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Systematic Analysis of Pyrazinamide-Resistant Spontaneous Mutants and Clinical Isolates of *Mycobacterium tuberculosis*

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Pyrazinamide (PZA) is a first-line antitubercular drug known for its activity against persistent *Mycobacterium tuberculosis* bacilli. We set out to systematically determine the PZA susceptibility profiles and mutations in the pyrazinamidase (*pncA*) gene of a collection of multidrug-resistant tuberculosis (MDR-TB) clinical isolates and PZA-resistant (PZA^r) spontaneous mutants. The frequency of acquired resistance to PZA was determined to be 10^{-5} bacilli *in vitro*. Selection at a lower concentration of PZA yielded a significantly larger number of spontaneous mutants. The methodical approach employed allowed for determination of the frequency of the PZA^r phenotype correlated with mutations in the *pncA* gene, which was 87.5% for the laboratory-selected spontaneous mutants examined in this study. As elucidated by structural analysis, most of the identified mutations were foreseen to affect protein activity through either alteration of an active site residue or destabilization of protein structure, indicating some preferential mutation site rather than random scattering. Twelve percent of the PZA^r mutants did not have a *pncA* mutation, strongly indicating the presence of at least one other mechanism(s) of PZA^r.

Pyrazinamide (PZA) was identified based on its structural activity relationship to nicotinamide, known for its antitubercular properties (28). PZA has the distinction of being identified directly *in vivo*, first in *Mycobacterium tuberculosis*-infected mice and guinea pigs and later in clinical cases (15, 33, 62). The *in vitro* activity of PZA was demonstrated subsequently, using media adjusted to a pH of 5.0 or 5.5 (36, 51, 66) because PZA has no detectable inhibitory activity against replicating bacilli at neutral pH.

PZA differs from most antitubercular drugs in having sterilizing activity on semidormant (persistent) *M. tuberculosis* bacilli. Its combination with rifampin and isoniazid in the standard tuberculosis (TB) treatment has reduced the duration of therapy to 6 months instead of the previous 9 to 12 months for otherwise healthy patients (65). PZA, like isoniazid and ethionamide, is a prodrug which must be converted into its active form for activity (12, 64). This enzymatic activation of PZA is catalyzed by the pyrazinamidase (PZase) encoded by the *pncA* gene in *M. tuberculosis* (54), and the active metabolite is pyrazinoic acid (POA). Interestingly, POA is active against PZA-resistant (PZA^r) isolates *in vitro* but displays no *in vivo* activity (19). The overall mode of action of PZA is rather unusual and remains poorly understood. No specific target has yet been identified for either PZA or POA, although a recent report indicating a possible interference in the *trans*-translation pathway shows a potential promising mechanism of action (57). The fatty acid synthase (FAS I) was proposed and challenged as a possible target of PZA and analogs (6, 68). This mechanism of action proved to be valid *in vitro* but not *in vivo*. Although the final target of activated PZA has yet to be found, a model for the mechanism of action has been proposed: PZA crosses the mycobacterial cell wall by passive diffusion and is converted by the PZase in the cytoplasm into POA, which is then released through either passive diffusion or a weak efflux pump (65). If the extracellular medium presents an acidic pH, the acidic POA form is protonated in part into an uncharged HPOA form, which is easily reabsorbed by the cell and redissociated intracellularly, releasing H^+ protons into the cytoplasm. Because the efflux mechanism of POA is inefficient or defective in *M. tuberculosis*, HPOA accumulates in the cytoplasm of the bacterium and causes cellular damage, resulting in cell death due to intracellular acidification. More recently, Shi and colleagues proposed that HPOA inhibits ribosomal protein S1 (RpsA). Inhibition of RpsA required for the *trans*-translation pathway leads to a decrease in stalled ribosome rescue and possibly an increase in the accumulation of toxic peptide waste (57). This model could explain the atypical characteristics of pyrazinamide, particularly its activity only at an acidic pH.

The PZase is a small protein of 186 amino acids that is encoded by the *pncA* gene. Mutations of the *pncA* gene or its putative promoter region are associated with most reported cases of PZA resistance in *M. tuberculosis* (54). Multiple mutations (substitutions, deletions, and insertions) have been described for this gene-promoter region (49, 52, 54, 59). A PZase-deficient strain can no longer metabolize the prodrug, resulting in PZA^r (54), an observation first reported by Konno and coworkers in the early 1960s (25); the relationship was confirmed through quantification of PZase activity (8). As such, *Mycobacterium bovis* strains are intrinsically resistant to PZA due to a distinctive phylogenetic single nucleotide polymorphism (SNP) (^{57}His → Asp [C169G]) of *pncA* resulting in an inactive PncA protein and hence in PZA^r (54). The number of reported

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pncA mutants associated with PZA^r varies from 70 to 100% (9, 17, 21, 22, 29, 58, 59), taking into account that PZA susceptibility testing has proven challenging (16).

Here we investigated the frequency of mutations in the *pncA* gene associated with PZA^r in a collection of well-characterized *M. tuberculosis* clinical isolates comprising a 14-year complete capture of multidrug-resistant (MDR) isolates and PZA^r spontaneous mutants. The correlations between PncA mutations, drug susceptibility, and structural analysis of the PncA protein were determined for selected PZA^r mutants. Most notably, the frequency of spontaneous acquired resistance to PZA was determined and found to be concentration dependent.

MATERIALS AND METHODS

***M. tuberculosis* clinical isolates.** One hundred thirty-eight of 174 strains were selected from the MDR-TB collection maintained at the Tuberculosis & Mycobacteria Centre of the Scientific Institute of Public Health, Belgium. This collection of 174 isolates comprises the first isolate from each MDR-TB patient identified in Belgium between 1994 and 2008. Twenty-three isolates were eliminated from the study because of poor growth, contamination, or nonviability (unable to confirm drug susceptibility testing [DST] results). A further 13 isolates with the PZA^r phenotype but carrying wild-type *pncA* were also eliminated from the study, as the DST results could not be reconfirmed due to accidental elimination of the isolates. The remaining 138 samples were included in the study regardless of PZA susceptibility profile. All were genotyped by spoligotyping and mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing (24 loci) in order to establish their genetic diversity (unrelated clinical isolates) or relatedness (clustered clinical isolates).

The resistance profiles of the clinical isolates for first- and second-line antibiotics were determined on solid medium by the proportion method of Canetti et al. (10) or in liquid medium in a radiometric Bactec 460 TB system (Becton, Dickinson Microbiology Systems, Cockeysville, MD) (since 2000) according to the manufacturer's instructions and the methods of Pfyffer et al. (47). Since 2005, a Bactec MGIT960 system has been used for DST of isoniazid, rifampin, and ethambutol, and it was expanded to PZA susceptibility testing in 2007 with the commercial availability of a PZA drug kit (Becton, Dickinson Microbiology Systems, Cockeysville, MD). Prior to 2007, all DST for PZA was performed by the method of Canetti et al. (10). DST confirmation of phenotypes was carried out by use of a Bactec MGIT960 PZA drug kit. MIC determination was performed by using this PZA drug kit, with the PZA provided in the kit diluted in a 1/4 volume (400 μg/ml) and subjected to 2-fold dilutions. The PZA susceptibilities of 15 samples (1 clinical isolate and 14 spontaneous mutants) displaying the PZA^r phenotype while carrying wild-type *pncA* and *rpsA* were reconfirmed by the method described above.

Selection of spontaneous PZA^r *M. tuberculosis* mutants. Spontaneous mutants resistant to PZA were selected on 7H11 plates containing either 100 or 500 μg/ml of PZA (Sigma-Aldrich) at pH 6.0. The protocol for selection of spontaneous mutants was adapted from the work of Luria and Delbruck, Mathys et al., and Morlock et al. (32, 35, 40). Briefly, a single colony of strain CDC1551 was cultured under standard conditions in 7H9 medium. At 3 weeks, the culture density was determined, adjusted to an optical density at 600 nm (OD_{600}) of ~0.02 (approximately 1×10^5 to 1×10^6 CFU/ml), and divided into 40 individual culture flasks (25 ml in each) in the absence of PZA or any other selective pressure. At day 32 postinoculation, the 40 cultures were adjusted to an OD_{600} of 1, and the cell density for 4 random samples was confirmed by serial dilution and plating for enumeration. All cultures were plated on 7H11 plates containing either 100 or 500 μg/ml PZA (pH 6.0), at concentrations of 10^7, 10^5, and 10^3 CFU/ml. Resistant colonies were picked and subcultured on 7H11 plates, pH 6.0, containing the same concentration used for the selection process, followed by stocking and DNA extraction. A maximum of 3 colonies per plate were picked and labeled according to the plate number for this study. A total of 112 colonies were sequenced. Statistical analysis was carried out using GraphPad Prism software. *P* values of <0.05 were considered significant.

Sequencing of drug target regions. The sense and antisense strands of the *pncA* gene and the corresponding 100 nucleotides (nt) upstream were sequenced for all isolates, using the primers P6 (5′-$^{-101}$CGCTTGCGGCGAGCGCTCCA^{-81}-3′) and P1 (5′-$^{+36}$GGTCATGTTCGCGATCGT^{+56}-3′) (adapted from reference 54). Nucleotide sequences were analyzed by using Sequencher software (Gene Codes Corporation).

Structural analysis and evaluation of mutation effects. The X-ray structure of the *M. tuberculosis* PncA enzyme was recently determined at 2.2-Å resolution (46) (Protein Data Bank entry 3PL1). This three-dimensional (3D) structure was used to estimate the impact of selected point mutations on structure and/or function of the enzyme. The prediction of changes in thermal protein stability for each observed PncA mutant was obtained from the CUPSAT (44) (http://cupsat.tu-bs.de) and PoPMuSiC (13) (http://babylone.ulb.ac.be/popmusic/) websites. Both programs evaluate the change in free energy of the protein folding-unfolding process upon mutations, i.e., the $\Delta\Delta G$. A positive or negative $\Delta\Delta G$ value indicates that the mutation is thermodynamically stabilizing or destabilizing, respectively, while the magnitude of $\Delta\Delta G$ indicates the extent of the alteration. Since mutations of a buried residue generally have more drastic consequences on protein structure, the solvent accessibility of mutated residues was calculated using the CUPSAT program.

RESULTS

Phenotypic resistance to PZA. Among the 138 clinical MDR isolates evaluated for PZA susceptibility, 60 proved to be PZA resistant and 78 were found to be susceptible. PZA susceptibility testing by use of a Bactec MGIT960 PZA drug kit was used to repeat DST for all isolates with borderline resistance or unexpected phenotypic-genotypic or clustering correlations. Since the MDR collection comprised 80.6% of all positive cultures identified in Belgium in the period 1994 to 2008 and reached 95.5% in 2003 to 2008, this 43% PZA^r observation for the 138 isolates is clinically significant.

Frequency of spontaneous mutations conferring pyrazinamide resistance on *M. tuberculosis*. Samples originating from 40 individual drug-free CDC1551 cultures were plated in parallel on 7H11 plates (adjusted to a pH of 6.0) containing 100 or 500 μg/ml of PZA in order to select for PZA^r spontaneous mutants. The frequencies of spontaneous mutants with resistance to PZA determined for all 40 flasks were found to be highly consistent, at ~1 and 1.5 mutants per 10^5 bacilli for selection on 100 and 500 μg/ml PZA, respectively. This difference in mutation frequency is statistically significant ($P < 0.001$) (see Table S1 in the supplemental material).

Polymorphism in the *pncA* gene and its correlation with PncA structure and activity. The *pncA* gene and corresponding ~100 nucleotides upstream were sequenced for the 151 MDR-TB clinical isolates and 112 PZA^r spontaneous mutants selected *in vitro* (43 selected on 100 μg/ml PZA and 69 selected on 500 μg/ml PZA). In total, the *pncA* gene and putative promoter region were sequenced for 263 samples. Thirteen of the 151 clinical samples had to be removed from the study postsequencing, as the original cultures were unavailable for DST reconfirmation.

The genetic analysis showed that 98.3% (59/60 isolates) of the Belgian MDR clinical isolates with the PZA^r phenotype presented a mutation in the *pncA* gene. We found that 1.7% (1/60 isolates) of the PZA^r MDR isolates carried wild-type *pncA* and its flanking region. A total (PZA^r and PZA^s) of 41 different amino acid changes, 3 protein truncations, and 5 frameshifts were observed, including 8 mutations

TABLE 1 Clustered clinical isolates sharing the same genotypes and *pncA* mutations[d]

Strain ID	PZA susceptibility[a]	nt polymorphism	aa change[b]	MIRU type	Spoligotype	Family[c]	SIT group[c]	Cluster
01MY0495	R	G436A	A146T	335247232234425113323832	034777777420771	ND	ND	1
06MY0899	R	G436A	A146T	335247232234425113323832	034777777420771	ND	ND	1
04MY1499	R	A146C	D49A	244233352644425173353723	000000000003771	Beijing	SIT1	2
08MY1099	R	A146C	D49A	244233352644425173353723	000000000003771	Beijing	SIT1	2
95MY0609	R	Del C530	FS	234125132234425113333732	777777404760731	LAM7_TUR	SIT1261	3
96MY0658	R	Del C530	FS	234125132234425113333732	777777404760731	LAM7_TUR	SIT1261	3
01MY1170	R	A128C	H43P	225233332244225153343822	777777777760731	T2	SIT52	4
06MY0371	R	A128C	H43P	225233332244225153343822	777777777760731	T2	SIT52	4
01MY0507	R	T515G	L172R	223263342334425143233613	777777777760771	T1	SIT53	5
03MY0423	R	T515G	L172R	223263342334425143233613	777777777760771	T1	SIT53	5
06MY0096	R	T515G	L172R	223263342334425143233613	777777777760771	T1	SIT53	5
03MY1173	R	G525A	M175I	225233332244225153343822	777777777760731	T2	SIT52	6
05MY1333	S	G525A	M175I	225233332244225153343822	777777777760731	T2	SIT52	6

[a] R, resistant; S, susceptible.
[b] FS, frameshift. Phenotypic determination was reconfirmed in 3 independent experiments for the M175I substitution.
[c] SIT, spoligo-international type; ND, not determined.
[d] There was an epidemiological link within every cluster identified.

previously not reported in the literature: Asp8Ala, Phe13Leu, Tyr64Ser, Glu107stop, Ala143Pro, Leu172Arg, and frameshifts starting in codons 55 and 82. For clarity and analytical purposes, samples were grouped according to mutation type and fingerprint cluster (Table 1), shared mutation type and strain diversity (Table 2), or susceptible isolates carrying mutations within *pncA* (Table 3). In this study, all cases with a shared mutation type also grouped genotypically (Table 1). Demographic data showed that the isolates in clusters were obtained from patients from the same country of origin (or residing there) (clusters 1-Georgia, 2-Chechnya, 4-Rwanda, 5-Belgium, and 6-Rwanda) or from patients living together or in proximity of each other (clusters 1, 2, and 5). It is noteworthy that one cluster of two isolates shared the same SNPs within *pncA* yet one was resistant and the other was susceptible to PZA. The implicated mutation, Met175Ile, has been described previously in the literature on PZA^r strains. The phenotypes were confirmed in two additional independent experiments with a Bactec MGIT960 PZA drug kit. Finally, 5 different *pncA* SNPs were identified in 7 susceptible isolates, with 4 of these SNPs previously correlated with drug resistance in the literature (Table 3). Given the incongruence in results for these isolates, the sequence and susceptibility profile were retested by use of the Bactec MGIT960 system and 100 μg/ml PZA and confirmed the original susceptibility result. In addition, the MIC was determined by use of 2-fold dilutions of PZA (12.5 μg to 200 μg/ml) and the Bactec MGIT960 system and was found to be >200 μg/ml or between 100 and 200 μg/ml. Three of these mutants carry a Cys14Gly substitution which was also observed in one PZA^r isolate.

In order to better understand the phenotypic-genotypic correlation of the isolates, CDC1551-derived PZA^r spontaneous mutants were selected at 100 and 500 μg/ml PZA in 7H11 medium

TABLE 2 Strains with distinct genotypes but common *pncA* mutations

Strain ID	PZA susceptibility	nt polymorphism	aa change	MIRU type	Spoligotype	Family	SIT group	Mutation cluster
02MY1010	R	A188C	D63A	223273342334425143233613	777777777760771	T1	SIT53	1
97MY0936	R	A188G	D63G	224233331334225153332422	777777743760771	LAM10	SIT61	1
08MY1582	R	G22A	D8N	22423332243222614333324?2	777777777760771	T1	SIT53	2
96MY0316	R	G22A	D8N	24324333223442512333383 2	700076777760771	X3	SIT92	2
97MY0555	R	G22T	D8Y	22425312233422515333552 2	777777770000771	U	SIT602	2
95MY0036	R	C211A	H71N	22422342242422514333342 2	757777037760771	ND	ND	3
02MY0668	R	C211T	H71Y	22323533253442525133443 2	777777777720771	Haarlem3	SIT50	3
03MY0478	R	C211T	H71Y	24423336264442515335362 3	000000000003771	Beijing	SIT1	3
03MY0092	R	C244G	H82D	22421413232411615253272 2	775777606060731	LAM11_ZWE	SIT1549	4
08MY1150	R	A245G	H82R	24423335264442515335382 3	000000000003371	Beijing	SIT265	4
01MY0507	R	T515G	L172R	223263342334425143233613	777777777760771	T1	SIT53	5[a]
02MY0982	R	T515G	L172R	23324533243442215333373 2	777777777720771	Haarlem3	SIT50	5
96MY0800	R	A29C	Q10P	22324533263442515323353 2	777741777720671	ND	ND	6
99MY0820	R	C28T	Q10Stop	225233332244225153343822	777777777760731	T2	SIT52	6
01MY0222	R	C260T	T87M	22422332222422514332442 2	777777777760731	T2	SIT52	7
99MY1429	R	C260T	T87M	22422332222422514332441 2	777777777760771	T1	SIT53	7
02MY1134	R	T464G	V155G	24421223242412616333222 2	601775607760771	LAM9	SIT1545	8
08MY1426	R	T464G	V155G	24323335264442517335372 3	000000000003771	Beijing	SIT1	8
05MY1333	S	G525A	M175I	225233332244225153343822	777777777760731	T2	SIT52	9[a]
06MY0999	R	A523G	M175V	24423335264442515335382 3	000000000003371	Beijing	SIT265	9

[a] Cluster 5, family T1, and cluster 9, family T2, include 3 and 2 samples, respectively.

TABLE 3 PZA-susceptible clinical isolates carrying mutations within the *pncA* gene

Strain ID	nt polymorphism	aa change	MIRU type	Spoligotype	Family	SIT group[a]
02MY1182	T40G	C14G	225233332234225154343723	777777777760731	T2	SIT52
09MY0190	T40G	C14G	234243162623235152332522	777777777760731	T2	SIT52
09MY0191	T40G	C14G	234243162623235152332522	777777777760731	T2	SIT52
06MY0826	A191C	Y64S	244233352644425173353723	000000000003771	Beijing	SIT1
05MY1333	G525A	M175I	225233332244225153343822	777777777760731	T2	SIT52
08MY1755	A403C	T135P	224242162?4225153332522	777777777760731	T2	SIT52
01MY1015	G427A	A143T	223245332434415153233732	777737777720171	ND	ND

[a] SIT, spoligo-international type.

adjusted to pH 6.0. Globally, 87.5% (98/112 mutants) of the PZA^r mutants presented a *pncA* mutation, but when the mutants were segregated into those selected at 100 and 500 μg/ml PZA, we found that only 67% (29/43 mutants) of the former were mutated, whereas all of the strains (69/69 mutants) selected on 500 μg/ml PZA displayed a *pncA* or putative promoter mutation (see Table S2 in the supplemental material). The MICs of the 14 *in vitro* mutants without any mutation in the *pncA* region were determined by use of 2-fold dilutions of PZA (50 to 400 μg/ml) and the Bactec MGIT960 method, and all were found to be >400 μg/ml. All 14 spontaneous mutants were subjected to whole-genome sequencing and found to carry a single SNP each. Most isolates were found to carry different mutations, but they were all within the same operon. These data will be communicated at a later date.

Twelve and 24 amino acid alterations were found uniquely in the 100- and 500-μg/ml PZA selections, respectively, while 5 were shared between the 100- and 500-μg/ml selections. Comparison of amino acid mutations identified in clinical isolates with those determined for the spontaneous mutants showed 9 polymorphisms and 17 codons in common. No polymorphisms were identified in putative promoter regions of the clinical isolates; however, a Del^{-4}A mutation and $^{-6}$G → C and $^{-7}$T → C substitutions were found in the spontaneous mutants selected at 500 μg/ml of PZA. The MICs for 2 of the isolates (Del^{-4}A and Del^{-7}C) carrying alterations within the putative promoter region were determined, and both were found to be resistant to >400 μg/ml of PZA with the MGIT960 system. Reverse transcription-PCR (RT-PCR) to evaluate the expression of the gene was not performed.

In spontaneous mutants, a mutation in codon 1 was the most frequently encountered mutation. The Met1Ile mutation was present in 10% (3/29 mutants) of the mutants selected on 100 μg/ml PZA and presenting a *pncA* mutation. Among the 69 mutants selected on 500 μg/ml PZA, 10 Met1Ile and 2 Met1Thr changes were encountered (17% of isolates). The second most frequently encountered mutated codon in the PZA^r spontaneous mutants was codon 8, represented by 7 isolates (4 Asp8Glu, 1 Asp8Asn, 1 Asp8Tyr, and 1 frameshift mutant) of the 69 mutants selected on 500 μg/ml PZA (10% of isolates).

We analyzed the recently published high-resolution structure of the *Mycobacterium tuberculosis* PncA protein (46) coupled with predictions of protein stability using both the PoPMuSiC (13) and CUPSAT (44) programs. Table S2 in the supplemental material lists the observed mutation types in this study and the corresponding stabilizing/destabilizing effects on the PncA structure, while Fig. 1 illustrates the dispersion and positions of the mutations in a three-dimensional model of the PZase. The data indicate that mutations of amino acid residues associated with either iron or substrate binding or the catalytic active site are most directly implicated in resistance. Most of the mutations in Table S2 affect buried amino acids and were determined to be energetically destabilizing, suggesting a reduction of protein stability and thereby a diminished or depleted PZase activity. Substitutions at codons 46 and 64 (^{46}Ala → Val and ^{64}Tyr → Ser) were predicted to be stabilizing, while substitutions at positions 82, 87, 103, and 175 (^{82}His → Asp, ^{82}His → Arg, ^{87}Thr → Met, ^{103}Tyr → His, and ^{175}Met → Ile) were found to be neutral. All except for the ^{46}Ala → Val and ^{103}Tyr → His substitutions were found in clinical isolates. The ^{175}Met → Ile mutation, which is predicted to be neutral in the 3D model, was also found in both PZA-resistant and -susceptible strains in this study (Tables 1 and 3).

DISCUSSION

Pyrazinamide is an essential first-line antitubercular drug used in TB treatment, yet much remains to be understood in regard to its biology and mechanisms of action. It has the distinct and unique characteristic that it was first discovered in an *in vivo* screen of nicotinamide derivatives in a structure-activity relationship study in 1948 (27). Today we know that PZA is a prodrug activated by the PZase encoded by *pncA*, a nonessential gene in *M. tuberculosis* (54). Like the case for some other nonessential target genes, mutations of *pncA* can often be deleterious for enzymatic activity, either due to a complete loss of function through a frameshift or truncation or due to SNPs in essential amino acid residues involved in catalysis, located at substrate or metal binding sites, or important for protein structure and stability. Surprisingly, PZA^r isolates carrying *pncA* mutations do not seem to be associated with a loss of bacterial fitness, either *in vivo* or *in vitro*, and while no selective pressure for this gene has been identified, little evidence of genetic drift has been reported. The lack of detectable effect on the overall fitness of the organism might be due to the fact that PZase is important in the recycling pathway of NAD, not in its synthesis.

In this study, MDR isolates captured over a 14-year period were sequenced for the *pncA* gene and flanking sequence, regardless of PZA susceptibility profile. Interestingly, 43% of the MDR strains analyzed proved to be additionally resistant to PZA. This is in agreement with the results of similar studies from Japan (53%) (2), Thailand (49%) (22), and South Africa (52.1%) (41). A study from India showed ~30.4% PZA^r in MDR strains, and one from Lisbon, Portugal, showed 82.7% (48/58 isolates) PZA^r in MDR isolates; however, the Portuguese isolates were also highly genotypically clustered and likely associated with outbreak situations (45, 60). Interestingly, one study showed ~6% PZA^r in otherwise pan-susceptible isolates (22).

The type and distribution of observed mutations were in agreement with previously published data and the data in the TB-

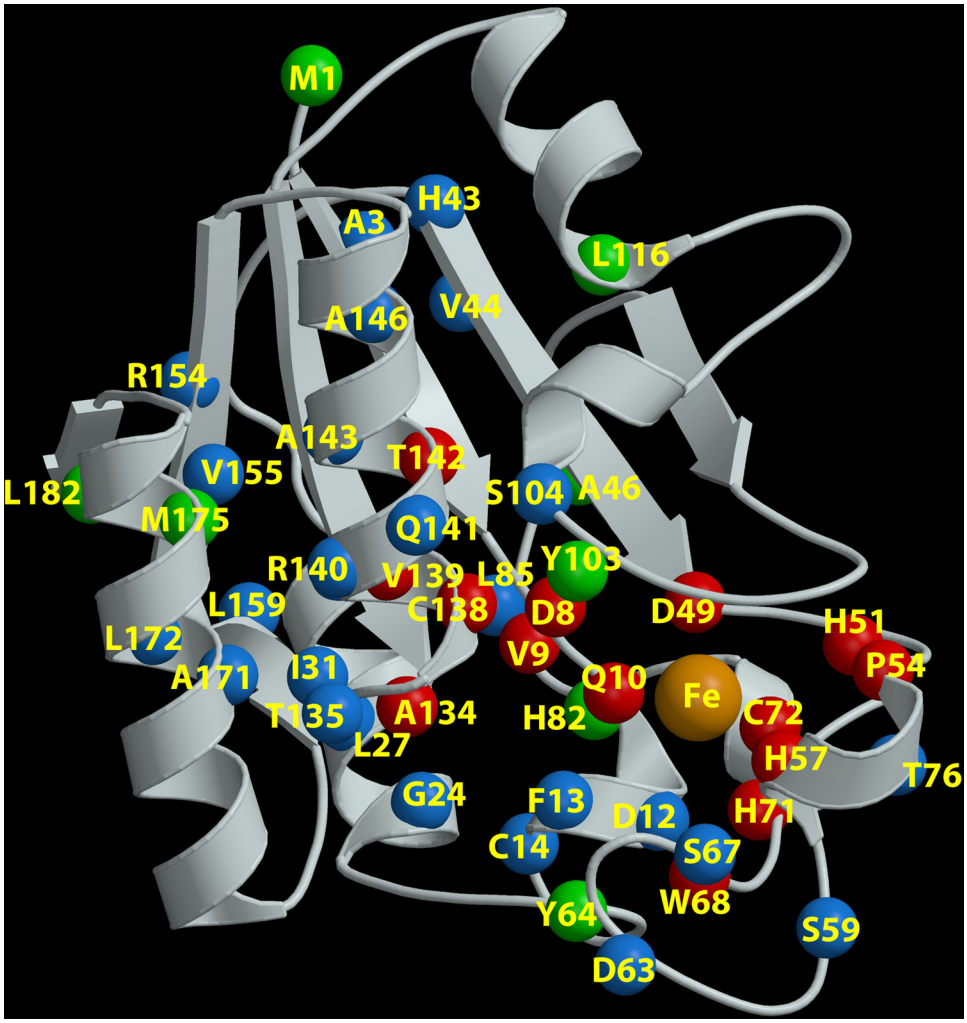

FIG 1 3D structure of pyrazinamidase showing that mutations cover the entire protein structure. The ribbon is shown in cream. The α-carbon atom of each mutated residue is shown as a colored ball. Green balls indicate neutral or stabilizing mutations (M1, A46, Y64, H82, T87, Y103, L116, T135, M175, and L182); the green residues A46, H82, and Y103 are in the vicinity of the active site, which could correlate with the loss of enzyme activity. Blue balls indicate destabilizing residues (A3, D12, F13, C14, G24, L27, I31, L35, H43, V44, S59, D63, S67, W68, T76, L85, S104, T135, D136, R140, Q141, A143, A146, R154, V155, L159, A171, and L172). Red balls indicate residues located in the active site pocket or involved in chelating the Fe atom (orange) (D8, V9, Q10, D49, H51, P54, H57, W68, H71, C72, G132, A134, C138, V139, and T142). Note that 4 α-carbon atoms (residues L35, T87, G132, and D136) are not visible, as they are behind the ribbon (on the bottom), and therefore they are not labeled in the figure. The figure was produced by successively using the MolScript (26) and Raster3D (37) programs.

DReaMDB database (http://www.tbdreamdb.com) (52). Comparison of mutations identified in clinical isolates with those determined from the spontaneous mutants showed 16 substitutions or sites of polymorphism in common (see Table S2 in the supplemental material). All 16 have also been reported previously in the literature, further underlining the probable functional importance of these amino acids, as previously described by Scorpio and coworkers (53). Mutations in the putative promoter region have also been reported widely. The most common promoter mutation is $^{-11}$A → G, which has been reported to occur in phylogenetically diverse strains (4, 11, 17, 18, 23, 29, 34, 38, 43, 48, 59). Other putative promoter mutations include $^{-12}$T → C (41, 59), $^{-10}$T → C (41), and $^{-7}$T → C (43). In this study, no polymorphisms were identified in putative promoter regions of the clinical isolates; however, a Del^{-4}A mutation and $^{-6}$G → C and $^{-7}$T → C substitutions were found in the spontaneous mutants selected at 500 μg/ml of PZA.

The mutations in the PncA protein associated with PZA^r are so diverse that the identification of the same mutation in two different isolates may suggest possible transmission and merits further genotyping and epidemiological investigation. Correlations between genotypic clustering and *pncA* mutations were reported in studies from Brazil and Portugal (4, 45, 50). Strain clusters sharing the same *pncA* mutation in South Africa grouped together by spoligotype but differed by MIRU-VNTR type (41). In this study, isolates sharing the same genotype and *pncA* mutation also shared epidemiological or demographic characteristics (Table 1). In contrast, genotypically distinct isolates carrying mutations in the same codon, whether resulting in the same or different substitutions, facilitated the identification of essential residues involved in the activation of PZA into pyrazinoic acid.

A puzzling observation in this study and previous reports (the TBDReaMDB database) is the appearance of scattered mutations throughout the *pncA* gene and putative promoter region, suggest-

ing multiple possibilities for inactivation or decreased activity and downregulation of the PZase. However, a closer look clearly identifies preferential sites. The mutations observed in the first codon have been reported in other studies (29, 39, 45, 48) and could very well interfere with the initiation of translation. The second most frequently encountered mutated codon in the PZA^r spontaneous mutants was codon 8 (10% of the mutants obtained with 500 μg/ml PZA). Asp8 is part of the catalytic triad Cys138-Asp8-Lys96 (46, 63), and mutations of this residue have frequently been reported in the literature on PZA^r strains. In order to better understand this "scattered" phenomenon, we analyzed the recently published high-resolution structure of the *M. tuberculosis* PncA protein (46) for the observed mutations in this study, coupled with predictions of protein stability. Most of the observed mutations affect buried amino acids and were determined to be energetically destabilizing, suggesting a reduction of protein stability and thereby a diminished or depleted PZase activity. Amino acids associated with either iron or substrate binding or catalytic active sites were most directly implicated in resistance. This is consistent with the literature and exemplified in the spontaneous mutants selected on 500 μg/ml PZA in this study. For instance, the four Fe-chelating residues (Asp49, His51, His57, and His71) were all found to be mutated among the PZA^r isolates. His57 also happens to be the intrinsic phylogenetic mutation found in all *M. bovis* PZA^r isolates. Likewise, modifications of the residues of the active site, such as Asp8, Val9, Gln10, Thr47, Gly132, Ala134, Cys138, Val139, and Thr142, were also all identified in the PZA^r isolates and/or spontaneous mutants. Almost all of these residues have already been identified by mutagenesis as essential for the enzymatic activity of the protein (46, 63).

The effect of neutral or stabilizing mutations on protein function is less evident. Substitutions at codons 46 and 64 were predicted to be stabilizing, while the substitutions at positions 82, 87, 103, and 175 were found to be neutral. One plausible explanation could be that substitutions at positions 46 (^{46}Ala → Val/Ser/Pro [22, 31, 42]), 82 (^{82}His → Asp/Arg/Leu [42, 50, 58]), and 103 (^{103}Tyr → His/Stop/Ser/Asp/Cys [29–31, 42, 50, 58, 61]), which have already been reported extensively in the literature, are located within close proximity of the substrate binding site and may impact the enzymatic activity while not affecting the overall protein structure. A puzzling challenge in this study was the observation that mutations at positions 14 and 175 (^{14}Cys → Gly and ^{175}Met → Ile) were found to be associated with both PZA-susceptible and -resistant isolates. Four clinical isolates were found to carry the ^{14}Cys → Gly substitution, but only one of these was found to be resistant. Likewise, 2 clinical isolates were found to carry the ^{175}Met → Ile substitution, with one being resistant and one being susceptible. Repeated reconfirmation by use of the standard Bactec MGIT960 PZA kit confirmed the phenotype, showing that the MICs for the 2 resistant isolates were borderline (>100 and <200 μg/ml), representing in part a possible source of the previously reported discrepancies. In the literature, these substitutions (^{14}Cys → Gly [56] and ^{175}Met → Ile [53]) have been associated with PZA resistance. These mutations were not identified in the spontaneous mutants (see Table S2 in the supplemental material). Furthermore, it is possible that these clinical isolates also carry other mutations interfering with PZA susceptibility. Interestingly, the latter mutation is predicted to confer a neutral effect, which might still allow for some enzymatic activity, although PZase activity was not explored in this study. Finally, it should be noted that the predicted values of stabilizing energy may not account for all influencing factors.

In this study, 9% (7/78 strains) of PZA-susceptible clinical strains also carried mutations within *pncA*, suggesting that some mutations either are phylogenetic (not associated with the resistance phenotype) or indicate genetic drift, notions that need to be evaluated further. Phylogenetic linkage for some neutral or synonymous mutations can be elucidated in some instances, while for most other SNPs it is not possible to assess given the restricted genotypic information required to draw firm conclusions. As such, the synonymous mutation ^{65}Ser → Ser has been identified in at least 7 different studies, but only 2 provide genotypic data which suggest that this mutation is found predominantly in some, but not all, members of the CAS spoligotype strain family (17, 60). The two CAS isolates investigated in our study did not have this silent mutation. In contrast, the PZA^r phylogenetic SNP ^{57}His → Asp (54, 59) demarcates the branching of *M. bovis* from *M. tuberculosis*. SNPs on codon 57 are not uniquely restricted to *M. bovis*, as they may also occur in *M. tuberculosis* isolates (17, 29). Alternative substitutions, such as ^{57}His → Pro and ^{57}His → Arg, are also possible (4, 7, 21). Another well-defined phylogenetic SNP in *pncA* is the synonymous substitution ^{46}Ala → Ala in *Mycobacterium canettii*.

Other studies also report synonymous or neutral mutations in PZA^s isolates (2, 9, 14, 17, 23, 55, 58). We identified 5 possible *pncA* alterations not associated with a drug resistance phenotype among 138 sequenced clinical isolates, even though the considered mutation or codon has been described in the literature as being associated with PZA^r: ^{14}Cys → Gly (56), ^{64}Tyr → Ser (29, 42), ^{135}Thr → Pro (17, 40, 56), ^{143}Ala → Thr (17), and ^{175}Met → Ile (29, 31, 53). The ^{175}Met → Ile mutation (previously described for PZA^r strains) (29, 31, 53) found in a Beijing SIT1 isolate was not present in another 29 strains belonging to the same strain family, suggesting a random event or subbranch rather than a phylogenetic demarcation.

Previously, the ^{47}Thr → Ala SNP, first reported for the W-MDR strain (5, 59), was later proposed erroneously to be a neutral phylogenetic mutation (16). In the present study, as in other works, we found no correlation between the ^{47}Thr → Ala mutation and the phylogeny of the wide W-Beijing strain family, whether strains were susceptible or resistant. Although the ^{47}Thr → Ala substitution was associated with the W-MDR outbreak isolate from New York City (5, 59), other W-Beijing strains most often carry a wild-type ^{47}Thr residue, and most unrelated PZA^r Beijing isolates display a variety of different mutations within the *pncA* gene (17, 23, 60). Most recently, the ^{47}Thr → Ala SNP was found to be the most common SNP associated with PZA^r in a large systematic study carried out by the Centers for Disease Control and Prevention (CDC) (9).

In this study, 1/60 PZA^r isolates was found to carry wild-type *pncA* and its flank. The resistant phenotype of this isolate was reconfirmed, and the MIC was found to be >400 μg/ml. Unfortunately, 13 other isolates sharing the same profile could not be included in the study, as the cultures were no longer available for reconfirmation. Consequently, an accurate determination of the frequency of PZA^r isolates displaying wild-type *pncA* could not be performed. Only 1 PZA^r clinical isolate was found to carry wild-type *pncA* and *rpsA*, involved in the recently identified additional mechanism of resistance (57).

The frequency of PZA^r mutants carrying wild-type *pncA* has

been a point of much contention and speculation, due primarily to the inherent complications in PZA susceptibility determinations, possible phylogenetic predisposition, and the limited number of unbiased population-based studies. Indeed, the frequency of PZA^r associated with *pncA* mutations has been reported to range from 70 to 100%, with reports of 70.58% (17), 72% (59), 75% (22), 84.6% (9), 91.4% (21), 97% (29), and 99.94% (58). In this study, PZA^r and PZA^s isolates of the MDR collection were evaluated genotypically and phenotypically. Within the spontaneous mutants, wild-type *pncA*-carrying PZA^r mutants were identified only among those selected at the lower concentration of 100 μg/ml of PZA, with none isolated at 500 μg/ml (solid medium). This difference may also be attributed to bias introduced in the selection of spontaneous mutants at different concentrations (100 versus 500 μg/ml PZA at pH 6.0) or to other unknown factors associated with the *in vitro* selection of PZA^r spontaneous mutants. Subsequent tests and reconfirmation of the MIC for all 14 wild-type mutants showed MICs of >400 μg/ml in liquid medium, although the mutants were originally selected at 100 μg/ml PZA. Mutations selected at low concentrations of a given drug can often result in higher levels of resistance in subsequent MIC determinations, as commonly observed with other antitubercular drugs, such as rifampin, isoniazid, and fluoroquinolones. This study clearly indicates that other targets or mechanisms are associated with PZA resistance. The 14 PZA^r mutants have been subjected to whole-genome sequencing, and mutations were found in a single operon, with none found within *rpsA* or *pncA* (57).

Finally, the simultaneous selection of PZA^r spontaneous mutants originating from 40 independent pan-susceptible cultures allowed for determination of frequencies of 1.5 and 1 PZA^r mutant per 10^5 bacilli for selection on 100 μg/ml and 500 μg/ml PZA, respectively, which are rather elevated values compared to those for rifampin or the fluoroquinolones. Different mutation frequencies between different concentrations of the same drug have been described previously for other antitubercular drugs, notably the fluoroquinolones (1, 24, 67). Our estimate differs radically from the value of 10^{-7} to 10^{-8} CFU/ml proposed by Bamaga et al. (3). This discrepancy is hard to explain, though various factors may have contributed, including pH, medium, and drug concentration. Scorpio et al. (53) and Hirano and coworkers (20) also generated spontaneous mutants but did not estimate the possible frequency of mutagenesis.

In conclusion, we obtained numerous interesting observations in this study: (i) the frequency of mutagenesis to PZA^r at pH 6.0 was found to be relatively high, at 10^{-5} CFU/ml; (ii) approximately 12% of the PZA-resistant spontaneous mutants did not carry mutations within *pncA* or its flank; (iii) approximately 43% of all clinical MDR-TB isolates investigated were additionally resistant to PZA; (iv) 8 novel substitutions in *pncA* were discovered; (v) protein destabilization may explain resistance patterns; and (vi) observed substitutions which did not confer a resistant phenotype could not be linked with genetic drift. Further studies are necessary to clearly correlate all *pncA* mutations to a PZA phenotype and to improve the resolution of PZA DST determination by molecular biology and overcome or complement the limitations of phenotypic susceptibility determinations.

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