



# Pyrazinoic Acid Inhibits a Bifunctional Enzyme in *Mycobacterium tuberculosis*

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**ABSTRACT** Pyrazinamide (PZA), an indispensable component of modern tuberculosis treatment, acts as a key sterilizing drug. While the mechanism of activation of this prodrug into pyrazinoic acid (POA) by *Mycobacterium tuberculosis* has been extensively studied, not all molecular determinants that confer resistance to this mysterious drug have been identified. Here, we report how a new PZA resistance determinant, the Asp67Asn substitution in Rv2783, confers *M. tuberculosis* resistance to PZA. Expression of the mutant allele but not the wild-type allele in *M. tuberculosis* recapitulates the PZA resistance observed in clinical isolates. In addition to catalyzing the metabolism of RNA and single-stranded DNA, Rv2783 also metabolized ppGpp, an important signal transducer involved in the stringent response in bacteria. All catalytic activities of the wild-type Rv2783 but not the mutant were significantly inhibited by POA. These results, which indicate that Rv2783 is a target of PZA, provide new insight into the molecular mechanism of the sterilizing activity of this drug and a basis for improving the molecular diagnosis of PZA resistance and developing evolved PZA derivatives to enhance its antituberculosis activity.

**KEYWORDS** PNPase, pyrazinoic acid, antibiotic resistance, drug target, ppGpp, pyrazinamide

In its most recent report on global tuberculosis (TB), the World Health Organization declared that TB killed 1.8 million people in 2015 (1). The report also reiterated an urgent need for new drugs with sterilizing activity that can prevent relapse and the development of drug resistance. Pyrazinamide (PZA) is considered a sterilizing drug in TB treatment regimens owing to its unique ability to act on *Mycobacterium tuberculosis* persists in the acidic environments of macrophages and inflammatory foci (2). It is a vital component of the modern short-course TB treatment regimen, as its inclusion shortens the treatment duration from 9 to 12 months to 6 months (3). Because the activity of PZA is synergistic with that of several new drugs and drug candidates presently in clinical development, it is considered a part of future regimens in combination with bedaquiline, pretomanid, and moxifloxacin (4). These new drug combinations are expected to further shorten the period of treatment for drug-susceptible as well as drug-resistant TB. Thus, PZA has proven to be a unique enhancer of a variety of current and emerging TB therapeutic regimens.

PZA is a prodrug that requires conversion into its active form, pyrazinoic acid (POA). The conversion is catalyzed by pyrazinamidase (PZase), an enzyme encoded by *pncA* of

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*M. tuberculosis* (5). Although the mechanisms of PZA activation by PZase and PZA resistance mediated by mutations of *pncA* and its flanking regions have been extensively studied, a complete picture of the molecular target(s) and cellular functions inhibited by POA that together prove lethal to *M. tuberculosis* has not been elucidated. The fatty acid synthase I (FAS I), encoded by *fasI*, has been reported to be one of the targets of PZA (6). The eukaryote-like *fasI* from *M. avium*, *M. bovis* bacillus Calmette-Guérin (BCG), or *M. tuberculosis* was found to confer resistance to the PZA analogue 5-chloro-pyrazinamide (5-Cl-PZA), when present in multicopy vectors in *M. smegmatis* (6). However, no mutations in *fasI* have been found in PZA-resistant *M. tuberculosis* strains, and FAS I was shown to be the target of 5-Cl-PZA but not of PZA (7). The ribosomal protein S1 (RpsA), a vital protein involved in the ribosome-rescuing process of *trans*-translation, has also been reported to be a target of POA (8). This followed an earlier identification of a low level of PZA resistance in *M. tuberculosis* clinical isolate DHM444 which had no mutations in *pncA* but which harbored a deletion of alanine at position 438 in RpsA (RpsA $\Delta$ A438) (8). Overexpression of RpsA rendered *M. tuberculosis* resistant to PZA, and unlike wild-type RpsA, RpsA $\Delta$ A438 did not bind POA (8). However, a strain defective for *trans*-translation was fully susceptible to PZA (9), suggesting that altered *trans*-translation does not fully account for PZA resistance. Recently, aspartate decarboxylase, encoded by *panD* and involved in the synthesis of the  $\beta$ -alanine precursor for pantothenate and coenzyme A biosynthesis, was also suggested to be an additional target of PZA in *M. tuberculosis* (10). However, an *M. tuberculosis* strain with *panC* (encoding pantothenate synthetase) and *panD* deleted was cultivated with pantothenate at a concentration that did not antagonize PZA activity (11). This finding uncouples pantothenate synthesis and the action of PZA, suggesting that pantothenate-mediated antagonism occurs via a novel mechanism that is independent of PanD. An ATP-dependent ATPase (ClpC1) involved in protein degradation was very recently reported to be associated with PZA resistance in *M. tuberculosis* (12).

In summary, the molecular targets and mechanisms described so far do not fully account for the activity of PZA, and additional mechanisms that are yet unknown exist. Herein, for the first time, we demonstrate that a mutation in Rv2783, a bifunctional enzyme, confers resistance to PZA and further explore the properties of this enzyme to develop insights into the mechanism that leads to PZA resistance.

## RESULTS

***M. tuberculosis* Rv2783c is associated with PZA resistance.** We hypothesized that an unknown mechanism conferred PZA resistance in four *M. tuberculosis* clinical isolates that we recently described (13), as they lacked mutations in previously described resistance determinants, namely, *pncA* and *rpsA*. We further hypothesized that molecular factors that bind POA or its metabolites but whose potential involvement in PZA resistance has not been identified could yield valuable leads in pursuing the resistance determinants. Therefore, we selected proteins that have been demonstrated to bind to a POA derivative, 5-hydroxyl-2-pyrazinecarboxylic acid (8), namely, those encoded by *Rv2731*, *Rv2783c*, *Rv3169*, and *Rv3601c*. Analysis of the sequences of these genes identified the same G199A mutation in *Rv2783c* in two clinical strains. Simultaneously, we amplified *pncA* and its flanking regions again and verified the pyrazinamidase (PZase) activities of the two strains. The PZase activity in the two strains was similar to that in wild-type *M. tuberculosis* (see Fig. S1 in the supplemental material), and the sequences of *pncA* and its flanking regions were identical to those in the wild type.

**Expression of Rv2783 with the Asp67Asn mutation (Rv2783<sub>Asp67Asn</sub>) in *M. tuberculosis* H37Rv causes PZA resistance.** To further explore the potential role of *Rv2783c* in PZA resistance, we engineered a recombinant *M. tuberculosis* strain to express *Rv2783c* with the G199A mutation (*Rv2783c*<sub>G199A</sub>) and compared its PZA susceptibility to that of wild-type *M. tuberculosis* (Table 1). Expression of the *Rv2783c*<sub>G199A</sub> mutant in *M. tuberculosis* H37Rv using the p60luxN vector (14) containing the strong *hsp60* promoter caused a 5-fold increase in the MIC of PZA (MIC = 500  $\mu$ g/ml) compared with the MICs for the *hsp60* vector control and the parental *M. tuberculosis* H37Rv strain (MICs = 100

**TABLE 1** PZA susceptibility testing of *M. tuberculosis* Rv2783c recombinant strains<sup>a</sup>

<i>M. tuberculosis</i> recombinant strain	<i>In vitro</i> PZA susceptibility	MIC ( $\mu\text{g/ml}$ )
Wild-type Rv2783c	Susceptible	150
Mutant Rv2783c <sub>D67N</sub>	Resistant	500
Hsp60Rv <sup>c</sup> (vector control)	Susceptible	100
Parental <i>M. tuberculosis</i>	Susceptible	100

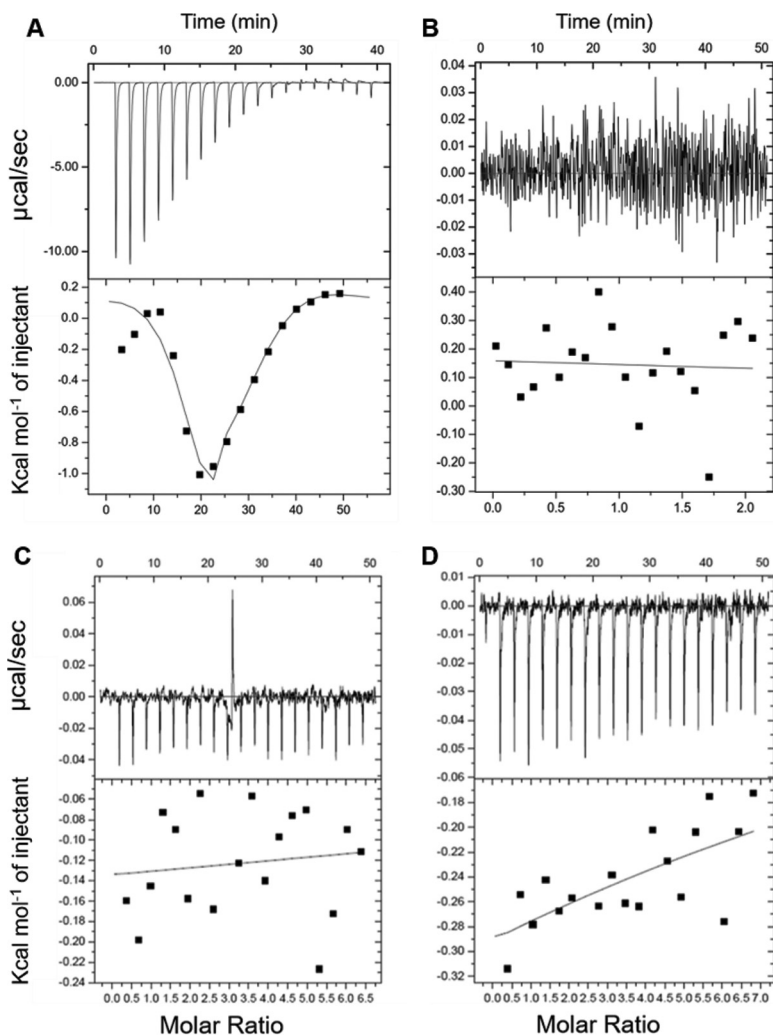
<sup>a</sup>The PZA susceptibility test, which was performed with the Bactec MGIT 960 system, was repeated 4 times. Expression of mutant Rv2783c<sub>D67N</sub> and wild-type Rv2783c in *M. tuberculosis* H37Rv was done using the extrachromosomal p60luxN vector (14) containing the strong mycobacterial *hsp60* promoter. The PZase assay result was positive for all strains.

$\mu\text{g/ml}$ ) at pH 5.5. On the other hand, the MIC of PZA against the recombinant *M. tuberculosis* strain containing additional wild-type Rv2783c in the same vector was 150  $\mu\text{g/ml}$ . In addition, both the wild-type Rv2783c and mutant Rv2783c<sub>G199A</sub> transformants were positive in the PZase activity assay, as were the parental *M. tuberculosis* strain and the *M. tuberculosis* empty vector control (Fig. S1).

**Rv2783 binds to POA but not to PZA.** To determine if POA binds to Rv2783 and whether the Asp67Asn substitution affects binding to POA, we used isothermal titration calorimetry (ITC) to measure the binding interactions of the purified wild-type Rv2783, the mutant Rv2783<sub>Asp67Asn</sub> and the *M. smegmatis* polynucleotide phosphorylase (PNPase) with POA or PZA. Wild-type Rv2783 bound to POA via a sequential binding sites model ( $n = 2$ ), with dissociation constant 1 ( $K_{d1}$ ) being equal to 1.05 mM and  $K_{d2}$  being equal to 3.17 mM (Fig. 1A), but did not bind to the prodrug PZA even at higher concentrations (Fig. 1B). On the contrary, the Rv2783<sub>Asp67Asn</sub> mutant and PNPase from naturally PZA-resistant *M. smegmatis* failed to bind to POA or PZA even at higher concentrations (Fig. S2). We observed, however, that POA bound to wild-type Rv2783 at relatively higher concentrations and only weakly at lower concentrations.

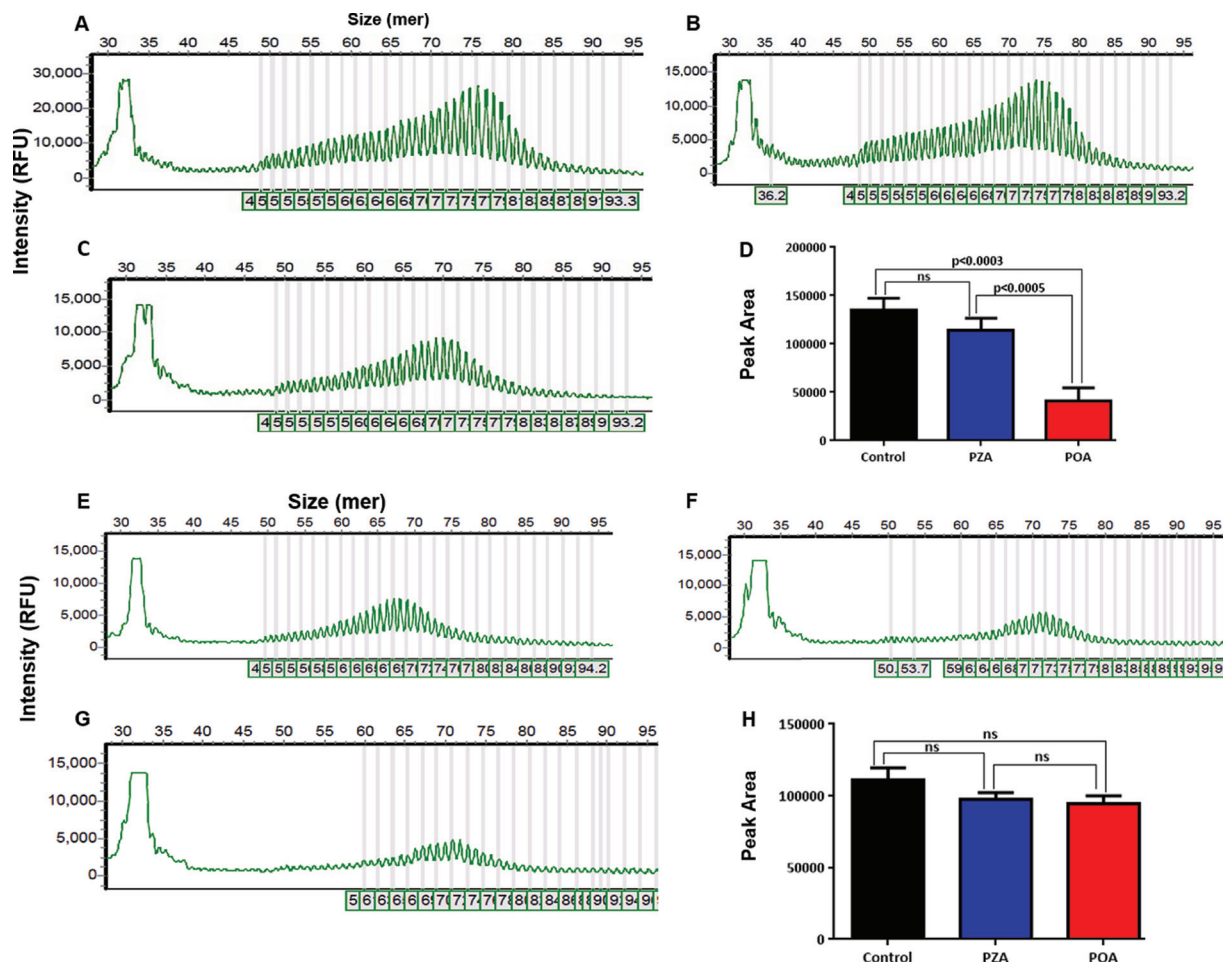
**ssDNA polymerization and phosphorolysis activities of Rv2783.** Rv2783 is predicted to function as a PNPase enzyme, which catalyzes synthetic and degradative activities in RNA metabolism (15). Exemplary bacterial PNPase enzymes have been reported to catalyze DNA activities *in vitro* when manganese or iron is provided in lieu of magnesium as the metal cofactor (16). Therefore, it was of considerable relevance to determine whether Rv2783 could catalyze single-stranded DNA (ssDNA) polymerization and phosphorolysis activities. Both the Rv2783 and Rv2783<sub>Asp67Asn</sub> proteins catalyzed the elongation of the ssDNA of a 5' 6-carboxyfluorescein (FAM)-labeled 36-mer ssDNA substrate in the presence of  $\text{Mn}^{2+}$  and dADP when incubated at 37°C for 60 min, yielding a bimodal distribution of fluorescently labeled end products ranging from 45-mer to 93-mer in size. We observed that the ssDNA polymerization activity of wild-type Rv2783 but not that of Rv2783<sub>Asp67Asn</sub> was significantly inhibited by POA, while PZA inhibited neither of them (Fig. 2). This implies that the mutant Rv2783<sub>Asp67Asn</sub> protein but not the wild-type Rv2783 protein can withstand the inhibitory effects of POA. However, there was no significant difference in the POA-free ssDNA polymerization activities of the wild-type Rv2783 and mutant Rv2783<sub>Asp67Asn</sub> proteins. On the other hand, upon substitution of dADP with inorganic phosphate, both proteins were able to degrade the 3' end of a 5' FAM-labeled 36-mer ssDNA substrate, resulting in the appearance of fluorescently 5' FAM-labeled decay products shorter than the input 36-mer ssDNA. However, unlike for wild-type Rv2783, where the inhibitory effect of POA on ssDNA phosphorolysis was significantly higher ( $P < 0.0001$ ), the inhibitory effect of POA on the ssDNA phosphorolytic activity of Rv2783<sub>Asp67Asn</sub> was comparatively lower ( $P = 0.0399$ ). Moreover, there was a significant difference ( $P = 0.0074$ ) in the POA-free ssDNA phosphorolysis activities of the two proteins (Fig. 3).

**RNA polymerization and phosphorolysis activities of *M. tuberculosis* Rv2783.** We studied the RNA polymerization and phosphorolysis activities of both wild-type



**FIG 1** Interaction of POA with wild-type Rv2783, mutant Rv2783<sub>Asp67Asn</sub> and *M. smegmatis* PNPase. (A) Representative results of an isothermal titration calorimetry (ITC) binding study indicate that POA binds to wild-type Rv2783 at high concentrations. (Top) Raw data. The data on the y axis indicate the amount of heat released per second during wild-type Rv2783 and POA binding. (Bottom) Integrated heat in each injection of POA together with the fit. The data on the y axis are expressed as the amount of heat released per mole in each injection. The association constants were obtained from fits of POA binding with wild-type Rv2783. (B) Representative results of an ITC binding study indicate that PZA did not bind to the wild-type Rv2783 protein. (Top) No heat was released during the wild-type Rv2783 and PZA interaction; (bottom) there was no integrated heat following each injection of PZA, and thus, there was no curve of the fit. (C and D) Representative results of an ITC binding study indicate that POA did not bind to the mutant Rv2783<sub>Asp67Asn</sub> protein (C) or to the *M. smegmatis* PNPase protein (D). (Top) Raw data indicating that no heat was released during the interaction between POA and the two proteins; (bottom) there was no integrated heat following each injection of POA, and thus, there was no curve of the fit.

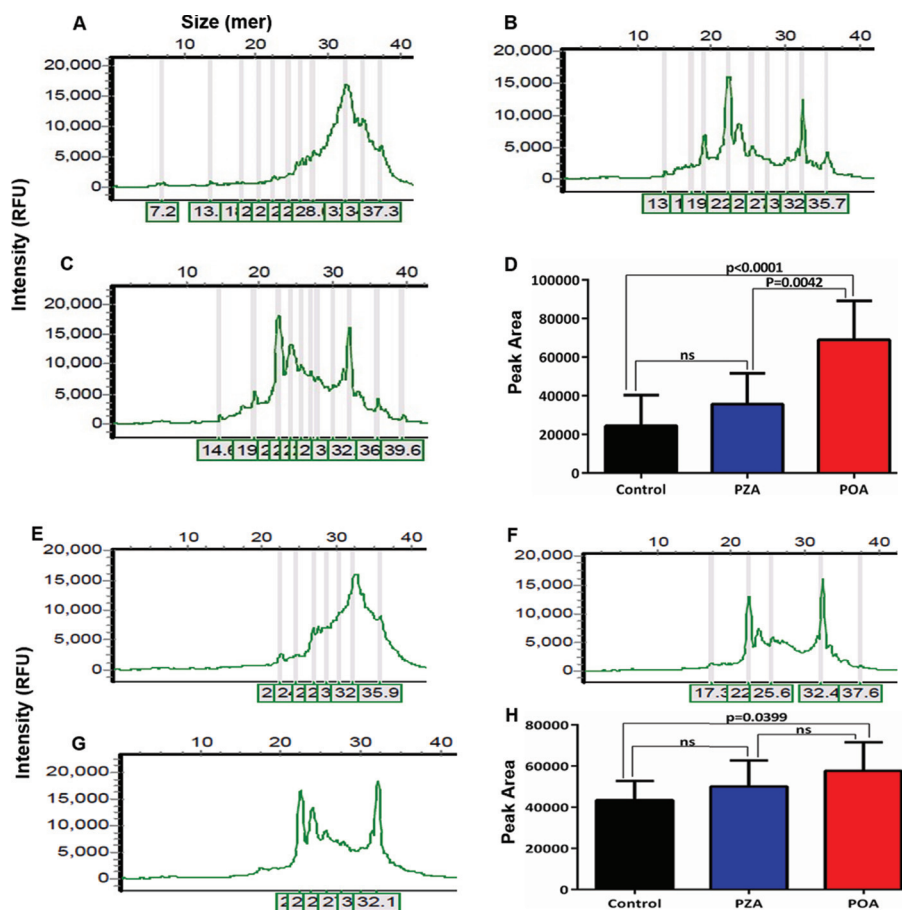
Rv2783 and Rv2783<sub>Asp67Asn</sub>. When reacted with a 5' FAM-labeled 24-mer single-stranded RNA (ssRNA) substrate in the presence of Mg<sup>2+</sup> and ADP (in lieu of Mn<sup>2+</sup> and dADP), the proteins catalyzed the extension of the input ssRNA into a polynucleotide tail. On the other hand, when the 5' FAM-labeled 24-mer ssRNA template was reacted with the proteins in the presence of Mg<sup>2+</sup> and phosphate (in lieu of ADP), most of the input ssRNA was degraded into a mixture of shorter nucleotide end products. Comparatively, as observed with the ssDNA activities, the ssRNA polymerization and phosphorolysis activities of wild-type Rv2783 but not those of the mutant protein were significantly inhibited by POA, while PZA did not significantly inhibit either activity (Fig. 4). In addition, there was no significant difference in the POA-free ssRNA metabolism activities between the wild-type and Rv2783<sub>Asp67Asn</sub> proteins.



**FIG 2** Capillary electropherograms and graphical presentations of the ssDNA polymerization activities of Rv2783. (A to C) Representative capillary electropherograms showing the uninhibited ssDNA polymerization activity of wild-type Rv2783 (A) and inhibition of the ssDNA polymerization activity of wild-type Rv2783 by PZA (B) and POA (C). (D) Comparison of the effects of POA and PZA on the ssDNA polymerization activity of the wild-type Rv2783 protein. (E to G) Representative capillary electropherograms showing the uninhibited ssDNA polymerization activity of mutant Rv2783<sub>Asp67Asn</sub> (E) and inhibition of the ssDNA polymerization activity of mutant Rv2783<sub>Asp67Asn</sub> by PZA (F) and POA (G). (H) Comparison of the effects of POA and PZA on the ssDNA polymerization activity of the mutant Rv2783<sub>Asp67Asn</sub> protein. For panels D and H, data are presented as means ± SEMs. Student's *t* test was used for the statistical analysis. ns, not significant; RFU, relative fluorescence units.

Taken together, these findings suggest the direct relevance of the Rv2783 protein in RNA metabolism.

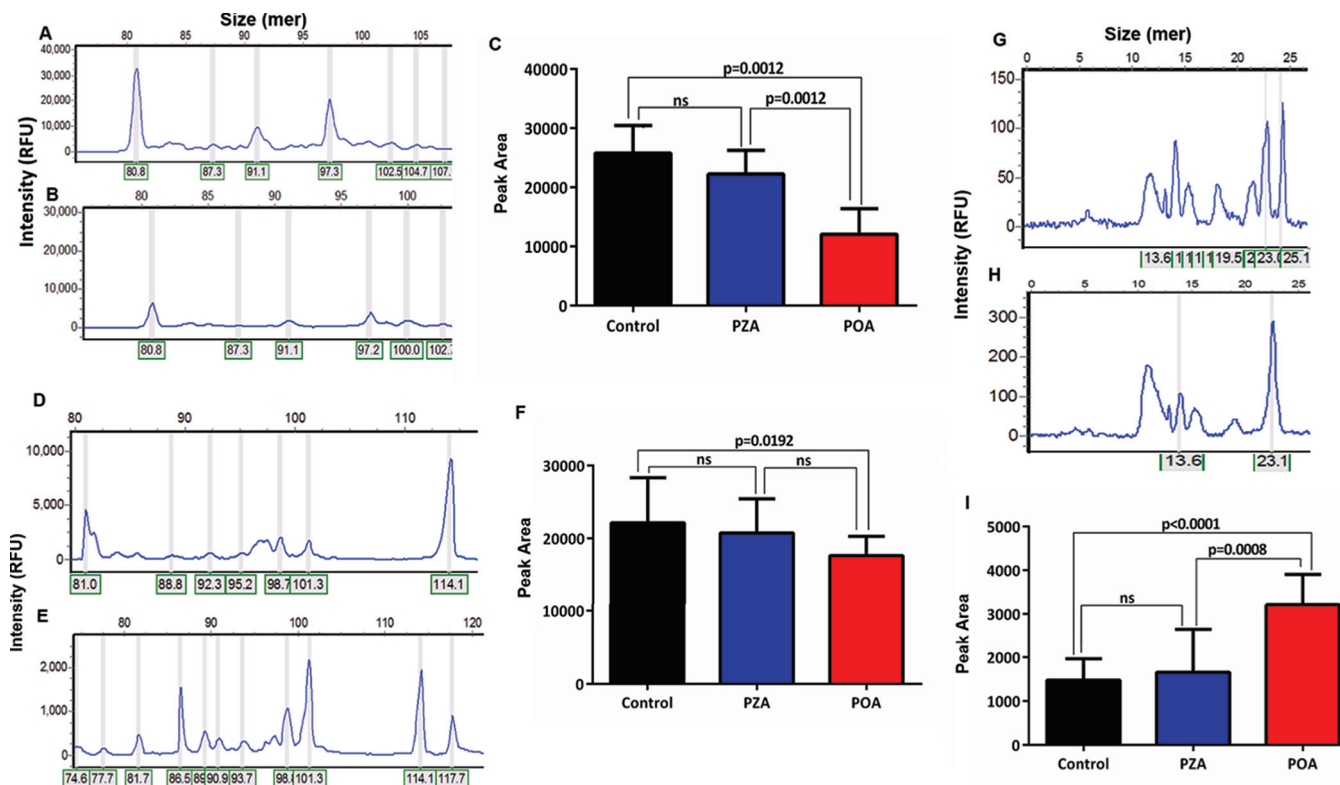
**ppGpp synthetase and hydrolase activities of Rv2783c.** On the basis of sequence homology, Rv2783 has been predicted to be a probable guanosine pentaphosphate synthetase (GpsI) that is involved in the synthesis and hydrolysis of (p)ppGpp (17) and the stringent response in many bacteria (18). Therefore, it was of considerable interest to determine whether the protein possessed ppGpp synthesis and ppGpp hydrolysis activities, as have been previously demonstrated for the *M. tuberculosis* Rel (Rel<sub>Mtb</sub>) protein (19). When reacted with various concentrations of ATP (phosphate donor) and GDP (substrate) in the presence of various concentrations of Mg<sup>2+</sup> and Na<sup>+</sup> at various pH values (5.5 to 8.0), both the wild-type Rv2783 and the Rv2783<sub>Asp67Asn</sub> proteins demonstrated only weak ppGpp synthesis activities, while Rel<sub>Mtb</sub> demonstrated strong ppGpp synthesis activity under similar reaction conditions (Fig. 5). On the other hand, when the same concentrations of the wild-type Rv2783 and Rv2783<sub>Asp67Asn</sub> proteins were reacted with 0.1 mM ppGpp in the presence of various concentrations of Mn<sup>2+</sup> and Na<sup>+</sup>, ppGpp was strongly hydrolyzed into GDP and ATP. However, while the wild-type Rv2783 protein exhibited relatively stronger ppGpp hydrolysis activity than Rv2783<sub>Asp67Asn</sub>, its activity, unlike that of the mutant, was significantly inhibited by POA (Fig. 6).



**FIG 3** Capillary electrophoresis and graphical presentations of the ssDNA phosphorolysis activities of Rv2783. (A to C) Representative capillary electrophoresis traces showing the uninhibited ssDNA phosphorolysis activity of wild-type Rv2783 (A) and inhibition of the ssDNA phosphorolysis activity of wild-type Rv2783 by PZA (B) and POA (C). (D) Comparison of the effects of POA and PZA on the ssDNA phosphorolysis activity of the wild-type Rv2783 protein. (E to G) Representative capillary electrophoresis traces showing the uninhibited ssDNA phosphorolysis activity of mutant Rv2783<sub>Asp67Asn</sub> (E) and inhibition of the ssDNA phosphorolysis activity of mutant Rv2783<sub>Asp67Asn</sub> by PZA (F) and POA (G). (H) Comparison of the effects of POA and PZA on the ssDNA phosphorolysis activity of the mutant Rv2783<sub>Asp67Asn</sub> protein. For panels D and H, data are presented as means  $\pm$  SEMs. Student's *t* test was used for the statistical analysis.

## DISCUSSION

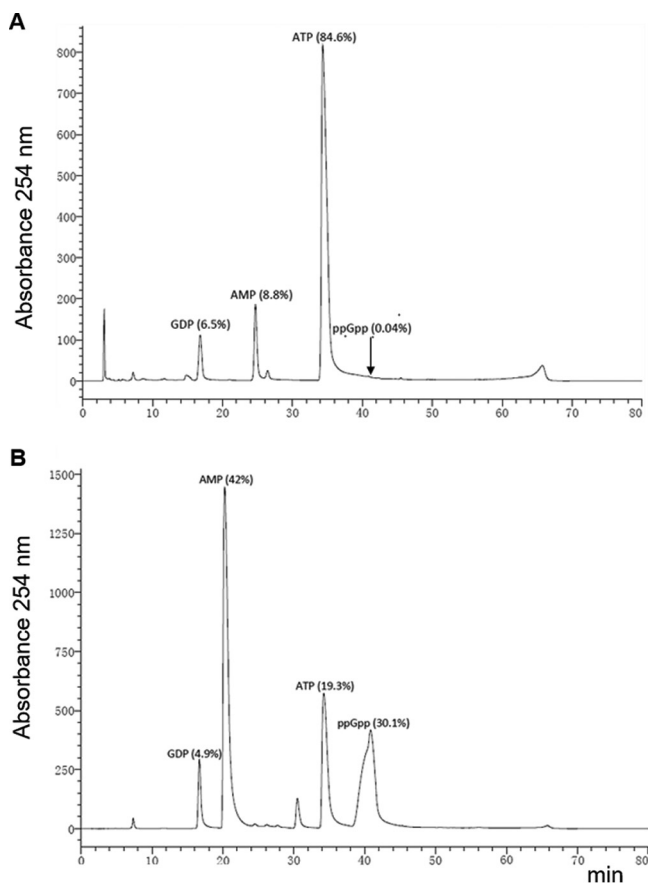
Herein, we demonstrated the involvement of the *M. tuberculosis* gene *Rv2783c* in PZA resistance. In our previous study (13), we identified four clinical strains that lacked mutations in genes reported to confer PZA resistance. This indicated the potential presence of an additional molecular mechanism that could confer PZA resistance. We sequenced the genes of *M. tuberculosis* proteins that were previously reported to bind with POA (8) as leads to seek a target(s) that conferred PZA resistance in these clinical strains. Analysis of the sequences of these genes indicated that two out of the four strains harbored the same G199A mutation in *Rv2783c*. The two PZA-resistant strains were found to be for positive PZase activity, and sequence analysis of their *pncA* genes, including the flanking regions, indicated no mutation. This allowed us to rule out the possibility of alterations in the potential regulatory regions of *pncA* that could result in a lack of PZase activity as a possible cause of the PZA resistance (13). Expression of Rv2783<sub>Asp67Asn</sub> increased the MIC of PZA in wild-type *M. tuberculosis*, providing direct evidence of the involvement of Rv2783 in PZA resistance. The finding that both the wild-type *Rv2783c* and Rv2783<sub>G199A</sub> transformants were positive in the PZase activity assay ruled out the possibility of a mutation in *pncA* as a possible cause of the PZA resistance in the transformant strain expressing Rv2783<sub>Asp67Asn</sub>. An increase in the level



**FIG 4** Capillary electrophoresis and graphical presentations of RNA metabolism activities of Rv2783. (A and B) Representative capillary electrophoresis showing the uninhibited RNA polymerization activity of wild-type Rv2783 (A) and inhibition of RNA polymerization activity of wild-type Rv2783 by POA (B). (C) Comparison of the effects of POA and PZA on the RNA polymerization activity of the wild-type Rv2783 protein. (D and E) Representative capillary electrophoresis showing the uninhibited RNA polymerization activity of mutant Rv2783<sub>Asp67Asn</sub> (D) and inhibition of the RNA polymerization activity of mutant Rv2783<sub>Asp67Asn</sub> by POA (E). (F) Comparison of the effects of POA and PZA on the RNA polymerization activity of the mutant Rv2783<sub>Asp67Asn</sub> protein. (G and H) Representative capillary electrophoresis showing the uninhibited RNA phosphorolysis activity of wild-type Rv2783 (G) and inhibition of the RNA phosphorolysis activity of wild-type Rv2783 by POA (H). (I) Comparison of the effects of POA and PZA on the RNA phosphorolysis activity of the wild-type Rv2783 protein. For panels C, F, and I, the data are presented as means ± SEMs. Student's *t* test was used for the statistical analysis.

of transcription of a target gene, leading to its higher level of expression, is a well-known drug resistance mechanism in bacteria (20). In this study, expression of Rv2783<sub>Asp67Asn</sub> in wild-type *M. tuberculosis* H37Rv was therefore possibly responsible for the observed PZA resistance *in vitro*. However, introducing wild-type Rv2783c under the control of a strong promoter on a multicopy extrachromosomal plasmid into the PZA-sensitive *M. tuberculosis* H37Rv strain only weakly increased the strain's resistance to PZA. It is possible that the increased number of copies of wild-type Rv2783c was not sufficient to withstand the inhibitory effects of the high concentrations of PZA. In addition, we observed a similar phenomenon in our recent study (14) in which the introduction of the wild-type target gene (*rpIc*) did not increase the level of resistance to linezolid, as was the case after introduction of a wild-type potential prothionamide target gene (unpublished data). Therefore, these findings illustrate that the Rv2783<sub>Asp67Asn</sub> mutation confers resistance to POA.

While wild-type Rv2783 bound to POA, it failed to bind to the prodrug PZA; this suggests that Rv2783 is a potential target of POA and confirms that, indeed, it is POA and not the prodrug PZA that has the anti-TB activity. We found, however, that POA bound to wild-type *M. tuberculosis* Rv2783 at a relatively higher concentration and bound only weakly at lower concentrations. This finding is concordant with an earlier observation (8) in which Rv2783 was also shown to bind only weakly to a POA derivative, 5-hydroxyl-2-pyrazinocarboxylic acid. This could probably explain why PZA (with a lower molecular weight of 123.11) must be administered at a higher dosage than other anti-TB drugs. On the other hand, the failure by Rv2783<sub>Asp67Asn</sub> and PNPase from naturally PZA-resistant *M. smegmatis* to bind to POA or PZA, even at higher

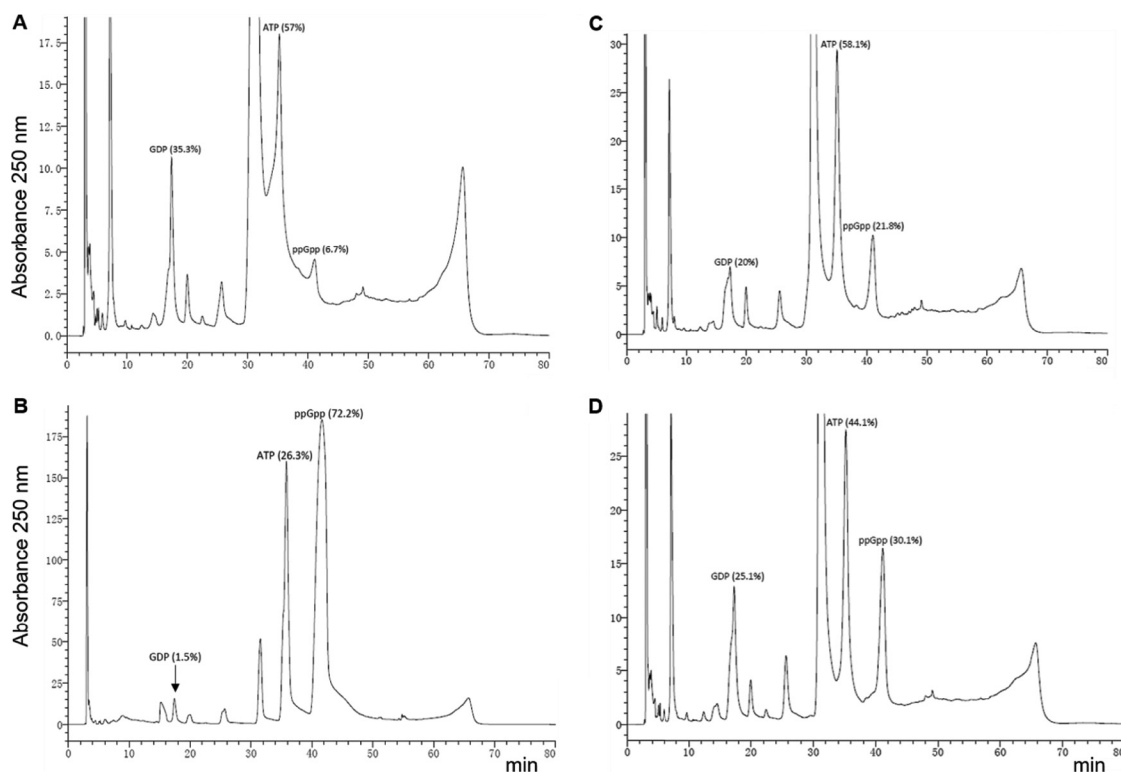


**FIG 5** ppGpp synthetase assays. Representative HPLC separation of ppGpp synthesis reaction products catalyzed by the wild-type Rv2783 protein (A) and the wild-type *M. tuberculosis* Rel protein (positive control) (B).

concentrations, further supports the suggestion that Rv2783 is a target of POA and that the Asp67Asn mutation affects its ability to bind to POA. We cannot exclude the possibility that the mutant Rv2783 is more stable or expressed at a higher level, but it did affect the susceptibility of *M. tuberculosis* to PZA. These findings suggest that POA may bind to Rv2783 and, in particular, to residue Asp67, as it was the altered site found in the two PZA-resistant strains and thus influences the PZA susceptibility of *M. tuberculosis*. PZA is a mysterious drug which is active only at acidic pH *in vitro* and has weak activity only at a very high dose *in vivo*, despite its lower molecular weight. In our unpublished works *in vitro*, even at 1 mg/ml, PZA exhibited no activity against *M. tuberculosis* at normal pH. In that case, the behavior of PZA in the biochemical assays and *in vivo* against *M. tuberculosis* can be very different. On the other hand, the binding of POA to wild-type Rv2783 is sequential ( $n = 2$  binding sites). The two binding sites are nonindependent, meaning that the binding by the first POA molecule is required to facilitate access and binding by the second POA molecule. These could probably explain POA's poor  $K_d$  (dissociation constant).

Our findings on the ssDNA polymerase and phosphorylase catalytic activities of Rv2783 contribute to an emerging picture of mycobacterial PNPases as catalysts of DNA metabolism, in addition to their synthetic and degradative roles in RNA metabolism (16). As suggested, Rv2783 is likely involved in conserving DNA integrity through DNA repair and mutagenesis *in vivo*. A similar PNPase-associated DNA metabolism in *Bacillus subtilis* has also been described (21). The study provided genetic evidence that *B. subtilis* PNPase participates in the homologous recombination and nonhomologous end-joining pathways of double-strand break repair in response to damage by hydrogen peroxide (21). Considering that mycobacteria possess three distinct pathways for





**FIG 6** ppGpp hydrolase assays. Representative HPLC separation of ppGpp hydrolysis reaction products catalyzed by the wild-type Rv2783 protein in the absence (A) and presence (B) of POA and the mutant Rv2783<sub>Asp67Asn</sub> protein in the absence (C) and presence (D) of POA.

the repair of DNA double-strand breaks (homologous recombination, nonhomologous end joining, and single-strand annealing), a similar participation by PNPase is highly possible. Alonso and colleagues suggested that PNPase reacts with broken DNA ends, either converting them from dirty breaks that cannot be ligated to clean ends that can be sealed by DNA ligase or adding nontemplated single-stranded 3' tails that can then influence the repair pathway choice (21, 22). The differential inhibitory effects of POA on the ssDNA polymerization and phosphorolysis activities of the wild-type and mutant Rv2783 proteins could help explain the difference in the PZA susceptibilities of their respective transformants. Taken together, it appears that the Asp67Asn substitution in Rv2783 alters the binding of POA, resulting in reduced susceptibility to POA. In such a case, the Rv2783<sub>Asp67Asn</sub> mutant strain would catalyze DNA activities (such as DNA repair) unimpeded under conditions that impact PZA susceptibility, especially during dormancy.

The RNA degradosome, a multienzyme complex comprised of proteins such as RNase E and PNPase, is involved in RNA metabolism and posttranscriptional control of gene expression in many bacteria (23–25). Although the mechanism of action of the RNA degradosome in *Escherichia coli* is relatively well understood, very little is known about the mechanisms involved in RNA metabolism in other bacteria (26, 27), including mycobacteria. Given the central role of the RNA degradosome in RNA metabolism in *E. coli*, it is conceivable that the RNA degradosome-dependent regulation of RNA stability in *M. tuberculosis* might also be a very important mechanism associated with cellular metabolism. During dormancy, *M. avium* exhibits a decline in susceptibility to antibiotics and an increase in RNase E levels (28). Likewise, having demonstrated the involvement of Rv2783 in RNA metabolism, we hypothesize an increased level of expression of Rv2783<sub>Asp67Asn</sub> and its subsequent involvement in DNA/RNA metabolism during dormancy, thereby influencing PZA susceptibility. This is likely true, as Rv2783<sub>Asp67Asn</sub> was able to catalyze RNA metabolism even in the presence of POA and

could, in addition, contribute toward sustaining RNA stability during dormancy. On the other hand, the ability of Rv2783 to catalyze RNA polymerization in a template-independent manner would possibly result in the production of inaccurate mRNAs, rRNAs, and tRNAs and further mistranslation. New protein variants with an adaptive function could be synthesized, producing some new bacillary phenotypes that could tolerate or resist various stresses and antibiotics (29). Specific mistranslation of the mycobacterial RNA polymerase, the target of rifampin (RIF), was demonstrated to be necessary and sufficient for phenotypic RIF resistance (30). In addition, we speculate that the potential diverse small RNAs synthesized by Rv2783 may regulate the gene expression of *M. tuberculosis* bacilli in different ways, enabling some of them to survive during persistence without a genome mutation.

The long-term survival of nonreplicating *M. tuberculosis* bacilli has been associated with Rel<sub>Mtb</sub>-mediated (p)ppGpp synthesis, which triggers the stringent response (19). The enzyme transfers a pyrophosphate from ATP to GDP or GTP to synthesize ppGpp and pppGpp, respectively, and also hydrolyzes (p)ppGpp into pyrophosphate and GDP or GTP (19). However, the strong ppGpp hydrolysis and weak ppGpp synthesis activities of Rv2783 demonstrated herein also strongly suggest its involvement in the regulation of intracellular concentrations of ppGpp in the bacilli under stressful conditions during dormancy. These catalytic activities of Rv2783 are similar to those of the *E. coli* SpoT protein, which is a bifunctional enzyme with strong (p)ppGpp hydrolase and only weak synthetase activities, which are activated under starvation conditions (31). PZA exhibits a preferential sterilizing activity against nonreplicating persisting bacilli during dormancy/persistence (2). Therefore, our findings that expression of Rv2783<sub>Asp67Asn</sub> caused PZA resistance and that the mutant protein retained the strong ppGpp hydrolysis and weak ppGpp synthesis activities even in the presence of POA strongly suggest the protein's vital role in the regulation of intracellular (p)ppGpp and, hence, the stringent response. The synthesis of (p)ppGpp without its hydrolysis directly impacts cellular pathways that are detrimental for the bacteria (32). The expression of a hydrolase-null Rel<sub>Mtb</sub> mutant incapable of hydrolyzing (p)ppGpp but still able to synthesize (p)ppGpp decreased the growth rate of *M. tuberculosis* and changed the colony morphology of the bacteria. Moreover, the expression of the Rel<sub>Mtb</sub> mutant during acute or chronic *M. tuberculosis* infection in mice was lethal to the infecting bacteria (32). Taken together, our findings further highlight the distinct importance of (p)ppGpp hydrolysis or regulation of its content that is essential for *M. tuberculosis* pathogenesis. We propose that Rv2783 plays an important role in the general homeostasis of (p)ppGpp during dormancy, and while this function is inhibited by POA in the wild type, the Asp67Asn mutation helps circumvent the effects of POA.

The Rv2783 protein plays many important roles in *M. tuberculosis*, so it could be essential. *Rv2783c* is located within a cluster of co-oriented open reading frames (see Fig. S3 in the supplemental material). The upstream *lppU* gene (encoding a lipoprotein), the downstream *pepR* gene (encoding a zinc protease), and the *Rv2781c* gene are deemed nonessential for *M. tuberculosis* growth, as was illustrated by the recovery of viable bacteria with a highly efficient transposon recognizing and inserting the binucleotide sequence TA within these respective open reading frames (33). In contrast, no transposon insertion within *Rv2783c* was recovered in several independent saturated *M. tuberculosis* mutation libraries using the same transposon combined with whole-genome sequencing (34, 35), suggesting the essentiality of *Rv2783c* for *M. tuberculosis* growth.

In conclusion, it is likely that Asp67 of Rv2783 is flexible and dispensable for enzymatic activity but is required for POA binding. POA binds to the Rv2783 protein, and the ensuing conformational changes inhibit its catalytic activities. The Asp67Asn mutation obstructs POA binding to the protein, and hence, the mutant protein proceeds with its catalytic activities. However, further studies by crystal structure analysis and functional analysis of some specific amino acid residues of Rv2783c are needed to confirm this hypothesis and delineate their roles. The limitation of this study is that the

**TABLE 2** DNA primers used in this study

Primer pair	Nucleotide sequence (5'-3') <sup>a</sup>
Rv2731F/Rv2731R	ATCGGCAAATATCGCG/GCACCATCGTGCAGCT
Rv2783cF/Rv2783cR	GGGAATTCATATGTCTGCCGCTGAAAT/CCCAAGCTTATTCGGTGACCACTCG
Rv3169F/Rv3169R	AACCTGGTGACCTGCA/CCGCATTTCCGGCGGTT
pncAF/pncAR	ATTTGTCGCTCACTACA/ATGCCCCACCTGCGGCT
panDF/panDR	TCGACTACCTGGAGCT/TGACTTCGGATTCCGGT
Ms2656F/Ms2656R1	GGGAATTCATATGTCTGTAGTCGAACT/AAAAGTACTCGGACCTGCGACGTTTC
HygF/HygR	AGAGACCAACCCCGTACTG/GTGAAGTCGACGATCCCGGT
Ms2656F/Ms2656R2	GGGAATTCATATGTCTGTAGTCGAACT/ATAAGAATCGGCGCCCGGACCTGCGACGTTTC
RelF/RelR	GGGAATTCATATGTGGCCGAGGACCAG/CCCAAGCTTAAATCAGCCCGCCCAAT

<sup>a</sup>Restriction enzyme sites are underlined.

binding experiment and enzyme activity assays carried out *in vitro* may not represent the native conditions in *M. tuberculosis*.

## MATERIALS AND METHODS

**General DNA techniques.** *M. tuberculosis* genomic DNA was extracted using the cetyltrimethylammonium bromide method as previously described (36) and used for subsequent PCRs. PCR amplification reactions were performed with *Pfu* DNA polymerase (TaKaRa, China), and 5% dimethyl sulfoxide was added due to the high G+C content of the mycobacterial genomes. The PCR-amplified products were analyzed by electrophoresis in agarose gels and purified using a DNA gel extraction kit (Bioflux). Plasmids were also extracted and purified using kits from the same company. Purified PCR products and recombinant plasmids were sequenced at The Beijing Genomics Institute, Shenzhen, China.

**PCR and DNA sequencing of new genes associated with PZA resistance.** To identify possible new genes associated with PZA resistance in four previously reported (13) PZA-resistant clinical strains lacking *pncA* and *ppsA* mutations, we amplified three genes (*Rv2731*, *Rv2783c*, and *Rv3169*), which encode proteins previously reported to bind a POA derivative, 5-hydroxyl-2-pyrazinocarboxylic acid (8), plus their flanking regions (FR), using primer pairs Rv2731F and Rv2731R, Rv2783cF and Rv2783cR, and Rv3169F and Rv3169R, respectively (Table 2). We also amplified *panD* (*Rv3601c*), another gene implicated in PZA resistance (10), plus its FR using primers panDF and panDR (Table 2). We amplified *pncA* plus its FR using primers pncAF and pncAR (Table 2) to verify the absence of any mutation. The PCR-amplified DNA products were then sequenced, and the gene sequences from the four PZA-resistant strains were compared against the respective wild-type gene sequences of *M. tuberculosis* H37Rv to identify potential mutations.

**Determination of PZase activities of the two PZA-resistant strains.** The PZase test of Wayne (37) was performed as previously modified (38). Briefly, two to three discrete colonies from the same batch were picked and transferred into 1 ml Middlebrook 7H9 medium supplemented with albumin-dextrose-catalase medium containing PZA (100  $\mu$ g/ml and 200  $\mu$ g/ml) in a 1.5-ml tube. The cells were incubated overnight at 37°C while shaking. Fifteen microliters of 2% Fe<sup>2+</sup> was added to the cells, and the cells were incubated at 4°C for 2 h for detection of a color reaction. Wild-type *M. tuberculosis* H37Rv was used as a positive control (PZA sensitive), while *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) Tice and a clinical strain with a *pncA* mutation were used as two negative controls (both strains were PZA resistant). PZA in the presence of the PZase enzyme from *M. tuberculosis* is converted to POA, which then reacts with ferrous ion to produce a brown compound, which can be detected as an indication of positivity for PZase activity.

**Rv2783 overexpression in *M. tuberculosis*.** The wild-type *Rv2783c* and the *Rv2783c*<sub>G199A</sub> mutant genes were amplified from *M. tuberculosis* H37Rv and the PZA-resistant mutant strains, respectively, using primer pair Rv2783cF and Rv2783cR (Table 2). The PCR fragments were digested with the same enzymes, ligated to the extrachromosomal plasmid p60luxN (14) downstream of the strong *hsp60* promoter, and transformed into *E. coli* DH5 $\alpha$  cells. An empty p60luxN vector was included as a control. Competent wild-type *M. tuberculosis* H37Rv cells were first incubated at 37°C for 10 min before electroporation, and transformation was performed at room temperature as previously described (39). Verification of the transformed *M. tuberculosis* cells was done by amplification of the 529-bp *hyg* marker gene open reading frame using the primer pair HygF and HygR (Table 2) and testing for PZase enzyme activity. Verified transformants were plated on 7H11 agar plates containing hygromycin (50  $\mu$ g/ml) and used for *in vitro* PZA susceptibility testing using the Bactec MGIT 960 system (BD, Franklin Lakes, NJ, USA).

**In vitro PZA susceptibility testing of *M. tuberculosis* transformants.** PZA susceptibility tests were carried out in MGIT PZA medium (pH 5.5; BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, a 0.5 McFarland suspension was diluted 1:5 and 1:50 in sterile distilled water. Dilutions of 1:50 were inoculated into MGIT PZA medium without drug, while 1:5 dilutions were inoculated into MGIT PZA medium supplemented with 25 to 500  $\mu$ g/ml PZA, and the dilutions were incubated in the MGIT instrument at 37°C. Results were read automatically within 14 and 21 days after inoculation of the medium. The *M. tuberculosis* H37Rv parental strain, which is susceptible to PZA, was used as a positive control, while BCG Tice, which is naturally resistant to PZA, was used as a negative control.

**Construction of protein expression plasmids.** The wild-type and mutant *M. tuberculosis* Rv2783c DNA fragments were PCR amplified from the genomic DNA of *M. tuberculosis* H37Rv and the clinical PZA-resistant mutant, respectively, using primer pair Rv2783cF and Rv2783cR (Table 2). The *M. smegmatis* PNPase protein, encoded by the *M.smeg\_2656* gene (2,292 bp), was expressed to serve as a positive control for the PNPase assays with the *M. tuberculosis* Rv2783 protein. Genomic DNA from wild-type *M. smegmatis* was used to amplify the *M.smeg\_2656* gene using primer pair Ms2656F and Ms2656R2 (Table 2). The *M. tuberculosis* Rel protein, encoded by the *Rv2583c* gene (2,373 bp), was also expressed to serve as a positive control for the ppGpp synthetase and hydrolysis assays with the Rv2783 protein. Genomic DNA from wild-type *M. tuberculosis* H37Rv was used to amplify the *Rv2583c* gene using primer pair RelF and RelR (Table 2). The PCR-amplified DNA fragments were digested with the appropriate enzymes and ligated to the pET-28a(+) plasmid vector that had been digested with the same enzymes to yield recombinant plasmids.

**Protein expression and purification of the mycobacterial proteins.** The mycobacterial proteins were overexpressed in *E. coli* strain BL21(ΔDE3) containing the recombinant plasmids described above after induction with isopropyl 1-thiogalactopyranoside (IPTG), which was added to a final concentration of 0.5 mM, at 16°C overnight. The proteins, expressed as His-tagged derivatives, were purified from soluble bacterial extracts by sequential nickel-affinity, anion-exchange, and gel filtration chromatography steps.

The bacterial cells were harvested by centrifugation at 4°C, and all subsequent procedures were performed at 4°C. The supernatants containing the recombinant proteins were purified on Ni<sup>2+</sup>-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) that had been equilibrated with buffer A (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 10 mM imidazole). The columns were washed with buffer A, and protein was eluted with buffer B (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 300 mM imidazole). The protein compositions of the fractions were monitored by SDS-PAGE. The appropriate protein fractions were pooled and loaded onto a Q-Sepharose column (180 ml) equilibrated with buffer C (20 mM Tris-HCl [pH 8.0], 5 mM NaCl) and eluted with buffer D (20 mM Tris-HCl [pH 8.0], 1 M NaCl) with a linear gradient (0 to 100%). The protein fractions were again monitored by SDS-PAGE, pooled, and gel filtered through a Superdex-200 size exclusion column equilibrated with buffer E (20 mM Tris-HCl [pH 8.0], 150 mM NaCl). The peak fractions were pooled, concentrated by centrifugal ultrafiltration, and stored at -80°C. The protein concentrations were determined using the Bio-Rad dye reagent with bovine serum albumin as the standard.

**Isothermal titration calorimetry (ITC) binding study.** Titration of the mycobacterial recombinant proteins with POA or PZA was performed in a VP-ITC 200 microcalorimeter (MicroCal, LLC, USA) at room temperature. The VP-ITC system was used to determine whether any interaction existed between Rv2783 and POA or PZA. Initially, protein solutions consisting of 10 μM wild-type Rv2783 or mutant Rv2783<sub>Asp67Asn</sub> and *M. smegmatis* PNPase in 10 mM phosphate-buffered saline (pH 7.5) were titrated with 100 μM POA or PZA (molar concentration ratio, 1:10). Blank titration of the drug solution in the same buffer in the absence of the proteins was performed. Further, the proteins were titrated with saturated POA or PZA (up to a 1:200 molar concentration ratio) at pH 5.5 and pH 6.5. The binding constants were estimated from the isotherms obtained using the calorimetric analysis origin software. Typically, 20 injections with 2 μl per injection were made at 300-s intervals, and the reaction temperature was 25°C. The heat of the reaction per injection (microcalories per second) was determined by integration of the peak areas.

**ssDNA and ssRNA catalytic activities of *M. tuberculosis* Rv2783.** The DNA/RNA polymerization and phosphorolytic activities of Rv2783 were determined according to previously published work on *M. smegmatis* PNPase (16), which served as a positive control, with some modifications.

**ssDNA polymerization assay.** Reaction mixtures (10 μl) containing 20 mM Tris-HCl (pH 7.5), 5 or 10 mM MnCl<sub>2</sub>, 2 mM dADP, 0.1 μM (1 pmol) 36-mer ssDNA substrate (5'-FAM-GCCCTGCTGCCGACCAACG AAGGTAAAAA-3'), and different concentrations of the wild-type and mutant Rv2783 and *M.smeg\_2656* (positive-control) proteins were incubated for 30 min at 37°C. The reactions were quenched by adding 10 μl of 90% formamide, 50 mM EDTA buffer. The samples were heated for 5 min at 100°C and then analyzed by capillary electrophoresis at BGI, Wuhan, China.

**ssDNA 3'-phosphorylase assay.** Reaction mixtures (10 μl) containing 20 mM Tris-HCl (pH 7.5), 5 mM MnCl<sub>2</sub>, 30 μM (NH<sub>4</sub>)<sub>3</sub>PO<sub>4</sub>, 0.1 μM (1 pmol) 36-mer ssDNA substrate (5'-FAM-GCCCTGCTGCCGACCAACG AAGGTAAAAA-3'), and different concentrations of the wild-type and mutant Rv2783 and *M.smeg\_2656* (positive-control) proteins were incubated for 60 min at 37°C. The reactions were quenched by adding 10 μl of 90% formamide, 50 mM EDTA buffer. The samples were heated for 5 min at 100°C and then analyzed by capillary electrophoresis at BGI, Wuhan, China.

**RNA polymerization assay.** Reaction mixtures (10 μl) containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 2 mM ADP, 0.1 μM (1 pmol) 24-mer ssRNA substrate (5'-FAM-GGUCGCAAUUGAUUCCGAUA GUG-3'), and different concentrations of the wild-type and mutant Rv2783 and *M.smeg\_2656* (positive-control) proteins were incubated at 37°C for 15 min. The reactions were quenched by adding 10 μl of 90% formamide, 50 mM EDTA buffer. The samples were heated for 5 min at 100°C and then analyzed by capillary electrophoresis at Sangon Biotech, Shanghai, China.

**RNA 3'-phosphorylase assay.** RNA phosphorylase assay reaction mixtures (10 μl) containing 20 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 0.5 mM (NH<sub>4</sub>)<sub>3</sub>PO<sub>4</sub>, 0.1 μM (1 pmol) 24-mer ssRNA substrate (5'-FAM-GGUCGCAAUUGAUUCCGAUA GUG-3'), and different concentrations of the wild-type and mutant Rv2783 and *M.smeg\_2656* (positive-control) proteins were incubated for 15 min at 37°C. The reactions were quenched by adding 10 μl of 90% formamide, 50 mM EDTA buffer. The samples were heated for 5 min at 100°C and then analyzed by capillary electrophoresis at Sangon Biotech, Shanghai, China.

**Inhibition of Rv2783 ssDNA and RNA catalytic activities by POA or PZA.** Both ssDNA and ssRNA polymerization and phosphorylation assays were repeated as described above but in the presence of POA or PZA (100  $\mu\text{g/ml}$ ).

**ppGpp synthetase and hydrolysis assays of the Rv2783 protein.** ppGpp synthetase and hydrolysis assays of the Rv2783 protein were done as described by Avarbock and colleagues on the ribosome-independent (p)ppGpp synthetase and hydrolase activities of Rel from *M. tuberculosis* (40) with some modifications.

**ppGpp synthetase assays.** Reaction mixtures (25  $\mu\text{l}$ ) containing 50 mM HEPES (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol (DTT), various concentrations of GDP (Sigma-Aldrich), ATP (Sigma-Aldrich),  $\text{MgCl}_2$ , and 5  $\mu\text{M}$  the wild-type and mutant Rv2783 proteins were incubated at 30°C for 1 h. The *M. tuberculosis* Rel protein was used as the positive control at a similar concentration. Three negative-control reactions in which the reaction mixtures included no wild-type or mutant Rv2783c protein, no GDP, and no ATP, respectively, were also run. The reactions were quenched by adding 1  $\mu\text{l}$  of formic acid. Additionally, the reactions were repeated in the presence of POA (100  $\mu\text{g/ml}$ ) at different pHs to evaluate the inhibitory effects of POA. After 5 min at room temperature, the reaction tubes were centrifuged, their contents were filtered to remove the precipitated proteins, and the proteins were analyzed by high-pressure liquid chromatography (HPLC) using ppGpp, ATP, GTP, GDP, and AMP substrates as standards for both reactions.

**ppGpp hydrolysis assays.** Hydrolysis reaction mixtures containing 50 mM HEPES (pH 8.0), 150 mM NaCl, 1 mM DTT, 0.1  $\mu\text{M}$  ppGpp (TriLink Biotechnologies), various concentrations of  $\text{MnCl}_2$ , and 5  $\mu\text{M}$  the wild-type and mutant Rv2783 proteins were incubated at 30°C for 20 min. The *M. tuberculosis* Rel protein was used as the positive control. The reactions were quenched by adding 1  $\mu\text{l}$  of formic acid. Additionally, the reactions were repeated in the presence of different concentrations of POA or PZA at different pHs to evaluate the inhibitory effects of POA and PZA. After 5 min at room temperature, the reaction tubes were centrifuged, their contents were filtered to remove the precipitated proteins, and the proteins were analyzed by HPLC as mentioned above.

**HPLC analysis of the ppGpp synthetase and hydrolysis products.** The chromatographic system consisted of a Waters in-line degasser, a Waters 600 controller connected to a Waters 600 pump, a Waters 486 tunable absorbance detector, and a Waters Millennium workstation (version 3.05) chromatography manager. The nucleotides were separated by reversed-phase chromatography using a Waters Symmetry  $\text{C}_{18}$  3.5- $\mu\text{m}$  (150- by 4.6-mm) column equipped with a NovaPak  $\text{C}_{18}$  Sentry guard column (Waters), adapting a prior method used for the separation of nucleotides (41). Separation was done using a method that started with a 70:30 concentration of buffer A-buffer B at a flow rate of 0.8 ml/min, which was changed in a linear gradient to 40:60 buffer A-buffer B after 30 min. From 30 to 60 min, the gradient was changed linearly from 40:60 buffer A-buffer B to 0:70:30 buffer A-buffer B-buffer C. The injection volume was 50  $\mu\text{l}$ , and detection was by UV at 254 nm. Buffer A consisted of 5 mM *t*-butyl ammonium phosphate (PicA reagent), 10 mM  $\text{KH}_2\text{PO}_4$ , and 0.25% methanol adjusted to pH 6.9. Buffer B consisted of 5 mM *t*-butyl ammonium phosphate, 50 mM  $\text{KH}_2\text{PO}_4$ , and 30% methanol (pH 7.0). Buffer C was acetonitrile. Buffers A and B were degassed, passed through a 0.45- $\mu\text{m}$ -pore-size filter to avoid the risk of microbial contamination, prepared fresh, and stored in the dark at 4°C prior to use. The Millennium workstation (version 3.05) chromatographic manager was used to pilot the HPLC instrument and to process the data throughout the method validation and sample analysis.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00070-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.1 MB.

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M. Njire, Jinsong Liu, W. W. Yew, E. Nuernberger, G. Lamichhane, and T. Zhang conceived and designed the experiments. M. Njire, N. Wang, B. Wang, J. Mugweru, Y. Tan, X. Cai, Y. Liu, J. Guo, and T. Zhang performed the experiments. M. Njire, N. Wang,

Jinsong Liu, J. Mugweru, Y. Tan, J. Guo, B. Wang, S. Tan, Jianxiong Liu, W. W. Yew, E. Nuernberger, G. Lamichhane, and T. Zhang analyzed the data. Y. Tan, Jinsong Liu, S. Tan, Jianxiong Liu, and T. Zhang contributed reagents, materials, or analysis tools. M. Njire, Jinsong Liu, W. W. Yew, H. M. A. Hameed, E. Nuernberger, G. Lamichhane, and T. Zhang wrote the manuscript.

The *Rv2783c* gene has been filed as a patent in China (201610564946.X) for the molecular diagnosis of PZA-resistant *Mycobacterium tuberculosis*.

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