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# In Vivo-Selected Pyrazinoic Acid-Resistant *Mycobacterium tuberculosis* Strains Harbor Missense Mutations in the Aspartate Decarboxylase PanD and the Unfoldase ClpC1

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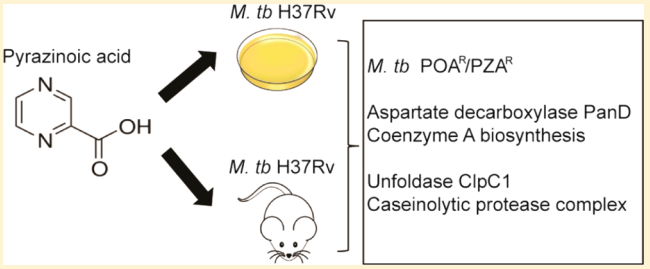
Supporting Information

**ABSTRACT:** Through mutant selection on agar containing pyrazinoic acid (POA), the bioactive form of the prodrug pyrazinamide (PZA), we recently showed that missense mutations in the aspartate decarboxylase PanD and the unfoldase ClpC1, and loss-of-function mutation of polyketide synthases Mas and PpsA-E involved in phthiocerol dimycocerosate synthesis, cause resistance to POA and PZA in *Mycobacterium tuberculosis*. Here we first asked whether these in vitro-selected POA/PZA-resistant mutants are attenuated in vivo, to potentially explain the lack of evidence of these mutations among PZA-resistant clinical isolates. Infection of mice with *panD*, *clpC1*, and *mas/ppsA-E* mutants showed that whereas growth of *clpC1* and *mas/ppsA-E* mutants was attenuated, the *panD* mutant grew as well as the wild-type. To determine whether these resistance mechanisms can emerge within the host, mice infected with wild-type *M. tuberculosis* were treated with POA, and POA-resistant colonies were confirmed for PZA and POA resistance. Genome sequencing revealed that 82 and 18% of the strains contained missense mutations in *panD* and *clpC1*, respectively. Consistent with their lower fitness and POA resistance level, independent *mas/ppsA-E* mutants were not found. In conclusion, we show that the POA/PZA resistance mechanisms due to *panD* and *clpC1* missense mutations are recapitulated in vivo. Whereas the representative *clpC1* mutant was attenuated for growth in the mouse infection model, providing a possible explanation for their absence among clinical isolates, the growth kinetics of the representative *panD* mutant was unaffected. Why POA/PZA resistance-conferring *panD* mutations are observed in POA-treated mice but not yet among clinical strains isolated from PZA-treated patients remains to be determined.




**KEYWORDS:** *tuberculosis, pyrazinamide, pyrazinoic acid, resistance, in vivo*

Treatment of tuberculosis (TB) involves a combination of the four first-line drugs: isoniazid, rifampicin, ethambutol, and pyrazinamide (PZA). PZA plays a critical role in this regimen by sterilizing lesions and preventing disease relapse. Inclusion of this drug in the treatment regimen in the 1980s resulted in shortening the duration of therapy from 9 to 6 months.[1] Since then, most new drug combinations in development include PZA.[2] However, its mechanism of action remains controversial.[3] Understanding how this critical drug works against TB may reveal new rational approaches for shortening TB treatment and preventing disease relapse.

PZA is a prodrug that requires conversion to its bioactive form, pyrazinoic acid (POA). Prodrug conversion is carried out by the bacterial pyrazinamidase PncA. The inactivation of PncA causes PZA resistance in vitro,[4] in vivo,[5] and in clinical isolates.[6] Host enzymes also metabolize PZA to POA, which could contribute to its activity in vivo.[7,8]

Recently, we[9,10] and others[11] isolated spontaneous POA-resistant *Mycobacterium tuberculosis* mutants in vitro by plating bacteria on agar containing POA, thereby avoiding selection of mutations in *pncA*. Missense mutations in the aspartate decarboxylase *panD*[9,11,12] and in the unfoldase/ATPase *clpC1*[10] were found to cause resistance to PZA and POA. Loss-of-function mutations of the phthiocerol dimycocerosate (PDIM) virulence factor producing polyketide synthases Mas and PpsA-E caused a lower level of resistance to PZA and POA.[9]

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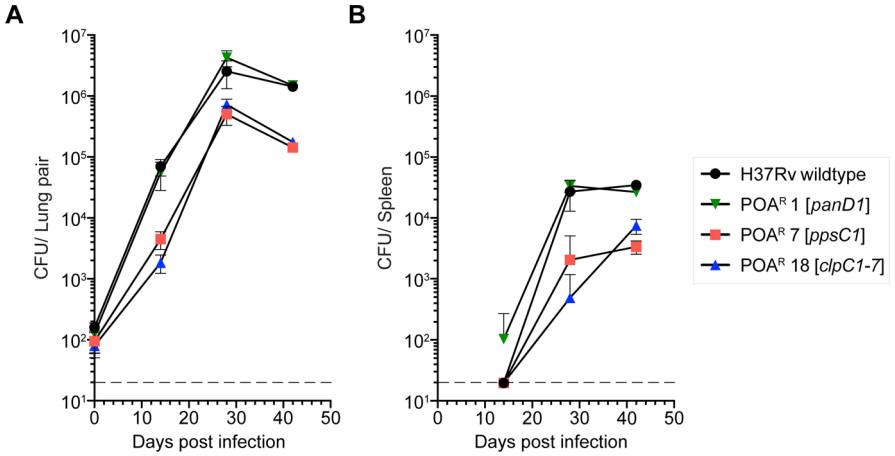


**Figure 1.** Growth in mice of wild-type *M. tuberculosis* H37Rv (ATCC 27294) and isogenic POA-resistant strains selected in vitro. Growth of POA$^R$ 1 [panD1], POA$^R$ 7 [ppsC1], and POA$^R$ 18 [clpC1-7] in the (A) lungs and (B) spleen of BALB/c mice is shown. Values represent the mean ± standard deviation of CFU counts obtained from four mice per group.

PanD is required for coenzyme A biosynthesis, and metabolic pathway analyses suggested PanD may be a direct target for POA; that is, inhibition of coenzyme A biosynthesis appears to be a mechanism of action of PZA.[9,11] Whether ClpC1, the unfoldase component of the caseinolytic protease complex, is a direct target of POA or whether missense mutations in this gene cause resistance by an indirect mechanism remains to be determined.[10] It also remains to be determined if the Mas-PpsA-E-based resistance is direct or indirect.[9]

An unsolved puzzle associated with PZA is the lack of clear evidence that mutations in genes other than *pncA* are associated with PZA resistance in clinical isolates.[12−15] Our finding that loss of PDIM virulence factor synthesis causes POA resistance abides with the hypothesis that spontaneous drug-resistant mutants that suffer a significant fitness cost in vivo may not survive or compete well with other drug-resistant mutants and hence cannot be identified in sputum isolates.[16] Here we first asked whether the in vitro isolated POA/PZA-resistant *panD*, *clpC1*, and *mas-ppsA-E* mutants are attenuated in vivo, therefore providing a possible explanation for the observation that these mutations are not prominent in clinical isolates. Then we asked whether the POA/PZA resistance mechanisms identified in vitro, that is, via selection of mutants on POA-containing agar, represent in vitro artifacts or whether these mechanisms can be recapitulated in vivo, via selection of mutants in POA-treated mice.

## RESULTS

**In Vitro Selected POA/PZA-Resistant *M. tuberculosis* Strains Harboring Mutations in *clpC1* and *mas/ppsA-E* Show Attenuated Growth in Vivo.** The current lack of evidence associating mutations in *panD*, *clpC1*, and *mas/ppsA-E* with PZA resistance in clinical isolates could be explained if such mutants suffer a fitness cost in vivo.[16] To address this hypothesis, we selected three isogenic POA/PZA-resistant *M. tuberculosis* mutants isolated previously in vitro (POA$^R$ 1 [*panD1*], POA$^R$ 18 [*clpC1-7*], POA$^R$ 7 [*ppsC1*]), representing the three different POA/PZA resistance-conferring genotypes with mutations in *panD*, *clpC1*, and *mas/ppsA-E*, respectively,[9,10] and carried out in vivo growth experiments in comparison with wild-type *M. tuberculosis*. Low-dose aerosol infection of BALB/c mice was performed, and colony-forming units (CFU) were quantified in lung and spleen homogenates obtained 2, 4, and 6 weeks postinfection. At 6 weeks postinfection, we confirmed that the POA-resistant strains POA$^R$ 1 [*panD1*] and POA$^R$ 18 [*clpC1-7*] had retained their relatively higher level resistance by plating lung homogenates on 7H11 agar containing 3 mM POA.

PDIMs, complex lipids residing in the cell wall of mycobacteria, have been well established as virulence factors, whereby loss of these lipids results in attenuation of *M. tuberculosis* in different animal models.[17−19] As expected, POA$^R$ 7 [*ppsC1*] containing a frameshift mutation in *ppsC*:Ins2674C as determined by whole genome sequencing in,[9] was significantly attenuated in both lungs and spleen as compared to the virulent wild-type strain (Figure 1), particularly during the early phase of infection in the lungs (Figure 1A). Interestingly, POA$^R$ 18 [*clpC1-7*] containing a mutation in *clpC1*:A625G/Lys209Glu, which has a higher level of POA resistance in vitro, mimicked the attenuation phenotype of POA$^R$ 7 [*ppsC1*] in both lungs and spleen (Figure 1), indicating that the mutation in *clpC1* causes in vivo fitness loss similar to loss of PDIMs over the first 6 weeks postinfection. In contrast, POA$^R$ 1 [*panD1*] containing a C-terminal mutation in *panD*:Δ380A displayed no defect in in vivo growth (Figure 1). H&E staining of one lung from four mice in each infection group revealed similar pathology in those infected with either wild-type or *panD* mutant strains throughout the course of infection. At 2 weeks postinfection, similar and diffuse lymphocytic infiltration was seen across all infection groups (not shown). At 4 weeks postinfection, mice infected with the *clpC1* and *ppsC* mutants had fewer visible granulomatous lesions and mostly perivascular lymphocytic infiltration, compared to the wild-type and *panD* mutant groups (Figure 2A). At 6 weeks postinfection, *clpC1*-infected mice had a lower number of granulomas ≥500 μm in diameter (Figure 2B). Ziehl−Neelsen staining showed clusters of acid-fast bacilli mostly in areas rich in foamy macrophages (Figure 2C−J). The abundance of bacilli in these clusters was clearly higher in mice infected with wild-type and *panD* mutant strains compared to those infected with *ppsC* and *clpC1* mutants (Figure 2G−J), which has been quantified in the Supporting Information, Table S1.

These results show that the previously in vitro selected POA/PZA-resistant strains harboring mutations in *clpC1* and *mas/ppsA-E* display attenuated growth in vivo, hence providing



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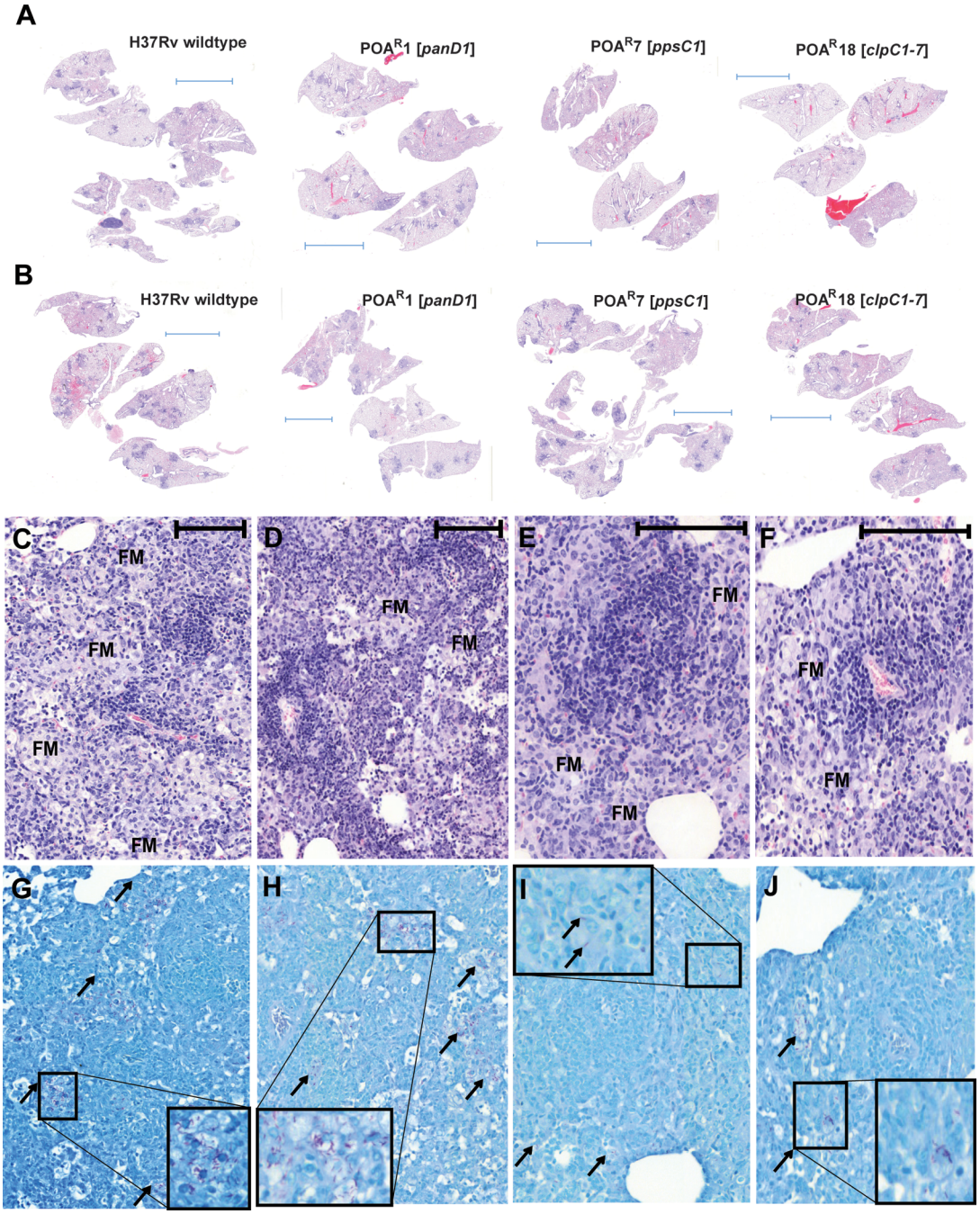


**Figure 2.** Comparative lung pathology induced by infection with POA-resistant mutants versus wild-type *M. tuberculosis* H37Rv (ATCC 27294). (A, B) Hematoxylin and eosin (H&E) staining of one lung from groups of four mice infected with H37Rv wild-type, POA$^R$ 1 [panD1], POA$^R$ 7 [ppsC1], and POA$^R$ 18 [clpC1-7] at 4 (A) and 6 (B) weeks postinfection. Blue scale bars for each group represent 5 mm. (C–J) High-power magnification images of representative individual granulomas in the corresponding infection groups at 6 weeks, stained by H&E (C–F) and Ziehl–Neelsen (G–J). (C–F) Typical granulomas contained lymphocyte aggregates with interspersed epithelioid histocytes and foamy macrophages on the outer rim of the granulomatous lesion. Black scale bars on top right for each group represent 100 $\mu$m. (G–J) Clusters of acid-fast bacilli were found mostly in areas rich in foamy macrophages (insets).

a possible explanation for their apparent absence in PZA-resistant clinical isolates. In contrast, the POA/PZA-resistant *panD* mutant displayed in vivo fitness indistinguishable from that of wild-type.

**In Vivo Selected POA/PZA-Resistant *M. tuberculosis* Strains Harbor Missense Mutations in *panD* and *clpC1*.** All reported *panD*, *clpC1*, and *mas/ppsA-E* POA/PZA resistance-conferring mutants have been selected on POA/PZA-containing agar,[9−12] that is, under in vitro culture conditions on rich 7H10/7H11 medium, and might hence represent in vitro artifacts. We therefore asked whether these resistance mechanisms can be recapitulated in selection experiments performed in vivo. BALB/c and C3HeB/FeJ mice were infected with *M. tuberculosis* wild type and treated with a range of POA and PZA doses for 8 weeks, and 28 apparent POA-resistant colonies were isolated from lung homogenates on POA-containing agar as described previously.[8] The details of the treatment and resistance mutation



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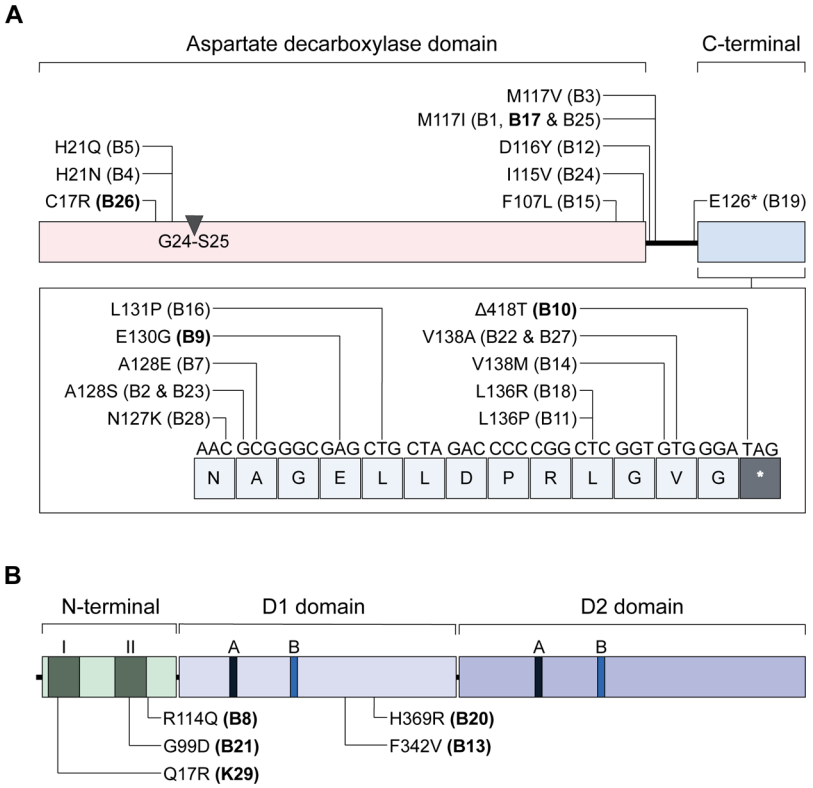


**Figure 3.** Location of amino acid sequence polymorphisms in (A) PanD and (B) ClpC1 of POA-resistant *M. tuberculosis* strains isolated from POA-treated mice. (A) Domain organization of PanD is shown as described in ref 20. The conserved autocleavage site between residues 24 (Gly) and 25 (Ser) in the aspartate decarboxylase domain is indicated. (B) Domain organization of ClpC1 is shown as described in ref 35. Within the N-terminal domain, two repeats are labeled I and II. A and B in the D1 and D2 domains indicate Walker A and Walker B motifs, respectively. The nine POA-resistant *M. tuberculosis* strains subjected to whole genome sequencing and described in Table 1 are labeled in bold.

frequencies are listed in the Supporting Information, Table S2.[8] To confirm POA resistance and carry out colony purification, the primary *M. tuberculosis* isolates were restreaked on agar containing 300 mg/L POA. As expected, all 28 suspected POA-resistant isolates grew, whereas the parental wild-type strain did not.

Because the in vivo-isolated strains displayed resistance to POA at a concentration higher than the MIC of in vitro-isolated low-level POA-resistant strains with loss of function mutations in *mas/ppsA-E*,[9] we expected them to harbor a resistance mechanism causing a higher level of POA resistance, such as mutations in *panD* or *clpC1* (with or without additional comutations in *mas/ppsA-E*).[9,10] On the basis of our observation that POA resistance-conferring *clpC1* mutations are attenuated in vivo, whereas *panD* mutations did not cause a growth defect in vivo, we hypothesized that *panD* mutations—if they do occur in vivo—may be found frequently in vivo. Targeted PCR sequencing of *panD* revealed that 23 (82%) of the 28 POA-resistant strains isolated after POA treatment of mice contained *panD* mutations observed in multiple selection experiments in vitro[9,11,12] (Supporting Information, Table S2). The parental strain (as expected) and the five remaining POA-resistant *M. tuberculosis* strains exhibited wild-type *panD* genes (Supporting Information, Table S2). Similar to the previously in vitro-selected *panD* mutations, the in vivo-selected *panD* mutations mainly cluster in the C-terminal region of PanD (outside the aspartate decarboxylase domain), with the exception of three strains (B4, B5, and B26) containing mutations near the autocleavage site (Gly24-Ser25[9]) and two strains (B15 and B24) with mutations at the C-terminal end of the aspartate decarboxylase domain (Figure 3A).[9,20,21]

Whole genome sequencing was carried out to identify the genetic basis of POA resistance in the five remaining in vivo-selected strains with wild-type *panD* genes (B8, B13, B20, B21, and K29). Whole genome sequencing was also performed for four strains containing various mutations in *panD* (B9, B10, B17, and B26) to detect potential common background mutations that may be contributing to POA resistance. As shown in Table 1, all five *panD* wild-type POA-resistant strains contained different missense mutations in *clpC1*. The mutations in *clpC1* were confirmed by targeted PCR sequencing. Similar to the previously in vitro-selected *clpC1* mutations, the in vivo-selected *clpC1* mutations are located in the N-terminal domain and in the D1 domain, outside the Walker A and Walker B motifs (Figure 3B).[10]

To verify that these strains display resistance levels similar to our previously in vitro selected POA-resistant *M. tuberculosis* strains containing *panD* or *clpC1* mutations, we performed MIC determinations for POA in broth as well as on agar in comparison with the susceptible parental H37Rv strain and representative in vitro isolated strains ($POA^R$ 1 [*panD1*] and $POA^R$ 18 [*clpC1-7*]). As shown in Table 1 and Supporting Information, Table S2 and Figure S1A, the strains selected in vivo displayed resistance to POA at levels similar to the in vitro-selected *panD* and *clpC1* mutant strains, that is, about a 4-fold increase in broth $MIC_{50}$ and at least a 4-fold increase in agar MIC values. It is interesting to note that one of the strains (POA B13) with a mutation in *clpC1*:T1024G/Phe342Val carries an additional frameshift mutation in *ppsA*:Ins2101C and


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**Table 1. Sequence Polymorphisms and Susceptibility to POA and PZA of POA-Resistant *M. tuberculosis* Strains Subjected to Whole Genome Sequencing**

| *M. tuberculosis H37Rv* strain | mutations | | | POA broth $MIC_{50}$[b] (mM) | PZA susceptibility result (S/R)[c] |
|---|---|---|---|---|---|
| | *panD*[a] | *clpC1*[a] | other genes | | |
| H37Rv parent | | | | 1.5 | S |
| POA B8 | | G341A/ Arg114Gln | | 5.5 | R |
| POA B9 | A389G/ Glu130Gly | | Rv0980c (PE-PGRS 18): C129A/ His43Gln | 6.0 | R |
| | | | Rv0980c (PE-PGRS 18): G217C/ Glu73Gln | | |
| | | | Rv0980c (PE-PGRS 18): A1088C/ Asn363Thr | | |
| | | | Rv2615c (PE-PGRS 45): G247A/ Gly83Ser | | |
| POA B10 | Δ418T | | Rv1230c: T1078G/Cys360Gly | 6.0 | R |
| POA B13 | | T1024G/ Phe342Val | *ppsA*: Ins2101C | 6.5 | R |
| | | | Rv1230c: T1078G/Cys360Gly | | |
| | | | *trpA*: A104G/Tyr35Cys | | |
| | | | *cycA*: A1297G/Thr433Ala | | |
| | | | *secA2*: G487T/Val163Leu | | |
| POA B17 | G351T/ Met117Ile | | Rv0007: G451A/Ala151Thr | 6.0 | R |
| | | | Rv3645: G502C/Ala168Pro | | |
| POA B20 | | A1106G/ His369Arg | Rv2061c: C39G/Tyr13* | 6.0 | R |
| | | | Rv2402: C1135A/Arg379Ser | | |
| POA B21 | | G296A/Gly99Asp | | 6.0 | R |
| POA B26 | T49C/Cys17Arg | | *ltp1*: C361A/Pro121Thr | 5.5 | R |
| | | | Rv3784: G965A/Gly322Asp | | |
| POA K29 | | A50G/Gln17Arg | *pitA*: Ins908G | 6.0 | R |
| | | | *embA*: A2695G/Thr899Ala | | |
| $POA^R$ 1 [*panD1*][d] | Δ380A | | | 6.0 | R |
| $POA^R$ 18 [*clpC1-7*][e] | | A625G/ Lys209Glu | | 6.0 | R |

[a]Polymorphisms were identified by whole genome sequencing and verified by targeted sequencing as described in the text. [b]$MIC_{50}$, POA concentration that inhibits 50% of growth compared to drug free control. Drug susceptibility tests were carried out three times independently, and mean values are shown. [c]BACTEC MGIT 960 test for susceptibility (S) or resistance (R) to 100 μg/mL PZA. [d]$POA^R$ 1 [*panD1*] was selected in vitro as described in ref 9. [e]$POA^R$ 18 [*clpC1-7*] was selected in vitro as described in ref 10.

that this double-mutant strain may display a marginally elevated level of POA resistance (Table 1).[9] An additional polymorphism in Rv1230c (T1078G/Cys360Gly) was observed in two POA-resistant strains isolated from different mice. Whether this polymorphism is involved in POA resistance is questionable as it does not appear to result in an increase of resistance to POA. Moreover, this polymorphism has been observed occasionally in isolates obtained from mice treated with other drugs unrelated to PZA in independent unpublished experiments by some of the authors (R.T., J-P.L., and E.N.) who used the same parental strain. Therefore, we believe that the appearance of this polymorphism in the present experiment likely represents genetic heterogeneity in the parental strain. To confirm that POA resistance correlates with resistance to the PZA prodrug, we demonstrated that all 28 POA-resistant strains isolated from POA-treated mice were also resistant to PZA using the BACTEC MGIT 960 PZA susceptibility test as well as agar MIC determination (Table 1 and Supporting Information, Table S2). In contrast, MICs of two other first-line TB drugs, rifampicin and isoniazid, were similar in POA-resistant strains and the wild-type H37Rv strain (Supporting Information, Figure S1B,C), indicating that the described mutations in *panD* and *clpC1* do not confer nonspecific antibiotic resistance.

Taken together, these results show that missense mutations in *panD* and *clpC1* do occur under POA pressure in vivo and, hence, do not represent an in vitro artifact.

**Selective Amplification of POA-Resistant Mutants in Vivo Occurs Only with High Systemic POA Exposures and Does Not Occur with a Standard Dose of PZA.** PZA is converted to POA by the host as well as *M. tuberculosis*.[7] Our previous mouse efficacy study showed that only POA doses producing systemic POA exposures higher than those observed in humans after administration of standard PZA doses produce bactericidal effects in mice and, even then, the magnitude of these effects is much lower than those observed with standard PZA doses.[5,8] However, we did not previously examine the effect of POA dose on the selective amplification of POA-resistant mutants. Therefore, we reanalyzed the drug-susceptible and POA-resistant CFU counts from the previous experiment and found that only POA doses exhibiting bactericidal activity against the total, drug-susceptible population (i.e., daily doses of 450−900 mg/kg) resulted in selective amplification of POA-resistant mutants compared to untreated animals (mean proportion of POA-resistant CFU after 8 weeks of POA treatment: $1.2 \times 10^{-6}$, $1.1 \times 10^{-6}$, and $6.5 \times 10^{-6}$ for mice receiving 0, 27.5−150, and 450−900 mg/kg of POA daily, respectively [$p < 0.0001$ for high-dose group vs other groups]) (Supporting Information, Figure S2). After 8



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weeks of treatment, 27 (90%) of 30 BALB/c mice treated with a range of POA doses harbored POA-resistant mutants in the lungs, compared to 1 (20%) of 5 mice treated with PZA at 150 mg/kg and 4 (80%) of 5 untreated mice ($p = 0.003$) for POA versus PZA treatment. The low proportion of mice harboring POA-resistant bacteria after treatment with PZA at 150 mg/kg contrasts markedly with results of another previous study,[5] in which selective amplification of PZA-resistant *pncA* mutants was observed after 8 weeks of treatment in five (100%) of five BALB/c mice receiving PZA at 100 mg/kg and in four (57%) of seven evaluable mice receiving 300−900 mg/kg/day. Thus, PZA treatment appears more likely to select for PZA-resistant *pncA* mutants than for *panD* and *clpC1* mutants that are resistant to both PZA and POA, and POA treatment only selectively amplifies the latter mutants at systemic POA exposures (i.e., mean plasma AUC > 300 μg·h/mL) above those observed in humans after standard PZA doses.[7,8]

## DISCUSSION AND CONCLUSION

PZA is a critical first-line TB drug owing to its treatment-shortening effects. Due to its remarkable properties, it is also a component of many novel drug combinations that are in development for treatment of both drug-susceptible and multidrug-resistant TB.[2] This is accompanied, however, by an alarming prevalence of PZA resistance in >50% of MDR-TB isolates.[22,23] The vast majority of PZA-resistant clinical isolates (70−97%) contain mutations in the *pncA* gene encoding the prodrug-activating pyrazinamidase,[24] where a highly diverse and scattered range of mutations has been described.[13−15] However, aside from evidence linking mutations in *rpsA* to PZA resistance,[25] the basis for resistance in the minority of PZA-resistant clinical isolates with wild-type *pncA* sequence remains undefined.

Previous in vitro selection of *M. tuberculosis* mutants resistant to POA, the bioactive component of PZA, identified three different resistance mechanisms. A higher level of resistance to POA is caused by (i) missense mutations in *panD*,[9,11] which encodes the aspartate decarboxylase involved in de novo pantothenate and coenzyme A biosynthesis, and (ii) missense mutations in *clpC1*, which encodes the unfoldase/ATPase of the caseinolytic protease complex.[10] A lower level of POA resistance is caused by frameshift mutations in *mas* and *ppsA-E*, which encode polyketide synthases responsible for PDIM synthesis.[9] However, to date, no significant association has been found between mutations in *panD* and PZA resistance in clinical isolates.[26,27] Similarly, we did not find any clinical isolates in the Genome-wide *M. tuberculosis* Variation Database,[28] containing the polymorphisms in *clpC1* that we identified in POA-resistant *M. tuberculosis* strains selected in vitro.[10] It has been speculated that acquisition of PZA resistance, including *pncA* mutations, results in a fitness cost that limits person-to-person transmission.[16] We further hypothesized that the PZA/POA resistance mechanisms identified in vitro compromise the fitness of *M. tuberculosis* and limit the selective amplification of these mutants in vivo. If this is true, it would provide one explanation for the current lack of evidence for such mutations among PZA-resistant clinical isolates. To test this hypothesis, we compared the growth of representative in vitro-selected *panD*, *clpC1*, and *ppsC* mutants with that of the virulent wild-type parental strain in BALB/c mice.

The PDIM-deficient strain *M. tuberculosis* POA$^R$ 7 [*ppsC1*] displayed a similar pattern of growth attenuation in lungs and spleens of BALB/c mice infected via aerosol as described previously resulting from the loss of PDIMs.[29,30] PDIMs are complex lipid virulence factors of *M. tuberculosis* and loss of these lipids on the bacterial cell surface has been known to cause attenuation in mice[18,19] and guinea pigs.[17] This is attributed to the critical role of PDIMs in multiplication of *M. tuberculosis* during the acute phase of infection,[29] whereby the lack of these lipids makes *M. tuberculosis* susceptible to killing by early innate immune host response.[30]

Interestingly, despite having a somewhat higher level of PZA/POA resistance, the strain POA$^R$ 18 [*clpC1-7*] displays a similar attenuation phenotype, implying that the polymorphism in *clpC1* affects the growth of *M. tuberculosis* during the early phase of infection. Our finding that a POA resistance mutation in *clpC1* imposes a fitness cost on *M. tuberculosis* in vivo suggests that these mutations may be at a competitive disadvantage with regard to amplification within the host and person-to-person transmission. It is noted that complementation studies are required to rule out that unidentified non-ClpC1 polymorphisms elsewhere in the genome of the strain POA$^R$ 18 [*clpC1-7*] may contribute to the observed growth behavior. On the other hand, our representative POA-resistant strain with a mutation in the C-terminal of *panD*:POA$^R$ 1 [*panD1*] displayed no growth defect in vivo. This finding suggests that *panD* mutants are more likely than *clpC1* or *mas*/*ppsA-E* mutants to be selectively amplified by POA treatment in vivo.

To test this hypothesis and to verify that the resistance mechanisms that we identified in vitro could be recapitulated in vivo, we characterized POA-resistant isolates previously obtained from *M. tuberculosis* wild-type infected mice treated with a range of POA doses.[8] Indeed, as predicted, sequencing of 28 POA-resistant *M. tuberculosis* strains isolated from different mice revealed that the vast majority of the strains (82%) contained various mutations in *panD*. PanD is involved in de novo production of pantothenate, which comprises the first stage of the biosynthesis of the essential cofactor coenzyme A. The *panD* gene was shown to be essential in vitro for *M. tuberculosis* growth.[31,32] Furthermore, an auxotrophic mutant of *M. tuberculosis* lacking *panC* and *panD* was highly attenuated in vivo,[32] implying that this pathway is crucial for the survival and growth of *M. tuberculosis* in vivo. Recently, we demonstrated that treatment with POA depletes intracellular pantothenate and coenzyme A levels,[9] later confirmed by ref 33. Resistance to POA/PZA and prevention of this POA-mediated coenzyme A depletion in vitro can be caused by mutations in *panD*[9,11,12] or by exogenous supplementation of pantothenate.[9,11,34] Mutations in *panD* causing POA/PZA resistance both in vitro and in vivo are almost exclusively localized in the C-terminal of PanD, which lies outside the aspartate decarboxylase domain and comprises a 13 amino acid tail, which is specific to mycobacteria,[20] indicating that this region of the protein may be involved in interaction with POA.[9] Despite some evidence suggesting that POA inhibits PanD enzyme activity,[11] whether POA directly interacts with PanD and how the different PanD mutations affect this interaction are currently under investigation.

In this study, we also confirmed the hypothesis that PZA/POA resistance causing mutations in *clpC1* are less likely to be found in vivo by demonstrating that only 5 of 28 POA-resistant strains (18%) selected in mice contained mutations in *clpC1*, whereas in vitro they were identified at more than twice this frequency.[10] *M. tuberculosis* ClpC1 works together with the



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ClpP1 and ClpP2 proteins of the caseinolytic protease complex and displays unfoldase and ATPase activities.[35,36] The Clp protease complex is crucial for viability of *M. tuberculosis* both in vitro and in vivo,[37,38] whereas the *clpC1* gene on its own has been demonstrated to be essential for growth in vitro[31] and within macrophages.[39] Thus, drugs targeting the Clp protease machinery are considered attractive.[40,41] Our finding that the mechanism of an established treatment-shortening TB drug, PZA, involves ClpC1 and that resistance mutations in this gene cause attenuation in vivo is of particular significance as novel antimycobacterials discovered recently, such as cyclomarin A,[42] lassomycin,[43] and ecumicin,[44] target ClpC1, and resistance against these compounds in vitro is attributed to mutations in *clpC1*. Curiously, one of the polymorphisms (Gln17Arg) identified in this work as causing PZA/POA resistance was previously found to cause resistance to lassomycin and lies in the highly acidic portion of the N-terminal repeat I. Gln17 was found to be the major interacting residue with lassomycin via formation of H-bonds.[43] However, the exact role of *clpC1* in the mechanisms of action and resistance involving PZA and POA remains to be established.

Our findings shed some light on why mutations in *panD*, *clpC1*, and *mas/ppsA-E* may be difficult to find among PZA-resistant clinical isolates. First, mutations in these three genes were associated with relatively small (between 2- and 8-fold) shifts in susceptibility to PZA and POA, whereas *pncA* mutations conferring PZA resistance are associated with much higher (≥20-fold) increases in PZA MIC.[4,45] To investigate whether previous studies may have missed *panD* or *clpC1* mutations in clinical isolates due to their relatively low-level PZA resistance, we analyzed 1849 publicly available genomes of *M. tuberculosis* clinical isolates from the Genome-wide *M. tuberculosis* Variation (GMTV) Database. None of the strains contained any of the polymorphisms in *clpC1* that we find associated with POA/PZA resistance in the current or previous study.[10] We, however, found eight isolates containing the C-terminal mutation G415T/Gly139Stop in *panD*. Seven of these isolates were categorized as PZA-susceptible, whereas one was described as PZA-resistant.[13] This identical polymorphism in *panD* was also observed in a PZA-susceptible clinical isolate from Russia, and the authors suggested that these strains may have been wrongly classified as PZA-susceptible due to the limitations of the BACTEC MGIT 960 PZA susceptibility test (which detects PZA resistance only at a single concentration of 100 μg/mL) in measuring low-level PZA resistance.[27] The greater level of PZA resistance conferred by *pncA* mutations is expected to give a greater survival advantage in the face of PZA treatment. This argument is reinforced by our data showing that PZA monotherapy in mice is more likely to select for *pncA* mutants than for *panD* or *clpC1* mutants.[5] Moreover, whereas *pncA* mutations do not appear to reduce *M. tuberculosis* fitness in vitro or in vivo,[14] our finding that mutations in *clpC1* and *ppsC* cause growth defects in vivo provides another selective advantage favoring amplification of *pncA* mutants under PZA treatment. Because PZA is converted to POA by the host in addition to the pathogen, even *M. tuberculosis* cells with *pncA* mutations are exposed to circulating concentrations of POA that might provide additional selective pressure for POA resistance mutations. For example, we previously showed comparable plasma POA AUCs whether mice were treated orally with PZA or POA at 150 mg/kg.[7,8] Host-metabolized POA exposures after standard PZA doses are similar to these mouse exposures but are likely too low to exert significant selection pressure in vivo,[7] because only POA doses of 450 mg/kg and higher produced bactericidal effects in mice and, even then, the magnitude of these effects is much lower than those observed with standard PZA doses.[5,8] We reanalyzed the quantitative drug-susceptible and POA-resistant CFU data from the prior mouse experiment[5,8] and found that only the POA doses that had bactericidal activity (i.e., daily doses of 450−900 mg/kg) resulted in selective amplification of POA-resistant mutants compared to untreated animals. Thus, the greater selective advantage of *pncA* mutations that prevent intrabacillary conversion of PZA to POA, the requirement for systemic POA exposures higher than those produced by host metabolism of PZA to POA to selectively amplify POA-resistant mutants in vivo, and the reduced fitness of *clpC1* and *mas/ppsA-E* mutants likely all interact to limit the selection of POA-resistant mutants in vivo. Finally, it must be noted that, due to the relatively small shift in PZA susceptibility conferred by *panD*, *clpC1*, and *mas/ppsA-E* mutations, current breakpoints for identification of PZA resistance in clinical isolates also may not identify these mutants as being PZA-resistant.

In conclusion, we show that the POA/PZA resistance mechanisms due to *panD* and *clpC1* missense mutations previously identified in vitro can be recapitulated in vivo. Strains harboring *clpC1* mutations were attenuated in mouse infection models, indicating loss of in vivo fitness and hence providing a possible explanation for the absence of such mutations in clinical isolates. Given the unimpaired fitness of *panD* mutants, we offer hypotheses relating to in vivo POA exposure and the clinical diagnosis of PZA resistance to explain why *panD* mutations are not observed more commonly among the small minority of PZA-resistant clinical isolates that do not harbor *pncA* mutations.

## MATERIALS AND METHODS

**Bacterial Strains, Culture Conditions, and Chemicals.** The parental strain *M. tuberculosis* H37Rv (ATCC 27294) was used to obtain representative POA-resistant strains: $POA^R$ 1 [*panD1*], $POA^R$ 7 [*ppsC1*], and $POA^R$ 18 [*clpC1-7*] as described in refs 9 and 10. Additional POA-resistant strains were selected by 8 weeks of POA treatment in BALB/c or C3HeB/FeJ mice infected with the parental strain as described in ref 8 and were shipped to the biosafety level 3 facility at the National University of Singapore, where the phenotypic and genotypic characterization detailed in this study was carried out. All strains were maintained in complete Middlebrook 7H9 medium (BD Difco) supplemented with 0.05% (v/v) Tween 80 (Sigma-Aldrich), 0.5% (v/v) glycerol (Fisher Scientific), and 10% (v/v) Middlebrook albumin−dextrose−catalase (BD Difco) at 37 °C with agitation at 80 rpm. Pyrazinamide, pyrazinoic acid, isoniazid, and rifampicin were purchased from Sigma-Aldrich and were freshly dissolved in 90% DMSO (Merck) and sterilized using 0.2 μm PTFE membrane filters (Acrodisc PALL). POA-resistant strains selected in mice were first subjected to colony purification and resistance confirmation on Middlebrook 7H10 agar (BD Difco) supplemented with 0.5% (v/v) glycerol and 10% (v/v) oleic acid−albumin−dextrose−catalase (OADC) (BD Difco) containing 300 mg/L POA. Isolated colonies were expanded in 7H9 to an $OD_{600}$ of 0.7−0.8 and stored in 25% glycerol in 1 mL aliquots at −80 °C.

**BALB/c Mouse Infection with *M. tuberculosis*.** Pathogen-free female BALB/c mice aged 8 weeks were purchased from Charles River Laboratories. Mice were group-housed in a biosafety level 3 animal facility and maintained with sterile


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ACS Infect. Dis. 2017, 3, 492−501

bedding, water, and mouse chow. This study was performed under strict accordance with recommendations outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health with protocol 140020D017 approved by Rutgers University's Institutional Animal Care and Use Committee.

The *M. tuberculosis* wild-type strain (H37Rv) and mutant strains POA$^R$ 1 [*panD1*], POA$^R$ 7 [*ppsC1*], and POA$^R$ 18 [*clpC1-7*] were used for infection. BALB/c mice were infected in a Glas−Col inhalation exposure system. Titered frozen bacterial stocks were diluted in phosphate-buffered saline (PBS), and $3 \times 10^6$ CFU/mL was added to the nebulizer to achieve the implantation of 100−300 CFU in the lungs. Three mice were sacrificed via cervical dislocation 24 h later to determine the starting bacteria load.

BALB/c mice ($n$ = 4 per group per time point) were sacrificed at 2, 4, and 6 weeks postinfection. Right lung and spleen were collected post-mortem and homogenized in 5 mL of PBS containing 0.05% Tween 80. CFU was determined by plating serial dilutions of homogenates onto Middlebrook 7H11 agar with OADC (GIBCO BRL). At week 6, lung homogenates were also plated on 7H11 agar containing 3 mM POA to determine resistance. Colonies were counted after at least 21 days of incubation at 37 °C.

At weeks 4 and 6 postinfection, formalin-fixed (Fisher Chemical) lung tissues of infected mice (one lung from four mice in each group) were paraffin-embedded and used for standard 5 $\mu$m sectioning. Tissue sections were stained with hematoxylin/eosin (H&E) for cellular composition or with Ziehl−Neelsen to reveal acid-fast mycobacteria. To quantify the acid-fast bacilli in lesions from mice infected with the different strains described above, five lesions were randomly picked from each group and acid-fast bacilli were counted at a magnification of 50× and field size 340 by 228 $\mu$m. Numbers are reported either as single bacilli or clusters, if there were too many bacilli per macrophage. Each cluster is representative of a single macrophage, and the range of numbers of bacilli within the respective clusters is reported.

**Susceptibility Testing.** MICs against POA, isoniazid, and rifampicin were performed by the broth dilution method. The strains were grown to mid log phase, spun down, resuspended in fresh 7H9 media, and adjusted to an $OD_{600}$ = 0.1. One hundred microliters of cell suspension was added into wells containing 100 $\mu$L of 2-fold serially diluted compound in transparent flat-bottomed 96-well plates (Corning Costar), sealed with Breath-Easy membranes (Sigma-Aldrich). The plates were incubated for 7 days at 37 °C with shaking at 80 rpm. After incubation, the cultures were manually resuspended and $OD_{600}$ was measured using a spectrophotometer (Tecan Infinite M200 Pro). Experiments were performed three times independently with technical replicates. $MIC_{50}$ values represent the concentration of drug that inhibits bacterial growth by 50% as compared to the respective drug-free control.

Susceptibility to PZA was assessed using the BACTEC MGIT 960 PZA susceptibility test following the guidelines as described in ref 46.

PZA and POA agar MICs were defined as the concentration of drug that suppresses colony formation upon plating $10^4$ CFU from mid log cultures on 7H10 agar plates in independent experiments and incubating them for 2 weeks at 37 °C as previously described.[10,47]

**Whole Genome Sequencing.** Genomic DNA was isolated from the different *M. tuberculosis* strains and subjected to whole genome sequencing on an Illumina MiSeq platform with AIT Biotech (Singapore). Sequencing and subsequent analysis was performed as described in ref 9.

***panD* and *clpC1* Sequencing.** The *panD* gene was amplified from the genomic DNA using the primers 5′-AGCTGCGCGATATCGGGCTT-3′ and 5′-TGCACGACC-TTTGCGTGCTCTT-3′ and *clpC1* with the primers 5′-ACATATGTTCGAACGATTTACCGACCGTGC-3′ and 5′-TGAATTCACCCATGTCAATCTGAATAAGCGC-3′ or 5′-GATGATGTCACCGCGGGTGTTG-3′ using Phusion High Fidelity DNA polymerase (Thermo Scientific) as per the manufacturer's instructions. The purified PCR products were subjected to capillary sequencing via BigDyeTerminator chemistry by AIT Biotech (Singapore) and analyzed using BioEdit (North Carolina State University) and BLAST (NCBI).

**Analysis of Selective Amplification of POA-Resistant Mutants in Vivo.** CFU count data obtained from lung homogenates plated on 7H11 plates with or without POA from the POA dose-ranging mouse efficacy experiment,[8] in which the POA-resistant mutants were selected, were reanalyzed to quantify and compare the selective amplification of resistant mutants during treatment of BALB/c mice with POA or PZA. Mice received a low-dose aerosol infection followed 4 weeks later by initiation of treatment with PZA 150 mg/kg alone or POA alone in one of the following doses (in mg/kg body weight): 37.5, 75, 150, or 450, once daily, or 75 or 450 twice daily. Total and POA-resistant CFU after 8 weeks of treatment were quantified by plating serial dilutions of lung homogenates on drug-free plates and plates containing POA 300 $\mu$g/mL (2−3× MIC for H37Rv parent), respectively. Mean frequencies of drug-resistant CFU were compared by one-way ANOVA with Dunnett's post-test to control for multiple comparisons. Additionally, the proportions of PZA-treated mice and POA-treated mice harboring POA-resistant mutants after 8 weeks of treatment were compared by Fisher's Exact test. Statistical analyses were performed using GraphPad Prism 6.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00017.

Growth inhibition dose response curves for POA-resistant *M. tuberculosis* strains, selection of POA-resistant mutants in vivo as a function of POA dose, abundance of acid-fast bacilli within lesions after infection with POA-resistant mutants versus wild-type *M. tuberculosis* H37Rv, phenotypic and genotypic characteristics of POA-resistant *M. tuberculosis* with details for 28 strains isolated from mouse lungs (PDF)

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### Author Contributions

P.G., V.D., E.N., and T.D. designed the experiments and wrote the manuscript. P.G. and M.Y. characterized the POA-resistant strains isolated from POA-treated mice. J.-P.L., R.T., and E.N. carried out the POA treatment experiments in mice, isolated POA-resistant strains, and initially characterized their POA resistance. J.S., G.R., L.L., and V.D. carried out the mouse infection experiments with in vitro isolated POA/PZA-resistant mutants.

### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

TB, tuberculosis; PZA, pyrazinamide; POA, pyrazinoic acid; PDIM, phthiocerol dimycocerosate

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