Open

Emerging Microbes and Infections (2013) 2, e34; doi:10.1038/emi.2013.38
© 2013 SSCC. All rights reserved 2222-1751/13
www.nature.com/emi

ORIGINAL ARTICLE

Mutations in *panD* encoding aspartate decarboxylase are associated with pyrazinamide resistance in *Mycobacterium tuberculosis*

Shuo Zhang[1,2,*], Jiazhen Chen[3,*], Wanliang Shi[1], Wei Liu[3], Wenhong Zhang[3] and Ying Zhang[1,3]

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). Resistance to PZA is most commonly associated with mutations in the *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 *pncA* or *rpsA*. To identify potential new mechanisms of PZA resistance, we isolated 174 *in vitro* mutants of *M. tuberculosis* H37Rv resistant to PZA to search for resistant isolates that do not have *pncA* or *rpsA* mutations. DNA sequencing revealed that 169 of the 174 (97.1%) PZA-resistant mutants had *pncA* mutations but 5 mutants lacked *pncA* or *rpsA* mutations. Whole genome sequencing analyses revealed that the 5 PZA-resistant mutants had different mutations all occurring in the same gene *panD* encoding aspartate decarboxylase, which is involved in synthesis of β-alanine that is a precursor for pantothenate and co-enzyme A biosynthesis. *panD* mutations were identified in naturally PZA-resistant *Mycobacterium canetti* strain and a PZA-resistant MDR-TB clinical isolate. Future studies are needed to address the role of *panD* mutations in PZA resistance and confirm PanD as a new target of PZA.
Emerging Microbes and Infections (2013) **2,** e34; doi:10.1038/emi.2013.38; published online 12 June 2013

Keywords: aspartate decarboxylase; mechanism of resistance; mode of action; *panD*; pyrazinamide

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.[1] PZA plays a critical role in modern TB chemotherapy[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 persister bacteria that are not killed by other TB drugs.[3] Because of its indispensible sterilizing activity, all new TB drug candidates in clinical trials are used together with PZA.[4–7]

PZA is an unconventional and paradoxical TB drug, characterized by lack of activity against growing bacteria under normal culture conditions,[8] but high activity for non-replicating persisters at acid pH (e.g. pH 5.5).[9] Even under acid pH conditions *in vitro*, PZA has a high minimal inhibitory concentration (MIC) of 50–100 μg/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 *pncA* encoding nicotinamidase/pyrazinamidase (PZase)[10] is the major mechanism for PZA resistance in *M. tuberculosis*.[10–12] Like isoniazid,[13] PZA is a prodrug which requires activation to its active form pyrazinoic acid (POA) by *M. tuberculosis* PZase enzyme.[10] Recently, we identified a new target of PZA as ribosomal protein S1 (RpsA, Rv1630), a vital ribosomal protein involved in trans-translation.[14] Trans-translation is involved in degradation of potentially toxic protein products formed in stressed bacteria required for persister survival. Mutations in *rpsA* have been found in some PZA-resistant strains without *pncA* mutations.[14–16] However, some PZA-resistant strains, which are typically low level PZA resistant (MIC=200–300 μg/mL, PH 6.0) and PZase positive do not have mutations in either *pncA* or the *rpsA* gene.[12,15,17] To identify new mechanisms of PZA resistance, in this study, we isolated a large number of *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 *pncA* or *rpsA* mutations indicate mutations in the *panD* gene encoding aspartate alpha-decarboxylase as a potential new mechanism of PZA resistance.

MATERIALS AND METHODS

Isolation of *M. tuberculosis* mutants resistant to PZA and PZA susceptibility testing

Mycobacterium tuberculosis H37Rv was grown in 7H9 liquid medium (Difco) supplemented with 0.05% Tween 80 and 10% bovine serum albumin-dextrose-catalase (ADC) enrichment at 37 ℃ for approximately

[1]Department of Molecular Microbiology and Immunology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205, USA; [2]Shandong Medicinal Biotechnology Centre, Shandong Academy of Medical Sciences, Jinan 250062, China and [3]Department of Infectious Diseases, Huashan Hospital, Fudan University, Shanghai 200040, China

*These authors contributed equally to this paper.
Correspondence: WH Zhang; Y Zhang
E-mail: zhangwenhong@fudan.edu.cn; yzhang@jhsph.edu
Received 28 May 2013; revised 3 June 2013; accepted 3 June 2013

10–14 days (mid- to late-exponential phase) with occasional agitation as described.[10] 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 ℃ 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.[18] Wild type *M. tuberculosis* H37Rv and a known PZA-resistant mutant PZA-R1 containing a *pncA* mutation (Q10P) were included as a drug susceptible control strain and a resistant control strain for the PZA susceptibility testing. PZA susceptible strain H37Rv did not grow on PZA containing plates while the PZA-resistant mutants and PZA-R1 resistant control strain grew on PZA containing plates.

Polymerase chain reaction (PCR) and DNA sequencing

The *pncA* PCR was performed using P1 primer (5′-GTCGGTCA-TGTTCGCGATCG-3′; from -105 base pair (bp) upstream of *pncA*) and P6 primer (5′-GCTTTGCGGCGAGCGCTCCA-3′; from 60 base pair downstream of the stop codon) as described.[11] 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 ℃ 15 min followed by 30 cycles of 94 ℃ 0.5 min, 55 ℃ 0.5 min, 72 ℃ 1 min followed by extension at 72 ℃ for 7 min. The PCR reaction was then cooled to 4 ℃. 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.[14] Primers (panD_F: 5′-TC-AACGGTTCCGGTCGGCTGCT-3′ and panD_R: 5′-TATCCGCC-ACTGCTGCACGACCTT-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,[19] 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 ℃ 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.[20] 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_009525) using SOAPaligner.[21] We used *M. tuberculosis* H37Ra genome sequence[20] 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.[22] 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,[14] 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

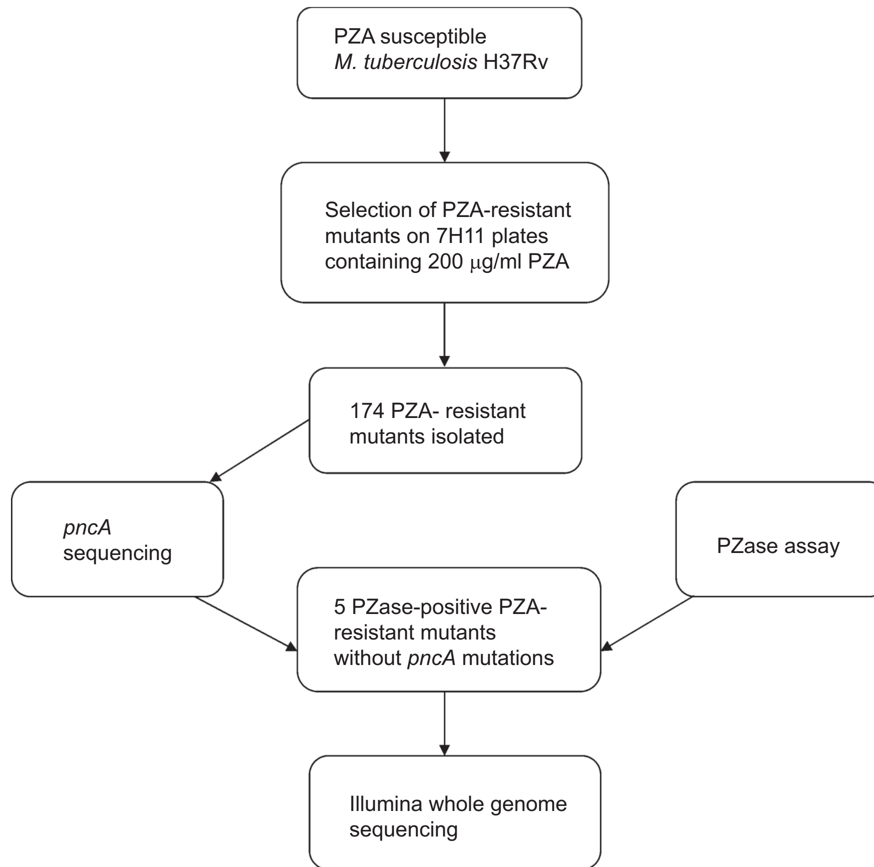

Figure 1 Flow chart of PZA-resistant mutant isolation and characterization.

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.

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. canettii 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.

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.[23] 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.

PZA resistance in *M. tuberculosis* is most commonly caused by mutations in *pncA* gene[10] encoding the PZase required for conversion of PZA prodrug to POA or occasionally caused by mutations in the target *rpsA* encoding ribosomal protein S1.[14] However, there are some PZA-resistant clinical isolates that do not have mutations in *pncA* or *rpsA*. It has been challenging to pin down the new mechanism of PZA resistance using clinical isolates because of the diverse genetic

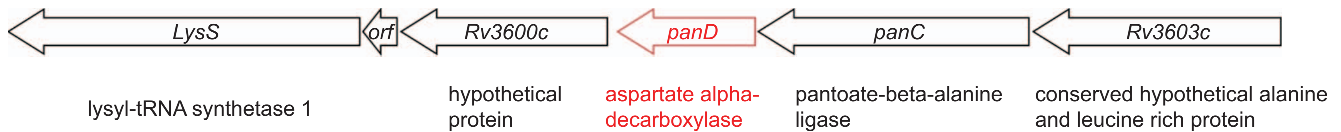

Figure 2 Genomic organization of *lysS-Rv3600c-panD-panC-Rv3603c* operon. *panD,* whose mutations are found to be associated with PZA resistance, is highlighted in red and is located in the operon *lysS-Rv3600c-panD-panC-Rv3603c*.

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 *rpsA* mutations,[16] 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)[12] 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,[24] 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 may inhibit pantothenate and CoA synthesis thereby interfering with diverse metabolic functions such as energy production and fatty acid metabolism in *M. tuberculosis* 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*.

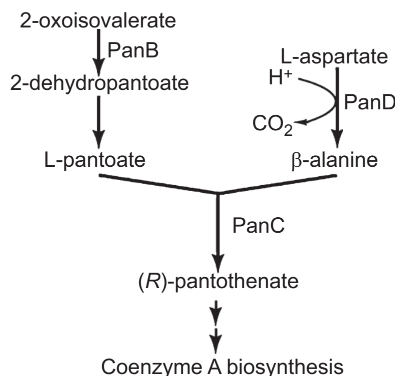

Figure 3 PanD is involved in synthesis of β-alanine, which is a precursor for pantothenate and coenzyme A biosynthesis in *M. tuberculosis*. Enzymes involved in the biosynthesis of pantothenate in *M. tuberculosis* include: PanB, ketopantoate hydroxymethyl transferase; PanD, L-aspartate alpha-decarboxylase; PanC, pantothenate synthetase.

ACKNOWLEDGEMENTS

The work was supported in part by NIH grant AI099512 and Major Project of the Twelfth Five-Year Plan (2013ZX10003008-003 and 2013ZX10003001-002).

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. *Tubercle* 1985; **66**: 219–225.
4 Andries K, Verhasselt P, Guillemont J *et al*. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. *Science* 2005; **307**: 223–227.
5 Rosenthal 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 and Chemothe* 2011; **55**: 5485–5492.
7 Tasneen R, Tyagi S, Williams K, Grosset J, Nuermberger 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 antituberculous drug pyrazinamide in tubercle bacillus. *Nat Med* 1996; **2**: 662–667.
11 Scorpio A, Lindholm-Levy P, Heifets L *et al*. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1997; **41**: 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 Soolingen D. Role of *rpsA* Gene Sequencing in Diagnosis of Pyrazinamide Resistance. *J Clin Microbiol* 2013; **51**: 382.
16 Feuerriegel S, Koser CU, Richter E, Niemann S. *Mycobacterium canettii* is intrinsically resistant to both pyrazinamide and pyrazinoic acid. *J Antimicrob Chemother* 2013; **68**:1439–1440.

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 Ioerger 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 Sambandamurthy 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.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivative Works 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/3.0