain. Although M. canettii which is naturally resistant to PZA was recently found to harbor panD mutations, it is worth noting that M. canettii also had an M117T amino acid substitution in the PanD. The relative contribution of the rpsA and panD mutations in the natural PZA resistance of M. canettii remains to be determined. The finding that panD mutations are closely associated with PZA resistance may offer yet a third mechanism of PZA resistance besides pncA and rpsA mutations. However, there may be other unidentified genes involved in PZA resistance, since we found that the PZA-resistant clinical isolate 9739 (PZA MIC= 200–300 μg/mL) does not have any mutations in pncA, rpsA, or panD (data not shown).

panD mutation in M. tuberculosis has been shown to cause higher attenuation of virulence in mice than BCG vaccine, indicating panD may be critical for survival and persistence of the bacilli in vivo. panD encoding aspartate alpha-decarboxylase is involved in synthesis of β-alanine which is in turn required for pantothenate and CoA synthesis. CoA has a central role in cellular metabolism. CoA is similar to nicotinamide adenine dinucleotide and flavin adenine dinucleotide (FAD) in structure and serves as an acetyl group carrier important for synthesis and oxidation of fatty acids and oxidation of pyruvate in the Tricarboxylic acid cycle to generate ATP. The possibility that PZA for synthesis and oxidation of fatty acids and oxidation of pyruvate in the TCA cycle to generate ATP. The possibility that PZA metabolism needs to be addressed in future studies.

Although a few other mutations such as mutations in HadC (β-hydroxyacyl- acyl carrier protein dehydratase) involved in cell wall mycolic acid elongation were identified in 3 of the 5 PZA-resistant mutants (Supplementary information Table 1), they are less likely causal in PZA-resistance. This is because mycolic acid synthesis mainly occurs in growing TB bacteria and inhibition by PZA of HadC responsible for mycolic acid elongation, while cannot be excluded, is inconsistent with the unique activity of PZA for non-growing persisters. Nevertheless, future studies are required to rule out the possibility of HadC mutations being involved in PZA resistance.

In summary, we identified a new gene panD whose mutations are closely associated with PZA resistance in PZA-resistant mutants and clinical isolates without pncA or rpsA mutations. panD may encode another target of PZA in addition to RpsA. Future studies are needed to assess the role of the identified panD mutations as a new mechanism of PZA resistance and confirm the role of PanD as a new target of PZA in M. tuberculosis.

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1 World Health Organization. Treatment of Tuberculosis: Guidelines, Fourth edition. Geneva: World Health Organization Press, 2010.

2 Zhang Y, Mitchison D. The curious characteristics of pyrazinamide: a review. Int J Tuberc Lung Dis 2003; 7: 6–21.

3 Mitchison DA. The action of antituberculosis drugs in short course chemotherapy. Tuberce 1985; 66: 219–225.

4 Andries K, Verhasselt P, Guillemont J et al. A diaryquinoline drug active on the ATP synthase of Mycobacterium tuberculosis. Science 2005; 307: 223–227.

5 Roseurnal IM, Zhang M, Williams KN et al. Daily dosing of rifapentine cures tuberculosis in three months or less in the murine model. PLoS Med 2007; 4: e344.

6 Tasneen R, Li SY, Peloquin CA et al. Sterilizing Activity of Novel TMC207- and PA-824-Containing Regimens in a Murine Model of Tuberculosis. Antimicrob Agents Chemother 2011; 55: 5485–5492.

7 Tasneen R, Tyagi S, Williams K, Grosset J, Nuernberger E. Enhanced bactericidal activity of rifampin and/or pyrazinamide when combined with PA-824 in a murine model of tuberculosis. Antimicrob Agents Chemother 2008; 52: 3664–3668.

8 Tarshis MS, Weed WA Jr. Lack of significant in vitro sensitivity of Mycobacterium tuberculosis to pyrazinamide on three different solid media. Am Rev Tuberc 1953; 67: 391–395.

9 McDermott W, Tompsett R. Activation of pyrazinamide and nicotinamide in acidic environment in vitro. Am Rev Tuberc 1954; 70: 748–754.

10 Scorpio A, Zhang Y. Mutations in pncA, a gene encoding pyrazinamidase/ nicotinamidase, cause resistance to the antituberculosis drug pyrazinamide in tubercle bacillus. Nat Med 1996; 2: 662–667.

11 Scorpio A, Lindholm-Lexy P, Heifets L et al. Characterization of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis. Extremophiles 1997; 1: 540–543.

12 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: 528–532.

13 Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. Nature 1992; 358: 591–593.

14 Shi W, Zhang X, Jiang X et al. Pyrazinamide inhibits trans-translation in Mycobacterium tuberculosis. Science 2011; 333: 1630–1632.

15 Simons SO, Mulder A, van Ingen J, Boeree MJ, van Soomslenger D. Role of rpsA Gene Sequencing in Diagnosis of Pyrazinamide Resistance. J Clin Microbiol 2013; 51: 382.

16 Feuerriegel S, Koset CI, Richter E, Niemann S. Mycobacterium canettii is intrinsically resistant to both pyrazinamide and pyrazinoric acid. J Antimicrob Chemother 2013; 68:1349–1440.
17 Alexander DC, Ma JH, Guthrie JL, Blair J, Chedore P, Jamieson FB. Gene sequencing for routine verification of pyrazinamide resistance in Mycobacterium tuberculosis: a role for pncA but not rpsA. J Clin Microbiol 2012; 50: 3726–3728.

18 Zhang Y, Permar S, Sun Z. Conditions that may affect the results of susceptibility testing of Mycobacterium tuberculosis to pyrazinamide. J Med Microbiol 2002; 51: 42–49.

19 Wayne LG. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am Rev Respir Dis 1974; 109: 147–151.

20 Zheng H, Lu L, Wang B et al. Genetic basis of virulence attenuation revealed by comparative genomic analysis of Mycobacterium tuberculosis strain H37Ra versus H37Rv. PLoS One 2008; 3: e2375.

21 Li R, Li Y, Kristiansen K, Wang J. SOAP: short oligonucleotide alignment program. Bioinformatics 2008; 24: 713–714.

22 Loeger TR, Feng Y, Ganesula K et al. Variation among genome sequences of H37Rv strains of Mycobacterium tuberculosis from multiple laboratories. J Bacteriol 2010; 192: 3645–3653.

23 Somoskovi A, Dormandy J, Parsons LM et al. Sequencing of the pncA gene in members of the Mycobacterium tuberculosis complex has important diagnostic applications: Identification of a species-specific pncA mutation in "Mycobacterium canettii" and the reliable and rapid predictor of pyrazinamide resistance. J Clin Microbiol 2007; 45: 595–599.

24 Sambandumurthy VK, Wang X, Chen B et al. A pantothenate auxotroph of Mycobacterium tuberculosis is highly attenuated and protects mice against tuberculosis. Nat Med 2002; 8: 1171–1174.

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17 Alexander DC, Ma JH, Guthrie JL, Blair J, Chedore P, Jamieson FB. Gene sequencing for routine verification of pyrazinamide resistance in Mycobacterium tuberculosis: a role for pncA but not rpsA. J Clin Microbiol 2012; 50: 3726–3728.

18 Zhang Y, Permar S, Sun Z. Conditions that may affect the results of susceptibility testing of Mycobacterium tuberculosis to pyrazinamide. J Med Microbiol 2002; 51: 42–49.

19 Wayne LG. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am Rev Respir Dis 1974; 109: 147–151.

20 Zheng H, Lu L, Wang B et al. Genetic basis of virulence attenuation revealed by comparative genomic analysis of Mycobacterium tuberculosis strain H37Ra versus H37Rv. PLoS One 2008; 3: e2375.

21 Li R, Li Y, Kristiansen K, Wang J. SOAP: short oligonucleotide alignment program. Bioinformatics 2008; 24: 713–714.

22 Loeger TR, Feng Y, Ganesula K et al. Variation among genome sequences of H37Rv strains of Mycobacterium tuberculosis from multiple laboratories. J Bacteriol 2010; 192: 3645–3653.