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Role of *pncA* and *rpsA* Gene Sequencing in Detection of Pyrazinamide Resistance in *Mycobacterium tuberculosis* Isolates from Southern China

Yaoju Tan,[a] Zuqiong Hu,[a] Tianyu Zhang,[b] Xingshan Cai,[a] Haobin Kuang,[a] Yanwen Liu,[a] Junyu Chen,[a] Feng Yang,[b] Ke Zhang,[b] Shouyong Tan,[a] Yanlin Zhao[c]

State Key Laboratory of Respiratory Diseases, Department of Clinical Laboratory, Guangzhou Chest Hospital, Guangzhou, China[a]; State Key Laboratory of Respiratory Diseases, Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences (CAS), Guangzhou, China[b]; National Tuberculosis Reference Laboratory, China Center for Disease Control and Prevention, Beijing, China[c]

We sequenced *pncA* and *rpsA* genes plus flanking regions of 161 *Mycobacterium tuberculosis* isolates and found 10 new *pncA* and 3 novel *rpsA* mutations in pyrazinamide-resistant strains determined by the Bactec MGIT 960 system. The 3′ end of *rpsA* might be added as the target of molecular detection of pyrazinamide susceptibility.

Pyrazinamide (PZA) is an indispensable first-line antituberculosis drug which exhibits unique sterilizing activity for both drug-sensitive and multidrug-resistant (MDR) tuberculosis. It can kill semidormant bacilli which persist in acidic-pH environments inside macrophages (1), where other drugs may not act so well. It is a prodrug that needs to be converted into its active form, pyrazinoic acid (POA), by the *Mycobacterium tuberculosis* pyrazinamidase (PZase), which is encoded by the 561-nucleotide (nt) *pncA* gene (2, 3). In 1996, the *pncA* gene was confirmed to be strongly associated with PZA resistance in *M. tuberculosis* (3), and in the following year, investigators found that *pncA* mutations constituted the primary mechanism of PZA resistance (4). In 2011, through systematic review with meta-analyses, Chang et al. drew the conclusion that molecular assays based on the *pncA* mutations were probably the way forward for detecting pyrazinamide resistance in *M. tuberculosis* (5). Molecular detection of PZA resistance-related mutations in the *pncA* gene is indeed more rapid than traditional mycobacterial susceptibility testing methods that depend on the growth of *M. tuberculosis* and are hampered by the complication of testing PZA activity (3).

Shi et al. recently confirmed that the ribosomal protein S1 (RpsA), encoded by the *rpsA* gene, was a target of POA which might be associated with PZA resistance in clinical *M. tuberculosis* isolates (6), so it is worth investigating the molecular characterization of *rpsA* gene mutations in both PZA-resistant and PZA-susceptible *M. tuberculosis* clinical isolates. However, some reports suggested that *rpsA* gene sequencing might not play a role in detecting PZA susceptibility by molecular methods (7).

Rapid and accurate detection of drug resistance contributes to early optimization of *M. tuberculosis* treatment, which is an effective way to further avoid the emergence of other drug resistance and potential PZA toxicity. However, the mutations in *pncA* are highly diverse in different regions (8–10), which hampers the development and application of molecular assays that are always based on the molecular characterization of gene mutations. In southern China, there are no data so far published describing *pncA* mutations in PZA-resistant clinical strains of *M. tuberculosis*, so it is necessary to analyze the profile of *pncA* and *rpsA* mutations of clinical *M. tuberculosis* strains in this region for the convenience of rapid and accurate detection of PZA resistance.

Drug susceptibility profile of clinical isolates. It is well known that testing for PZA susceptibility (3) *in vitro* is very hard, and the CLSI-recommended Bactec 460TB radiometric system (460TB; Becton, Dickinson, Sparks, MD) was replaced recently in many laboratories by the nonradiometric Bactec MGIT 960 system (BT960; Becton, Dickinson, Sparks, MD) (11). In the present study, 161 clinical *M. tuberculosis* strains isolated from January 2011 to October 2012 from southern China, mostly (115 of 161) from Guangdong province, were selected to determine their susceptibilities to PZA by BT960 as recommended by the manufacturer with modified Middlebrook 7H9 broth (pH 5.9) containing 100 μg/ml PZA. *Mycobacterium bovis* BCG ATCC 34540 and *M. tuberculosis* H37Rv ATCC 27294 were used as PZA-resistant and PZA-susceptible controls, respectively. The susceptibilities of these isolates to rifampin (RIF), isoniazid (INH), ethambutol (EMB), and streptomycin (STR) were previously determined by BT960 when the patients were hospitalized in Guangzhou Chest Hospital, the biggest tuberculosis treatment center located in southern China (12). Of the 161 *M. tuberculosis* clinical isolates, 109 isolates were PZA susceptible and 52 were PZA resistant. Their susceptibilities to RIF, INH, EMB, and STR are summarized in Table 1 and Table 2.

Sequencing *pncA* and *rpsA* genes of the clinical isolates. As *pncA* and *rpsA* genes are the only known genes related to PZA susceptibility and their corresponding flanking regions (FR) may contain regulatory elements which may affect gene expression, it is reasonable that mutation in FR may alter the mutants' susceptibility to PZA. We analyzed the *pncA* and *rpsA* genes and their corresponding FR. No typical promoter was found upstream of the *pncA* gene from nt −1 to nt −358 including its upstream gene

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Address correspondence to Tianyu Zhang, zhangtianyugz2013@163.com, or Shouyong Tan, tanshyo@163.com.
T.Z., S.T., and Y.Z. contributed equally to this article.

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TABLE 1 Drug susceptibility and genotypic characteristics of PZA-resistant clinical isolates[a]

Resistance	*pncA* + FR mutation(s)	*rpsA* + FR mutation	No. of isolates
INH, RIF	D8A[b] (GAC→GCC)	—	1
INH, RIF	Q10P (CAG→CCG)	—	1
INH, RIF	F13L (TTC→CTC)	—	1
INH, RIF	S18P[b] (TCG→CCG)/P54L (CCG→CTG)	—	1
INH, RIF	G24D (GGC→GAC)	—	2
INH, RIF	L27[b] deletion	—	1
INH, RIF	T47A (ACC→GCC)	—	1
INH, RIF	H57Y (CAC→TAC)	—	2
INH, RIF	F58S[b] (TTC→TCC)	—	1
INH, RIF	S66P (TCG→CCG)	—	1
INH, RIF	T76P (ACT→CCT)	—	2
INH, RIF	F81V (TTC→GTC)	—	1
INH, RIF	H82R (CAT→CGG)	—	1
INH, RIF	E91 deletion[b]	—	1
INH, RIF	F94L (TTC→CTC)	—	2
INH, RIF	F94S (TTC→TCC)	—	1
INH, RIF	K96E (AAG→GAG)	—	2
INH, RIF	E111 deletion[b]	—	1
INH, RIF	Q122 deletion[b]	—	2
INH, RIF	VVG130-132 deletion[b]	—	1
INH, RIF	V131 deletion[b]	—	2
INH, RIF	G132A (GGT→GCT)	—	1
INH, RIF	T135P (ACC→CCC)	—	2
INH, RIF	D136G (GAT→GGT)	—	2
INH, RIF	V139G (GTG→GGG)	—	1
INH, RIF	V139A (GTG→GCG)	—	1
INH, RIF	V139L (GTG→CTG)	—	1
STR, RIF	Q141P (CAG→CCG)	—	1
INH, RIF	L159R (CTG→CGG)	—	1
INH, STR, EMB	G162D (GGT→GAT)	—	1
INH	T168P (ACC→CCC)	—	3
INH, RIF	E174G[b] (GAG→GGG)/M175R[b] (ATG→AGA)	—	2
INH, RIF	—	R474W (CGG→TGG)	1
INH	—	R474L (CGG→CTG)	1
STR	−11 (A→G)	—	1
INH, STR, EMB	—	E433D (GAG→GAC)	1
STR	—	—	4
Total	45	3	48 + 4

[a] Abbreviations and symbol: INH, isoniazid; RIF, rifampin; STR, streptomycin; EMB, ethambutol; FR, flanking region, which means the upstream and downstream regions of the gene; —, no mutation.
[b] First nonsynonymous mutations of *pncA* found.

TABLE 2 Drug susceptibility and genotypic characteristics of PZA-sensitive clinical isolates[a]

Resistance	*pncA* + FR mutation(s)	*rpsA* + FR mutation	No. of isolates
—	—	—	57
INH, RIF	—	—	17
INH, RIF	—	Q162R (CAG→CGG)	1
INH, STR	—	—	8
INH	—	—	6
RIF	—	—	4
STR	—	—	14
EMB	—	—	1
INH, STR, EMB	—	—	1
Total	108	1	109

[a] Abbreviations and symbol: INH, isoniazid; RIF, rifampin; STR, streptomycin; EMB, ethambutol; FR, flanking region, which means the upstream and downstream regions of the gene; —, no mutation.

Rv2044c. Though there are only 162 nt between *rpsA* and its upstream gene (*Rv1629*), a typical putative prokaryotic promoter was found as CCGAGTTTGTCCAGCGTGTACCCGTCGAGTA GCCTCGTCA***G***GTACCAATC (nt −90 to nt −41 of *rpsA*), which was predicted with an online program (http://www.fruitfly.org/seq_tools/promoter.html). The score was 0.86, with the total score being 1.0. The bold italic G was supposed to be the transcription starting site. There were only 25 nt between *rpsA* and its downstream gene, and no regulatory element was found. We designed primers to amplify and sequence the whole *pncA* and *rpsA* genes plus FR of the clinical strains according to sequences of *M. tuberculosis* H37Rv (GenBank accession no. NC000962) using the software Primer Premier 5.0 (Premier, Canada). The primers included the following: for the *pncA* gene plus FR, primers F1, 5′-TGCCACTCGCCGGTAACCGG (nt 321 to 340 downstream of *pncA*), and R1, 5′-GGTGGCCGCCGCTCAGCTGG (nt −119 to −100 of *pncA*); for the *rpsA* gene plus FR, primers F2, 5′-GGCC GCAGCTGGGACGCGGC (nt −192 to −173 of *rpsA*), and R2,

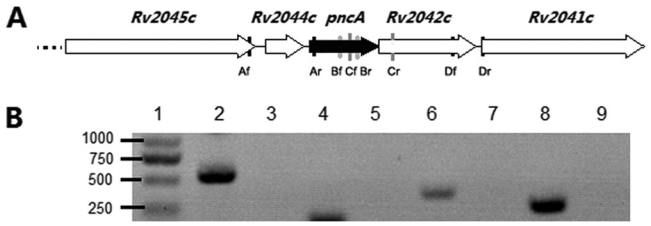


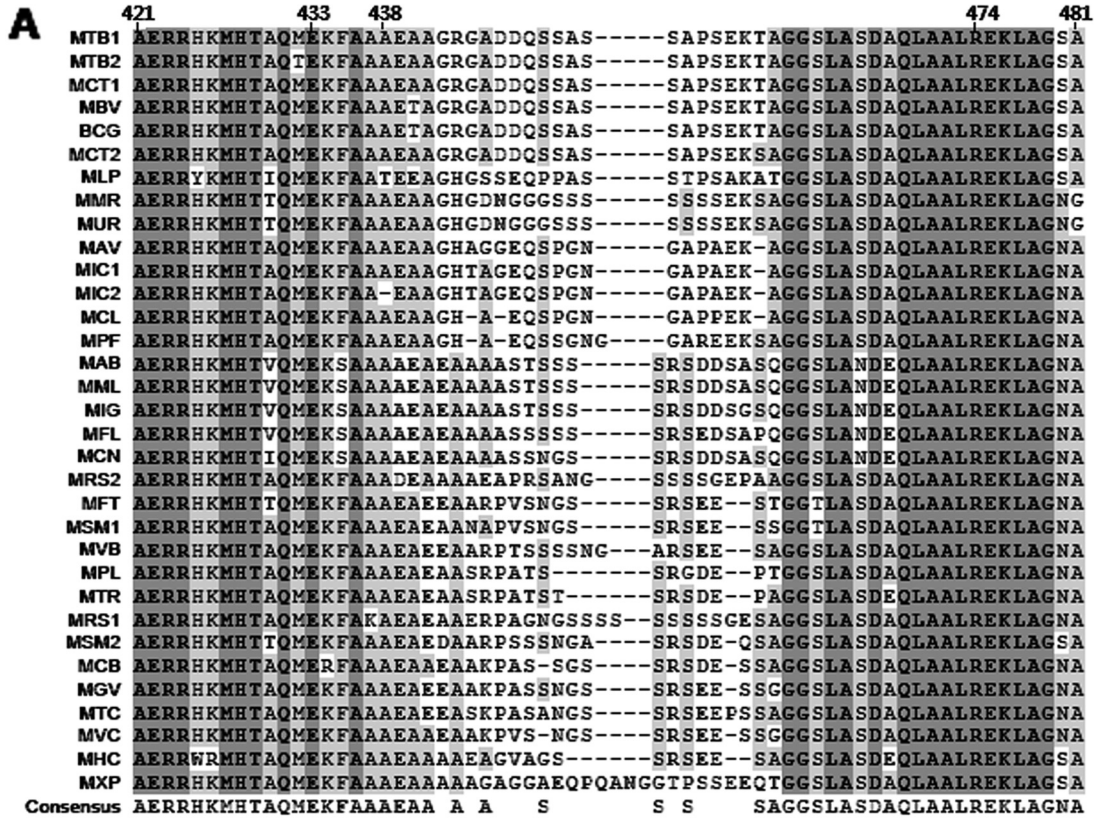
FIG 1 (A) Diagram of *pncA* gene and its surrounding genes on the *M. tuberculosis* chromosome. Af-Ar, Bf-Br, Cf-Cr, and Df-Dr were primer pairs for the amplification of the corresponding regions. (B) Electrophoresis results of the reverse transcription-PCR experiments. Lane 1, DNA markers (bp); lanes 2, 4, 6, and 8, reverse transcription-PCR products with primer pairs A, B, C, and D, respectively, using the cDNA as the templates; lanes 3, 5, 7, and 9, PCR products with primer pairs A, B, C, and D, respectively, using the reverse transcription products as the control templates without adding the reverse transcriptase.

5′-CGGTCCAGCGCTCCGTCTGC (nt 203 to 222 downstream of *rpsA*). The additional *rpsA* sequencing primers (HC-0815-3-R1, 5′-GTCCTCATTGGCTTGC; HC-0815-3-R2, 5′-CGTTGTTGCGGTTCTTGTC) and all other primers in this study were synthesized at BGI, China, where DNA sequencing was performed. Susceptibility testing was repeated for all the PZA-resistant isolates that had no *pncA*-plus-FR mutation, and their *rpsA* genes and *pncA* genes were resequenced in a double-blind form. The results were exactly the same as the first time.

Transcription of *M. tuberculosis pncA* gene in a polycistron. Only 40 nt exist between *pncA* and *Rv2044c*, and 1 nt overlapped between *pncA* and its downstream gene (*Rv2042c*), so we inferred that *pncA* might be cotranscribed with its surrounding genes in a polycistron (Fig. 1A). We thus carried out a reverse transcription-PCR (RT-PCR) experiment to prove this suggestion. Four pairs of primers were designed: Af (5′-ATCCGCATCAGCACCG)-Ar (5′-CCCTCGCAGAAGTCGTTC), Cf (5′-GGCACGCCACTGCTGAA)-Cr (5′-CGAACCCACCGGGTCTT), and Df (5′-ATGACTTTAGGCGAGGATGA)-Dr (5′-TCGAACGGCTTATTGACC) were used for amplifying the fragments spanning *Rv2045c-Rv2044c-pncA*, *pncA-Rv2042c*, and *Rv2042c-Rv2041c*, respectively; primer pair Bf (5′-CAATCGAGGCGGTGTTC)-Br (5′-CAATGATCGGTGGCAATAC) was used to amplify a fragment in *pncA* as a positive control. Total RNA was extracted from *M. tuberculosis* H37Ra or the clinical nt −11 mutant at its log phase by the RNAiso Plus kit. Then, the samples were treated with recombinant DNase I to remove the possible contaminated genomic DNA. RT-PCR was performed using the DRR036S kit. PCR products amplified with the above 4 pairs of primers using the RT-PCR products from *M. tuberculosis* H37Ra as the templates were electrophoresed. All the reagents for these experiments were from TaKaRa, China, and the corresponding procedures were carried out according to the manufacturer's instructions. The sizes of the PCR products with these 4 pairs of primers were all exactly as expected: Af-Ar, 543 bp; Bf-Br, 148 bp; Cf-Cr, 356 bp; and Df-Dr, 255 bp (Fig. 1B). The samples with templates from RT-PCR produced without adding reverse transcriptase all produced no bands, so there was no genomic DNA contamination after the treatment with recombinant DNase I (Fig. 1B). We verified that the *pncA* gene was transcribed in a polycistron in *M. tuberculosis*. So, an accurate description of the mutations at −11 and the nearby region should be that the mutation is at a putative upstream regulatory region of the *pncA* gene, but not at its promoter.

***pncA* mutations.** There was no *pncA* mutation in all 109 PZA-susceptible isolates (0%), but 44 out of 52 (84.6%) PZA-resistant isolates showed *pncA* mutations (Table 1), which showed the strong correlation between mutations in *pncA* and phenotypic resistance to PZA and supported the finding that a *pncA* mutation could cause PZA resistance in *M. tuberculosis* (2). The *pncA* gene mutation frequency in the PZA phenotypically resistant strains here is much higher than those reported previously in other provinces of China (27 to 48.1%) (13–17) but similar to those from other countries (69% to 98.8%) in which the PZA phenotypic resistance was determined by the BT960 or 460TB system (18–21). It is well known that testing for the susceptibility of PZA *in vitro* is very hard (3) because PZA only has very weak activity even in an acid-pH environment, in which even the control without PZA could not grow well. Many factors, such as the inoculum size and the metabolic state of the culture, can have a great influence on the susceptibility testing results. In the previously published papers from China, an absolute concentration method was used to determine the PZA phenotypic susceptibility. This method may have both higher false-positive and higher false-negative resistance results than the BT960/460TB systems do, which may contribute to the disparities in mutation frequencies in *pncA*, especially in the PZA phenotypically sensitive ones from different regions. This is partially supported by the finding that obvious discrepancies existed between BT960 and 460TB, even when they were manipulated by the same persons working on the same samples (7, 11). Furthermore, a 90.5% *pncA* mutation rate in the PZA-resistant strains in eastern China determined using the BT960 system was reported very recently, which was very similar to our finding here (22).

Among the 44 mutant isolates, 8 harbored deletions and 3 exhibited double point mutations, while the remaining 33 isolates had single point mutations. The mutations were randomly distributed almost along the entire 561-bp-long *pncA* gene, from nt 23 to nt 525 in the clinical isolates. This was similar to other reports (23–25) and similar to the "all *pncA* mutations" collected and analyzed in the TB Drug Resistance Database (http://www.tbdreamdb.com/PZA_Rv2043c_AllMutations.html), in which mutations were randomly distributed from nt 1 to nt 554. Only 13 of 44 (29.5%) mutants in this study located in the clustering of mutations of G132-T142 and P69-L85 found by previous investigators (25), which showed that the location of mutations in the *pncA* gene was more variable than expected before (25) and that the mutations were not clustered as in other genes associated with drug resistance, such as the *rpoB* gene (12). None of the isolates with identical *pncA* mutations were epidemiologically linked, so the PZA-resistant strains might not have been obtained from transmission. For the mutations in *pncA*, 23 types of point mutations had been reported previously (8, 9, 21, 26–29), and to our best knowledge, 10 novel types of mutations (13 strains) were first found in this study. The latter included all six types of the deletion mutations (L27, 91E, 111E, 122Q, 130-132VVG, and 131V) and four types of amino acid substitutions (D8A, S18P, F58S, and E174G+M175R double mutation) in PZases as summarized in Table 1. Such novel mutants accounted for 25% (13 of 52) of all the PZA-resistant isolates. The PCR–single-strand conformational polymorphism (PCR-SSCP) and line probe assay (LiPA) developed previously (30, 31) could detect DNA mutations quickly, but they could not tell if the mutation was synonymous or nonsynonymous. No silent mutations were found in the *pncA*


A
421
433
438
474
481
MTB1 AERRHKMHTAQMEKFAAAEAAGRGADDQSSAS-----SAPSEKTAGGSLASDAQLAALREKLAGSA
MTB2 AERRHKMHTAQTEKFAAAEAAGRGADDQSSAS-----SAPSEKTAGGSLASDAQLAALREKLAGSA
MCT1 AERRHKMHTAQMEKFAAAEAAGRGADDQSSAS-----SAPSEKTAGGSLASDAQLAALREKLAGSA
MBV AERRHKMHTAQMEKFAAAETAGRGADDQSSAS-----SAPSEKTAGGSLASDAQLAALREKLAGSA
BCG AERRHKMHTAQMEKFAAAETAGRGADDQSSAS-----SAPSEKTAGGSLASDAQLAALREKLAGSA
MCT2 AERRHKMHTAQMEKFAAAEAAGRGADDQSSAS-----SAPSEKSAGGSLASDAQLAALREKLAGSA
MLP AERRYKMHTIQMEKFAATEEAGHGSSEQPPAS-----STPSAKATGGSLASDAQLAALREKLAGSA
MMR AERRHKMHTTQMEKFAAAEAAGHGDNGGGSSS-----SSSSEKSAGGSLASDAQLAALREKLAGNG
MUR AERRHKMHTTQMEKFAAAEAAGHGDNGGGSSS-----SSSSEKSAGGSLASDAQLAALREKLAGNG
MAV AERRHKMHTAQMEKFAAAEAAGHAGGEQSPGN-----GAPAEK-AGGSLASDAQLAALREKLAGNA
MIC1 AERRHKMHTAQMEKFAAAEAAGHTAGEQSPGN-----GAPAEK-AGGSLASDAQLAALREKLAGNA
MIC2 AERRHKMHTAQMEKFAA-EAAGHTAGEQSPGN-----GAPAEK-AGGSLASDAQLAALREKLAGNA
MCL AERRHKMHTAQMEKFAAAEAAGH-A-EQSPGN-----GAPPEK-AGGSLASDAQLAALREKLAGNA
MPF AERRHKMHTAQMEKFAAAEAAGH-A-EQSSGNG----GAREEKSAGGSLASDAQLAALREKLAGNA
MAB AERRHKMHTVQMEKSAAAAEAEAAAASTSSS-----SRSDDSASQGGSLANDEQLAALREKLAGNA
MML AERRHKMHTVQMEKSAAAAEAEAAAASTSSS-----SRSDDSASQGGSLANDEQLAALREKLAGNA
MIG AERRHKMHTVQMEKSAAAAEAEAAAASTSSS-----SRSDDSGSQGGSLANDEQLAALREKLAGNA
MFL AERRHKMHTVQMEKSAAAAEAEAAAASSSSS-----SRSEDSAPQGGSLANDEQLAALREKLAGNA
MCN AERRHKMHTIQMEKSAAAAEAEAAAASSNGS-----SRSDDSASQGGSLANDEQLAALREKLAGNA
MRS2 AERRHKMHTAQMEKFAAADEAAAAEAPRSANG----SSSSGEPAAGGSLASDAQLAALREKLAGNA
MFT AERRHKMHTTQMEKFAAAEAEEAARPVSNGS-----SRSEE--STGGTLASDAQLAALREKLAGNA
MSM1 AERRHKMHTAQMEKFAAAEAEAANAPVSNGS-----SRSEE--SSGGTLASDAQLAALREKLAGNA
MVB AERRHKMHTAQMEKFAAAEAEEAARPTSSSSNG---ARSEE--SAGGSLASDAQLAALREKLAGNA
MPL AERRHKMHTAQMEKFAAAEAEAASRPATS-------SRGDE--PTGGSLASDAQLAALREKLAGNA
MTR AERRHKMHTAQMEKFAAAEAEAASRPATST------SRSDE--PAGGSLASDEQLAALREKLAGNA
MRS1 AERRHKMHTAQMEKFAKAEAEAAERPAGNGSSSS--SSSSSGESAGGSLASDAQLAALREKLAGNA
MSM2 AERRHKMHTTQMEKFAAAEAEDAARPSSSNGA----SRSDE-QSAGGSLASDAQLAALREKLAGSA
MCB AERRHKMHTAQMERFAAAEAAEAAKPAS-SGS----SRSDE-SSAGGSLASDAQLAALREKLAGNA
MGV AERRHKMHTAQMEKFAAAEAEEAAKPASSNGS----SRSEE-SSGGGSLASDAQLAALREKLAGNA
MTC AERRHKMHTAQMEKFAAAEAEEASKPASANGS----SRSEEPSSAGGSLASDAQLAALREKLAGNA
MVC AERRHKMHTAQMEKFAAAEAAEAAKPVS-NGS----SRSEE-SSGGGSLASDAQLAALREKLAGNA
MHC AERRWRMHTAQMEKFAAAEAAAAEAGVAGS------SRSEE--SAGGSLASDEQLAALREKLAGSA
MXP AERRHKMHTAQMEKFAAAEAAAAAGAGGAEQPQANGGTPSSEEQTGGSLASDAQLAALREKLAGSA
Consensus AERRHKMHTAQMEKFAAAEAA A A S S S SAGGSLASDAQLAALREKLAGNA
B
MTB complex and MBV
slow growth
fast growth except for MCT
MTB1 t
MTB2 n
MCT1?
MBV n
BCG n
MCT2?
MLP ?
MMR n
MUR n
MAV n
MIC1 t
MIC2 n
MCL t
MPF n
MAB ?
MML n
MIG n
MFL ?
MCN ?
MRS2 n
MFT t
MSM1 n
MVB t
MPL n
MTR t
MRS1 ?
MSM2 n
MCB n
MGV n
MTC n
MVC n
MHC t
MXP ?

gene in our study, which indicated that PCR-SSCP and LiPA might be used to detect PZA resistance quickly with a high success rate by detecting the *pncA* mutation in southern China.

There were still 8 PZA-resistant *M. tuberculosis* isolates that had no mutation in the *pncA* gene. Four of them did not harbor any mutation, including FR; three of them showed an *rpsA* single point mutation (see below); and one other strain had a point mutation in the putative regulatory region (A-11G) of the *pncA* gene, which was a very common mutation found in isolates from other countries (10, 23–25). To explore the possible reason why this can affect PZA resistance, we checked the transcription levels of *pncA* genes in this clinical isolate and the laboratory control *M. tuberculosis* H37Ra strain. RT-PCR products were prepared as described in the section demonstrating transcription of *pncA* gene in a polycistron. Real-time PCR with SYBR green dye was used for detecting the initial concentration of *pncA* mRNA with *sigA* as inner reference. The primers used were Bf-Br for *pncA* and sigAF (5′-CTCGACGCTGAACCAGACCT)-sigAR(5′-AGGTCTTCGT GGTCTTCGTC) for *sigA* (32). Levene's test for equality of variances and the *t* test for equality of means were calculated using the software SPSS13.0 (SPSS Inc., Chicago, IL). This A-11G mutation may not affect *pncA* expression at the transcription level, because it can still be transcribed in a polycistron with flanking genes in the clinical −11 mutant (data not shown) and its expression level showed no difference from that in *M. tuberculosis* H37Ra. This mutation, however, may disturb the translation of the *pncA* gene by affecting the ribosome movement, because the nt −11 mutation is close to the ribosome binding site.

These new findings displayed the regional disparities in *pncA* mutations and the highly diverse patterns in *pncA* mutations (4, 5, 22), which partially make molecular detection of PZA susceptibility difficult to be adopted worldwide. Therefore, systematic establishment of the relationship between the mutation characterization of PZA resistance-related genes and the PZA resistance phenotype is highly needed for development and application of rapid molecular assays.

Of multidrug-resistant (MDR) strains, 68.4% (39/57) were PZA resistant, but only 12.5% (13/104) of non-MDR strains harbored such resistance. The former was significantly higher than the latter ($P \ll 0.01$), which implied that the MDR strains were more likely to be PZA resistant than the drug-sensitive ones. This result is similar to those in South Africa and Thailand (4, 27). The importance of PZA in treating MDR-TB has been proved recently in a murine model in that all PZA-containing regimens tested showed better activities than those corresponding regimens without PZA when combined with second-line drugs (33). All these findings emphasized the importance of rapid detection of PZA resistance in controlling MDR-TB.

***rpsA* mutations.** Three PZA-resistant clinical isolates with wild-type *pncA* (*pncA*^{WT}) harbored single mutations R474L, R474W, and E433D, respectively. One PZA-susceptible clinical isolate also contained one Q162R single mutation. The frequency of *rpsA* mutations showed no significant statistical difference ($P = 0.099$) between PZA-resistant and PZA-susceptible isolates, which may be because of the small sample size for PZA-resistant isolates. The low frequency of *rpsA* mutations proved again that the *rpsA* mutation was not the main mechanism of PZA resistance in *M. tuberculosis*, though it can affect the susceptibility to PZA (6). Alexander et al. (7) could not find any *rpsA* mutations in PZA-resistant isolates, but one RpsA protein mutation (A364G) in 13 PZA-sensitive strains was discovered. In addition, the *rpsA* gene plus FR is long and not very convenient for amplification and sequencing, so the authors concluded that analysis of the *rpsA* gene had no role in the detection of pyrazinamide resistance (7). This suggestion was further supported by Simons and colleagues, who reported that only 1 of 5 PZA-resistant isolates with *pncA*^{WT} had an RpsA (V260I) mutation (34) and that this so-called mutation was even questionable based on the disparity of the reported mutated codon and the amino acid that it encoded. However, in the current study, 3 of 7 phenotypically PZA^{r} but genotypically *pncA*^{WT} + FR^{WT} isolates had *rpsA* mutations which were all clustered in the C terminus of RpsA protein (3′ of *rpsA* gene). This was concordant with the findings by Shi et al. (6), who reported that deletion of amino acid 438 in the C terminus of RpsA (Fig. 2A) could cause PZA resistance in an *M. tuberculosis* clinical isolate with *pncA*^{WT}. We noticed that *rpsA* mutations in the PZA-sensitive strains found until now were mostly away from the C-terminal end of RpsA. We then made an alignment of C-terminal amino acid sequences of ribosomal protein S1 (RpsA) from 33 strains of 28 mycobacterial species (Fig. 2A) and found the same phenomenon as described by Shi et al. (6) that, unlike other parts, C-terminal ends of RpsA proteins were highly variable. At the same time, we drew a phylogenetic tree according to the C-terminal amino acid sequences of RpsAs from these mycobacterial species (Fig. 2B) using Vector NTI Suite 7.0 (Invitrogen, USA). It is similar to the phylogenetic trees based on either the nearly complete 16S rRNA gene sequence or the concatenated hypervariable sequences of 16S rRNA and *rpoB* and *hsp65* genes (35). Two different species, for example, *Mycobacterium marinum* and *Mycobacterium ulcerans*, have RpsAs that are identical not only in the C

FIG 2 (A) Alignment of C-terminal amino acid sequences of ribosomal protein S1 (RpsA) from different mycobacterial species. Species are abbreviated as follows with GenBank accession numbers indicated in parentheses: MTB1, *M. tuberculosis* H37Rv (NP_216146.1); MTB2, *M. tuberculosis* CTRI-2 (YP_005916702.1); MCT1, *M. canettii* CIPT 140070008 (YP_007287527.1); MBV, *M. bovis* AF2122/97 (NP_855309); BCG, *M. bovis* BCG Pasteur 1173P2 (NP_855309.1); MCT2, *M. canettii* CIPT 140070010 (YP_007264352.1); MLP, *M. leprae* TN (NP_301983.1); MMR, *M. marinum* M (YP_905584.1); MUR, *M. ulcerans* Agy99 (YP_905584.1); MAV, *M. avium* subsp. *paratuberculosis* K-10 (NP_960259); MIC1, *M. intracellulare* 13950 (YP_005338525.1); MIC2, *M. intracellulare* (WP_009954955.1); MCL, *M. colombiense* CECT 3035 (WP_007776137.1); MPF, *M. parascrofulaceum* (WP_007169711.1); MAB, *M. abscessus* (YP_001703031.1); MML, *M. massiliense* GO 06 (YP_006520985.1); MIG, *M. immunogenum* MTCC 9506 (YP_006730099.1); MFL, *M. franklinii* (AEI54876.1); MCN, *M. chelonae* (AEI54878.1); MRS2, *M. rhodesiae* NBB3 (YP_005002313.1); MFT, *M. fortuitum* (WP_003884756); MSM1, *M. smegmatis* MC^{2} 155 (YP_888124.1); MVB, *M. vanbaalenii* PYR-1 (YP_954159.1); MPL, *M. phlei* (WP_003884756.1); MTR, *M. thermoresistibile* (WP_003923841.1); MRS1, *M. rhodesiae* (WP_005149060); MSM2, *M. smegmatis* JS623 (YP_007292760.1); MCB, *M. chubuense* NBB4 (YP_006453027.1); MGV, *M. gilvum* PYR-GC (YP_001134823.1); MTC, *M. tusciae* (WP_006246193.1); MVC, *M. vaccae* (WP_003931928.1); MHC, *M. hassiacum* (WP_005627902.1); and MXP, *M. xenopi* (WP_003920384.1). Amino acid numbers are indicated on the top according to the order of RpsA from *M. tuberculosis*. The identical amino acids from all species are highlighted in dark gray, and the similar resides are highlighted in light gray. (B) The phylogenetic tree drawn according to the C-terminal amino acid sequences of RpsA from the above mycobacterial species. t, type strain; n, nontype strain; ?, not clearly indicated in web sources as type strain or nontype strain (http://www.bacterio.net/ [updated and expanded on 9 July 2013] and http://www.atcc.org).

terminus, while different strains of the same species, for example, *Mycobacterium smegmatis* MC^2 155 and *M. smegmatis* JS263, or *Mycobacterium rhodesiae* NBB3 and another strain, have very different RpsA C-terminal ends but are even closer to other species in the phylogenetic tree. This agrees with the fact that the genus *Mycobacterium* has very limited interspecies genetic variability (35). In the current study, we found that all 3 mutated sites (E433, A438, and R474) were highly conserved among all the *Mycobacterium* species. It is interesting that the A438 deletion that took place at AAA (436 to 438) had the same effect as did A436, which seemed more conserved than A438 (Fig. 2A). If E433 and R474 mutations did cause PZA resistance as the A438 deletion did, sequencing the C-terminal end of the *rpsA* gene product in isolates with $pncA^{WT}$ may be needful for more rapid and accurate detection of PZA susceptibility, especially in southern China, though the role of this region is modest. Of phenotypically PZA-resistant isolates, 86.5% (45 of 52) could be predicted by sequencing only *pncA* plus FR and 92.3% (48 of 52) could be predicted if sequencing the 3′ end of *rpsA* was added in our study. In the meantime, it is needful to point out that not all mutations at the C-terminal ends of RpsA may cause PZA resistance. For example, *M. bovis* BCG has a nonfunctional *pncA* and an *rpsA* gene encoding mutated RpsA (A440T) and is naturally resistant to PZA. *M. smegmatis pncA* and *pzaA* both encode proteins with PZase activity and can transform PZA into POA. Introduction of *M. smegmatis pncA* or *pzaA* into *M. bovis* BCG can restore its sensitivity to PZA (36). Therefore, the resistance of *M. bovis* BCG to PZA is possibly because of the *pncA* mutation but not the *rpsA* mutation, and so RpsA (A440T) may not play a role in PZA resistance. Another strain, *M. tuberculosis* CTRI-2, contained a mutated RpsA (M432T) at its C-terminal end and was PZA sensitive (37). In this regard, therefore, the actual role of E433 and R474 mutations of RpsA found here in PZA resistance needs to be verified further.

In conclusion, the *pncA* mutations were randomly dispersed along with the entire gene and observed at a high ratio in PZA-resistant clinical isolates in southern China. Of clinical isolates with *pncA*-plus-FR mutations, 28.9% (13 of 45) were new, which displayed the high diversity of mutations in the *pncA* gene in different regions. It was first shown here that the *pncA* gene was transcribed in a polycistron and that the A-11G mutation of *pncA* did not affect its expression at transcription level. The 3′ end of the *rpsA* gene, if not the whole *rpsA* gene plus FR, should be added as the target for a two-step approach, where only $pncA^{WT}$ isolates from southern China are subjected to sequencing of this fragment. However, the influence of these *rpsA* mutations on PZA susceptibility needs to be proved further in the future, as well as that of many *pncA* mutations. For more accurate results, phenotypic PZA susceptibility testing, especially using the BT960 system, is needed for *M. tuberculosis*, and in particular MDR *M. tuberculosis* clinical isolates with no known gene mutations.

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