Tuberculosis chemotherapy is dependent on the use of the antibiotic pyrazinamide, which is being threatened by emerging drug resistance. Resistance is mediated through mutations in the bacterial gene \textit{pncA}. Methods for testing pyrazinamide susceptibility are difficult and rarely performed, and this means that the full spectrum of \textit{pncA} alleles that confer clinical resistance to pyrazinamide is unknown. Here, we performed in vitro saturating mutagenesis of \textit{pncA} to generate a comprehensive library of PncA polymorphisms resultant from a single-nucleotide polymorphism. We then screened it for pyrazinamide resistance both in vitro and in an infected animal model. We identify over 300 resistance-conferring substitutions. Strikingly, these mutations map throughout the PncA structure and result in either loss of enzymatic activity and/or decrease in protein abundance. Our comprehensive mutational and screening approach should stand as a paradigm for determining resistance mutations and their mechanisms of action.
Tuberculosis (TB) is a global public health challenge that caused 1.4 million deaths in 2015. Antibiotic chemotherapy is a mainstay of TB control programs, however, the emergence of drug resistance is a significant problem, with an estimated 580,000 cases in 2015. Standard chemotherapy for TB requires a 6-month regimen of a combination of four antibiotics: rifampicin (RIF), isoniazid (INH), ethambutol, and pyrazinamide (PZA). PZA is a cornerstone of current and future, TB treatment regimens. Its introduction into TB chemotherapies was critical for reducing treatment to 6 months. PZA is also under evaluation in new regimens designed to further reduce treatment duration and more effectively treat drug resistant TB. Despite its central role and ubiquitous use in TB therapy, PZA drug-susceptibility testing (DST) is not widespread, nor part of the World Health Organization recommendations. To improve treatment outcomes and reduce transmission, prompt diagnosis of PZA drug resistance is paramount. The development of rapid and reliable DST is therefore a priority for TB control.

The current gold-standard for PZA DST is a whole-cell phenotypic approach using the BD BACTEC MGIT 960 system. Unfortunately, testing is notoriously challenging and unreliable, especially in clinical isolates. The limitations and inconsistencies result from the difficulty of growing TB under acidic (pH < 6.0) conditions required by the assay. Inoculum size effects (large inoculums increase false resistance rates) and growth-phase-dependent activity (limited activity against rapidly growing bacilli) have been found in susceptible isolates. As a result, no genetic “hot-spot” region comprising highly penetrant mutations has been identified. An attractive molecular diagnostic alternative is full gene sequencing. However, because PZA susceptibility testing is rare, the entire spectrum of pncA alleles conferring clinically significant PZA resistance has not been defined and polymorphisms in PncA have been found in susceptible isolates. Thus, a molecular diagnostic for PZA resistance is currently impractical and would require a comprehensive and systematic assessment of mutations conferring PZA resistance.

Here, we took an unbiased approach to assess the impact of all pncA single-nucleotide polymorphisms (SNPs) on PZA susceptibility. We generated a comprehensive library of pncA mutations in Mycobacterium tuberculosis (Mtib) using random PCR mutagenesis, and screened the pooled library for PZA resistance both in vitro and in infected mice. We identified a large repertoire of resistance-conferring substitutions, some previously seen in clinical isolates and, importantly, many that have not. We find

**Fig. 1** Generation of a comprehensive pncA mutant library in *M. tuberculosis*. a Frequency (%) of pncA single-nucleotide polymorphisms in five pncA libraries generated by random PCR mutagenesis using different template concentrations (DNA ng) and cycle numbers (n). n corresponds to the number of single colonies sequenced from each library and the library selected for screening. b The mean pncA SNP frequency (%) from three biological replicates at each nucleotide of our candidate library relative to the mean of three biological replicates of an unmutagenized wild-type control determined using Illumina sequencing. c Minimum inhibitory concentration (MIC, μg ml⁻¹) of pyrazinamide (PZA) and pyrazinoic acid (POA) for three isogenic control strains, WT, pncA-null, and Comp, in pH 5.9 (Acid) and pH 6.8 (Std) media. d Growth inhibition of four isogenic control strains, WT, pncA-null, Comp and a vector control (VC), and the pncA library with increasing concentrations of pyrazinamide in pH 5.9 (acidic) media. Time-to-positivity (TTP) ratio is the mean of the time-to-positivity of the test condition relative to the mean of the no-drug control. A minimum of three biological replicates per strain per test condition was performed. Error bars represent the standard deviations derived from the propagation of error using the quotient of the coefficient of variation from each condition. NG no growth
that resistance results largely from a loss of PncA enzymatic activity and/or protein abundance, which can be empirically determined but not easily predicted from the primary sequence. Furthermore, we find many substitutions that do not confer resistance to PZA. This catalog of PncA substitutions thus provides a comprehensive roadmap for the accurate molecular characterization of PZA resistance. Our comprehensive mutational and screening approach should stand as a paradigm for determining resistance mutations and their mechanisms of action.

**Results**

**PncA mutant library.** To systematically assess the phenotype of all pncA SNPs, we constructed libraries of pncA mutants using error-prone, random PCR mutagenesis (Fig. 1a). A candidate library, containing the highest proportion of SNPs, was transformed into an H37Rv pncA null strain (pncA-null). The resultant library was characterized by Illumina sequencing of full-length pncA amplicons, with reads containing multiple mutations, or indels discarded. SNPs were markedly enriched within the coding region of pncA relative to an unmutagenized wild-type (WT) control (Fig. 1b), averaging 4.5-fold higher at each nucleotide. Therefore, despite the presence of WT pncA within our library, our sequencing was sufficiently sensitive to detect mutations above the background error rate throughout the gene.

**In vitro selection of pyrazinamide resistant substitutions.** As many factors influence the reproducibility of in vitro PZA DST, we used the BD BACTEC MGIT 960 system with standardized inoculums from titered frozen stocks (Supplementary Fig. 1). We first defined the minimum inhibitory concentrations (MICs) (Fig. 1c) for our control strains. In acid (pH 5.9) media, the pncA-null strain was highly resistant with a MIC of >1000 μg ml−1. In contrast, a pncA-null strain complemented with a WT pncA gene (Comp) was hypersusceptible to PZA relative to the parental H37Rv strain (WT), with MICs of 1 and 20 μg ml−1, respectively. RT-qPCR confirmed a 4-fold over-expression of pncA (Supplementary Fig. 2), consistent with our use of the strong mycobacterial optimized promoter (MOP) to drive pncA transcription. The PZA hypersusceptibility in our complement strain increases the dynamic range in which PZA susceptibilities can be tested. To define this further we measured growth inhibition in the presence of increasing PZA concentrations (Fig. 1d). The growth of the Comp strain was inhibited even at the lowest concentration tested, with all growth suppressed at concentrations greater than 100 μg ml−1. There were no growth differences in any strain after treatment with POA (Supplementary Fig. 3), indicating that PZA susceptibility is solely due to enzymatic activation by PncA. Growth inhibition was greatest in acid media (Fig. 1d and Supplementary Fig. 3) and was thus the conditions used for our in vitro screen.

To enrich for resistant clones, we exposed our pooled pncA mutant library to six sequential rounds of in vitro selection using a range of PZA concentrations (Fig. 2a). As expected, the rate of growth, determined by the time-to-positivity (TTP), increased with each successive selection (Fig. 2b and Supplementary Fig. 4), consistent with the enrichment of resistant clones. After Illumina sequencing of full-length pncA amplicons, the average mutation frequency at pncA nucleotides changed progressively after each successive PZA selection (Supplementary Fig. 5). Importantly, there was minimal variation in the average mutation frequency at pncA nucleotides in the absence of PZA (Supplementary Fig. 6), indicating that selection only occurred in the presence of drug.

We then identified polymorphisms under positive PZA selection across all drug concentrations and selections. We first excluded mutations that were both under-represented and showed no evidence of selection (Supplementary Fig. 7), yielding a library representation of ~88% (N = 977) of all non-synonymous amino acid substitutions resultant from a SNP (Supplementary Data 1 and 2). We thus identified a total of 264 enriched SNPs (Supplementary Data 1), resulting in 191 SNPs, we constructed libraries of pncA mutants using error-prone, random PCR mutagenesis (Fig. 1a). A candidate all pncA mutant library

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unique non-synonymous amino acid substitutions (Supplementary Data 2). An additional 210 non-synonymous amino acid substitutions whose abundance decreased after PZA selection were also observed (Supplementary Data 1 and 2).

Selection of pyrazinamide resistant substitutions in mice. It is unknown whether in vitro PZA selection faithfully recapitulates susceptibilities observed during infection. We therefore subjected our pncA mutant library to PZA selection in mice. We infected BALB/c mice with the pooled pncA mutant library by tail-vein injection, resulting in an average implantation of $5.97 \times 10^5$ colony-forming units (CFU) in the spleen (Fig. 2c), corresponding to >300-fold library coverage. The average CFUs in the spleens increased over 42 days in the mock control group (phosphate-buffered saline (PBS) treatment) but declined from day 21 in the PZA treated mice, although the difference was not statistically significant (Fig. 2c).

We identified a total of 320 enriched SNPs that were positively selected by PZA after either 21 or 42 days of treatment corresponding to 251 unique non-synonymous amino acid substitutions (Supplementary Data 1 and 2). For those enriched after both 21 and 42 days of PZA treatment the degree of selection was on average higher after 42 days of treatment, indicating ongoing selection (Supplementary Fig. 8). An additional 219 non-synonymous amino acid substitutions whose abundance decreased after PZA treatment were also observed (Supplementary Data 1 and 2). Importantly, after 42 days of mock treatment, no amino acid substitutions were significantly increased in abundance relative to the implantation library demonstrating pncA SNPs have minimal fitness costs during infection and positive selection only occurred in the presence of drug (Supplementary Fig. 9).

Enriched mutations confer pyrazinamide resistance. To determine if enrichment during selection in our screens correlated with PZA susceptibility, we isolated single clones from our pncA mutant library and assayed their MICs to PZA using the BD BACTEC MGIT 960 system (Supplementary Table 1). We found that 100% of clones that were enriched either in vitro or during infection in mice had an elevated MIC (>4-fold increase compared to the Comp control). Similarly, for clones not enriched in either screen, 90% were found to be susceptible. These results demonstrate that our PZA susceptibilities predicted from both screens accurately identifies substitutions that confer resistance to PZA.

Catalog of pyrazinamide resistant amino acid substitutions. Having confirmed that enrichment in our screens accurately predicts PZA resistance we constructed a comprehensive catalog of amino acid substitutions that result in PZA resistance (Supplementary Data 2). We compared amino acid substitutions enriched in vitro to those enriched after infection in mice (Fig. 3a). After exclusion of 26 amino acid substitutions that were enriched in one screen but depleted in the other, we found a high concordance. However, more resistant substitutions were found only after infection in mice than after selection in vitro, suggesting PZA selection in vitro does not fully recapitulate sensitivities observed during infection, with greater selective pressure observed during infection in mice.

In total, of a possible 977 non-synonymous amino acid substitutions resulting from a SNP, 301 (31%) were classified as conferring PZA resistance based on enrichment after in vitro and/or mouse selection. A further 310 (32%) were depleted in one or both selections and we can be confident these do not confer resistance (Supplementary Data 2). The remaining 366 non-synonymous amino acid substitutions, not selected after PZA treatment in our screens, are also likely to be susceptible based on our validation of individual clones (Supplementary Table 1). However, it is possible that some of these substitutions will confer a weak PZA resistance phenotype not apparent using conventional in vitro DST. Importantly, of 37 possible nonsense mutations 32 were classified enriched while 0 were depleted. As nonsense mutations are expected to confer PZA resistance, we can be confident in our selection criteria. Therefore, while many PncA amino acid substitutions confer PZA resistance, the majority of mutations do not.

We then compared our catalog to mutations that have previously been associated with PZA resistance in clinical isolates. Such an analysis is problematic because of variability in the genetic background of clinical isolates and the inherent limitations in MGIT DST across laboratories. Nevertheless, we compared our results to two recent retrospective studies\(^{32, 47}\) where PZA susceptibilities were carried out by global reference laboratories. Despite differences between these two studies, a total of 84 (71%) PZA resistant non-synonymous amino acid substitutions found in clinical isolates were similarly cataloged PZA resistant in this study (Fig. 3b). However, our study identified an additional 217 resistant conferring substitutions, highlighting the comprehensiveness of our catalog.

Mechanisms of pyrazinamide resistance. Although we, like others\(^{31–33}\), found PZA resistance-conferring substitutions occur throughout the entire length of PncA (Fig. 4a), many other substitutions do not produce resistance to PZA. What then, is the mechanism of resistance? Mutations that disrupt the active site of PncA are expected to result in PZA resistance and we do find many substitutions in the catalytic triad\(^3\) (amino acids 8, 96, and 138) and iron coordinating residues\(^8\) (amino acids 49, 51, 57,
we investigated the which have low solvent accessibility, will be the most critical for stability. Amino acids buried within the interior of the protein, substitutions suggests interference with protein folding and PncA catalytic activity as well as reduced protein levels can mechanistically contribute to PZA resistance.

We hypothesized that alterations in protein structure may lead to decreased protein abundance and reduced activation of PZA. In support of this we found a greater percentage of cataloged resistant substitutions are non-conservative substitutions compared to susceptible substitutions (Fig. 4b, c). To experimentally test this hypothesis, we measured protein abundance and enzymatic activity in isolated single clones from our pncA mutant library.

We used mass spectroscopy to quantify the abundance of PncA relative to two housekeeping proteins, RpoB (Fig. 5a) and DnaK (Supplementary Fig. 10). We found that 19 of 23 resistant mutants had decreased PncA protein levels compared to WT. In general, susceptible strains had levels of PncA similar to those of WT cells. These results indicate that the majority of resistance-conferring substitutions tested result in decreased protein abundance.

Three amino acid substitutions (C138G, C138S, and D8N) had comparable levels of PncA to WT, but are cataloged as resistant. These substitutions all occur within the catalytic triad, suggesting they could impact enzyme activity. To confirm this we used a modified quantitative Wayne assay49 to determine catalytic activity. Of the three PZA resistant clones tested all had reduced PZase activity, relative to the Comp strain (Fig. 5b). In contrast, susceptible isolates had equivalent enzymatic activity. This indicates, for the subset of PncA mutations tested, that loss of catalytic activity as well as reduced protein levels can mechanistically contribute to PZA resistance.

The reduction in protein abundance caused by non-active site substitutions suggests interference with protein folding and PncA stability. Amino acids buried within the interior of the protein, which have low solvent accessibility, will be the most critical for proper protein folding.50 To test this, we investigated the predicted solvent accessibility51, obtained using the previously reported Mtb PncA crystal structure52, of amino acids that confer resistance. We found a consistent negative correlation ($r = -0.53$) between the solvent accessibility and the frequency of resistance-conferring substitution at each amino acid (Fig. 5c and Supplementary Fig. 11). Furthermore, all PncA amino acids at which no substitution resulted in PZA resistance generally map to surface exposed residues that do not overlap with the active site (Fig. 6a, b). Substitutions at these amino acids are therefore predicted to have little or no effect on protein folding or catalytic activity. Combined, these results suggest that resistance results largely from a loss of enzymatic activity and/or protein abundance, which can be empirically determined but not easily predicted from the primary sequence.

Discussion

PZA resistance prevents the use of this critical drug in shortening TB treatment, both in current therapy and possible future regimens. Unfortunately, the current methods of testing for PZA resistance are phenotypic, which are difficult and unreliable. In fact, it has been proposed that any non-synonymous mutation within pncA can be considered a PZA resistant allele52. In this study, we find that this is not the case. We used saturating mutagenesis of pncA to systematically screen for amino acid polymorphisms that confer resistance to PZA. While many mutations are associated with resistance to PZA, most substitutions were susceptible. Nonetheless, our results have significantly expanded the repertoire of PZA resistance-conferring mutations, as most of these mutations have not been observed in clinical isolates.

Importantly, our catalog of PZA susceptibilities shows that a molecular sequencing based diagnostic of pncA could be used to both rule in and rule out patients as candidates for successful PZA therapy, a key development in determining and designing successful treatment regimens. This study generates a comprehensive reference for interpreting the PZA susceptibility of pncA alleles and is an essential advance in developing rapid molecular diagnostics for PZA resistance.

Our assays relied on statistical tests to evaluate PZA susceptibility, so we, like others, cannot rule out all false negative and, to
The propagation of error using the quotient of the coefficients for each mutant relative to the mean of 10 biological replicates for Comp is shown. Essential acid substitutions that confer pyrazinamide resistance at each amino acid in PncA. Linear trend line is shown in Fig. 5.

Fig. 5 PncA protein abundance and catalytic activity result in pyrazinamide resistance. a Quantitation of PncA protein abundance relative to RpoB in cataloged pyrazinamide susceptible (brown) and resistant (black) isolates determined using mass spectrometry. Protein abundance in each mutant strain is relative to the Comp strain. *P ≤ 0.01 (t-test, two-sided). † corresponds to isolates tested for enzymatic activity. The mean fold-change of three biological replicates for each mutant relative to the mean of 10 biological replicates for Comp is shown. Error bars represent the standard deviations derived from the propagation of error using the quotient of the coefficient of variation. b Mean PncA catalytic activity relative to the Comp strain is shown. *P ≤ 0.05 (t-test, two-sided). Error bars represent the standard deviations derived from three biological replicates for each strain by the propagation of error using the quotient of the coefficient of variation. c Correlation between the solvent accessibility (%) and the proportion (%) of all possible non-synonymous amino acid substitutions that confer pyrazinamide resistance at each amino acid in PncA. Linear trend line is shown in red (r = −0.53).

The importance of understanding the mechanisms of antibiotic resistance cannot be understated. Strikingly, mutations conferring resistance to PZA occur throughout the entire length of PncA. The majority of the substitutions we evaluated using proteomics were associated with reduced PncA protein levels which may account for the widespread intragenic distribution of drug resistance-conferring mutations. Those with little or no effect on protein abundance did map to active site regions and had reduced enzyme activity indicating disruption of catalysis is also a mechanism of resistance. Crystallographic studies will be needed to confirm these findings. Furthermore, despite multiple selections some codons, mapping almost exclusively to surfaced exposed residues, failed to enrich for any PZA resistant substitution. Interestingly, while it is known that pncA is non-essential, its in vivo function is poorly understood. Consistently, we observed no generalizable fitness defects for pncA mutations in the absence of PZA treatment. However, given the synergistic drug interactions that PZA exhibits, it would be intriguing to explore the effects of PncA resistant substitutions generated in this study, on these interactions. These results underscore the value of a comprehensive mutational and functional analysis of drug resistance mechanisms.

We assessed the direct correlation between PZA susceptibility in vitro and during infection in mice. Reassuringly, amino acid substitutions found to be resistant in vitro were also identified in our mouse model. However, many more PZA resistance substitutions were detected after treatment in mice than observed in vitro. When individual mutants, classified as resistant in the mouse screen, were assayed using standard DST we were able to confirm they were truly resistant. Studies have shown that the activity of PZA is augmented by host immunity during treatment in a mouse and is bactericidal in a three dimensional cellular model in contrast to the bacteriostasis seen in vitro. A stronger antibiotic selective pressure due to the enhanced activity of PZA in the host is therefore the most likely reason why we identified more mutations in the mouse. It is also conceivable, but unlikely, that phenotypic resistance or antibiotic tolerance may be implicated. It is clear from our single-colony validations that resistance to PZA is directly linked to specific genetic mutations contained within different alleles of pncA.

Our murine model does not perfectly replicate infection in a human, and we had to recover bacilli from the spleen to ensure representation of our library after inoculation. Nevertheless, PZA treatment in mice is certainly closer to human chemotherapy...
than in vitro treatment in acid media. These results thus highlight the limitations of only using an in vitro assay for predicting PZA resistance in clinical samples. For these reasons we are confident that our comprehensive catalog of PncA substitutions, generated from both in vitro and mouse infection screens, does predict clinically relevant PZA resistance. For most anti-TB drugs, and certainly for PZA, there are few data associating in vitro antibiotic susceptibility of specific mutations with treatment outcomes in humans. Further studies in animal models and human cohorts are therefore needed to determine how individual mutations influence treatment efficacy particularly in the context of combination therapy. Our comprehensive mutational and screening approach should stand as a paradigm for identifying resistance mutations. The dual approach of DST both in vitro and in mice may represent a new model to comprehensively screen for the most clinically relevant mutations that includes a readout of therapeutic success.

**Methods**

**Bacterial strains, plasmids, and media.** We used *E. coli* DH5α and *Mtb* H37Rv (sAY101) in this study. Strains, plasmids, and primers are listed in Supplementary Figs. 2–5. Unless otherwise noted, *Mtb* strains were grown using Middlebrook 7H9 (0.2% glycerol and 0.05% Tween 80) or 7H10 agar (0.5% glycerol) (Difco, BD) and supplemented with OADC (BBL, BD). *Mtb* from the in vivo screen were plated on Middlebrook 7H11 (0.5% glycerol) (Difco, BD) and supplemented with OADC (BBL, BD), 200,000 U/ml polymixin B, 10 mg/ml amphotericin B, 50 mg/ml cabanecillin, and 20 mg/ml trimethoprim. Zeocin was used at 50 µg/ml and 20 µg/ml for *E. coli* and *Mtb*, respectively. Hygromycin was used at 50 µg/ml for *Mtb*. All strains were grown at 37°C.

**WT pncA and MOP** were PCR amplified using ANY_P55/ANY_P57 and ANY_P149/ANY_P150, respectively, and sequentially cloned into ZeoR, L5 integrating vector pAY99 using Clal/NotI and Clal/NotI, respectively, to generate pAY107 then pAY108 (Comp). The vector control (pAY111) was generated by PCR amplification of Emerald using ANY_P147/ANY_P148 and cloning into pAY107 using NotI/XbaI. GeneMorph II Random Mutagenesis Kit (Agilent Technologies) was used according the manufactures protocols using ANY_P153/ANY_P154. Amplicons were subsequently cloned into pAY112 using NotI/XbaI to generate our library of pncA SNPs. 50,000 *E. coli* clones were harvested by scraping to create our pooled pncA SNP library (pAY230). Approximately 34,000 *Mtb* transformants were scrapped to constitute sAY260.

**RT-qPCR.** RNA was extracted from 25 ml, mid-log phase (OD600 = 0.5–0.6) cultures. Pelleted cells were suspended in 1 ml of TRIzol (Ambion, Life Technologies). Five repeated cycles of 1 min bead-beating using MagNAlyser (Roche) and 1 min at –20°C with 0.1 mm sterile zirconia/silica beads (Biospec) was performed followed by chloroform extraction and ethanol precipitation. RNA was DNase (Fermentas) treated prior to cDNA synthesis using Script Advanced cDNA Synthesis Kit (Bio-Rad) according to the manufactures protocol. qPCR was performed on CFX 96 Real Time PCR detection system (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad) and KM, P15/P15/KM, P36 and sigA, F1sigA/R for pncA and sigA, respectively. All samples were performed in biological triplicate, externally normalized to sigA, and the mean fold-change and standard deviations evaluated relative to WT (sAY101).

**In vitro drug-susceptibility screening.** PZA and pyrazinamid acid susceptibility were evaluated using the BD BACTEC MGIT 960 system with manufacturer supplied PZA medium/supplement and BBL MGIT medium/growth supplement, respectively. Mycobacterial growth indicator tubes (MGITs) were inoculated with 50,000 CFU directly from a thawed, titered glycerol stocks. PZA (Sigma Aldrich) or pyrazinamid acid Sigma Aldrich) was added to a final concentration of 0, 1, 4, 20, 100, 500, or 1000 µg/ml. The mean TTP and standard deviations of three biological replicates relative to the no-drug control is reported.

The sequential PZA susceptibility screen was performed using the BD BACTEC MGIT 960 system with manufacturer supplied PZA medium/supplement containing 4, 20, 100, or 500 µg/ml PZA (Sigma Aldrich). The first selection was made with 50,000 CFU directly from the titered glycerol stocks of sAY260. After ≤24h of individual MGITs reaching their TTP, bacterial cells were collected by centrifugation and suspended in 1 ml 7H9 broth. An aliquot of 30 µl was re-inoculated to a new MGIT containing the same PZA concentration. The remaining bacteria were pelleted by centrifugation and frozen at –20°C. This was repeated for a total of 6 selection rounds. Genomic DNA from pellets of each selection round was extracted using the Hain GenoLyse Kit according to the manufactures protocol. Three biological replicates were performed per drug concentration.

**Drug-susceptibility testing.** PZA and pyrazinamid acid DST was performed using the BACTEC MGIT 960 system with manufacturer supplied PZA medium/supplement and the five place antimicrobial susceptibility test (AST) set carrier and protocol with the following modifications. PZA concentrations ranged from 0.2 to 500 µg/ml. Pyrazinamid acid concentrations ranged from 1 to 1000 µg/ml. PZA and pyrazinamid acid containing MGITs were inoculated with 25,000 CFU directly from thawed, titered glycerol stocks. No drug MGITs were inoculated with 2500 CFUs directly from a thawed, titered glycerol stocks. After the AST no-drug control MGIT reached positivity, the MICs were recorded as the lowest drug concentration with a growth index <100.

**Pyrazinamide screening in mice.** sAY260 was grown to OD600 = 1.0 then washed and suspended in PBS. A total of 6–8 week-old female BALB/c mice (~20 g each; supplied by the University of KwaZulu-Natal) were infected with the same PZA and pyrazinamid acid concentration (106 CFUs). Mice were arbitrarily randomized into two cohorts, receiving 150 mg kg–1 PZA or PBS by oral gavage 5 days per week for 42 days, four days after infection (day 0). Five mice from each cohort were randomly selected for sacrifice and the spleens harvested on day –3, day –0, day 21 and day 42 to determine the CFUs and quantify pncA SNPs. Five mice per time-point provided sufficient statistical comparisons within each group and to mitigate loss of samples due to contamination. No mice or samples were excluded from the analysis. The authors were not blinded to cohort allocation or assessment of the outcomes. Spleens were homogenized in 2 ml PBS using a mortar and pestle. CFUs were reported as mean and standard deviations of total bacterial burden in the organ. The coefficient of variation (CV) between control groups at day –3 and day 0 were highly similar, indicating technical reproducibility in infection. The CV between PZA and mock treated mice increases after 21 and 42 days, consistent with variety in how mice innately control the initial burden of infection. Greater than 10,000 estimated CFU per mouse were scraped, pooled, and frozen for guard, the thioscynate (TCA) supernatant of which were evaluated using the BD BACTEC MGIT 960 system with manufacturer supplied PZA medium/supplement and the five place antimicrobial susceptibility test (AST) set carrier and protocol. PZA concentrations ranged from 0.2 to 500 µg/ml. Pyrazinamid acid concentrations ranged from 1 to 1000 µg/ml. PZA and pyrazinamid acid containing MGITs were inoculated with 25,000 CFU directly from thawed, titered glycerol stocks. No drug MGITs were inoculated with 2500 CFUs directly from a thawed, titered glycerol stocks. After the AST no-drug control MGIT reached positivity, the MICs were recorded as the lowest drug concentration with a growth index <100.
from a sequenced unselected WT pncA clone was subtracted from the observed frequencies in each sample. Enrichment was calculated as the log2 mean fold-change of each PZA selection relative to the corresponding no-drug control of the same round (in vitro) or treatment length (mouse infection).

Statistical significance was determined by fitting a negative binomial distribution to each replicate of normalized read counts in each condition, using empirical Bayes estimation of variances, and comparing the differences in scaled means across conditions using a Student’s t-test implemented in DESeq2. The resulting p-values were adjusted for multiple comparisons using the Benjamini–Hochberg procedure. For each in vitro PZA concentration, SNPs exhibiting ≥2 fold-change (adjusted P ≤ 0.05) in two or more selection rounds were identified. Mutations were then collated across all conditions and compressed to their respective amino acid substitutions. Mutations identified as both enriched and depleted at any point were ignored. In mice, SNPs exhibiting ≥2 fold-change (adjusted P ≤ 0.05) after either 21 or 42 days of PZA treatment were identified. Mutations were then collated across both selection days and compressed to their respective amino acid substitutions. Mutations identified as both enriched and depleted at any point were ignored. Any amino acid substitution enriched or depleted after either in vitro and/or mice selection, were cataloged as PZA resistant or susceptible, respectively.

**PncA proteomics.** The proteome of sAY245 (Sup) was uniformly 13C labeled by growing cells to OD600 = 1 in a modified Hartmans-de Bont (HDB) media: 3.4 mM EDTA, 49 mM MgCl2•6H2O, 0.7 mM CaCl2•2H2O, 0.07 mM ZnSO4•7H2O, 1.8 µM FeSO4•7H2O, 0.08 µM CuSO4•5H2O, 890 µM K2HPO4, 708 µM NaH2PO4, 15 mM (NH4)2SO4, 27.4 mM glycerol-1,3,5-Tr, and 0.05% (v/v) xylanol. Lysates for 13C labeled sAY245 and unlabeled samples were prepared from 30 ml bacteria grown to mid-log phase (OD600 = 0.7). Pelleted cells were washed three times with 10% glycerol and suspended in PBS containing protease inhibitor cocktail (Roche) and 100 µl 0.1 mm sterile zirconia/silica beads (Biospec). 4 repeated cycles of 1 min bead-beating at 7000 rpm using MagNA Lyser (Roche) and 5 min at 20 °C was used to rupture cells. A final concentration of 2% SDS was added and samples were incubated at 65 °C for 30 minutes. Lysates were cleared twice by centrifugation, passed through 0.2 µm Corning Costar SpinX columns (Sigma Aldrich), and stored at −80 °C. Lysates from 13C labeled sAY245 and unlabeled samples were mixed 1:1 in total volume of 200 µl. Proteins were extracted by the addition of 600 µl methanol, 130 µl chloroform, and 450 µl H2O and centrifugation. Proteins were then precipitated by addition of 400 µl methanol to the organic phase of each sample followed by centrifugation. Protein was suspended in 100 µl 6 M urea, 25 µl 200 mM DTT, and 20 µl 200 mM iodoacetamide. Samples were digested overnight with 4 ng trypsin (Promega) in total volume of 1 ml. A total of 10 µl acetic acid was added and each sample was column purified using Sep-Pak Vac 6cc 500 mg C18 cartridges (Waters, WAT043395), washed with 10 µl 2% acetonitrile with 0.1% formic acid and eluted with 2 ml 65% acetonitrile with 0.1% formic acid. Columns were equilibrated and prior to the addition of each sample, by sequential addition of 5 ml acetonitrile, 5 ml 65% acetonitrile with 0.1% formic acid, and 10 ml 2% acetonitrile with 0.1% formic acid, with each sample was suspended in 50 µl 2% acetonitrile with 0.1% formic acid and stored at 4 °C. Unique pncA, rpoB, and dnaK peptides (Supplementary Table 7) were targeted using a Q Exactive MS coupled to a Dionex RSLC 3000 nano LC system (Thermo Scientific), using a 30 min gradient and mobile phase from 1% acetonitrile and 0.1% formic acid to 50% acetonitrile and 0.1% formic acid, with a regeneration phase to clean up and equilibrate the column. Separation was carried out on a 15 cm by 75 µm ID fused silica column, backed with 3 µm C18 stationary phase, and a flow rate of 0.300 µl min−1. The nano-spray source was operated at a capillary temperature of 275 °C, an emitter voltage of 1.6 kV and a lens voltage of −55 V. A targeted-MS2 method was employed with an AGC target of 1.0e5 ions, an isolation width of 2 m/z 1 and an HCD setting of 27. Spectral traces were quantified using Skyline proteomics software. The area-under-the-curve (AUC) for each peptide transition was exported using the MS1 plug-in. Any transitions containing the mutation of interest or with an AUC < 8 were excluded for each sample, the quantified light (L) AUC was then converted to the 13C-heavy (H) AUC for each transition (L/H). The mean L/H for all peptides was then normalized to the mean L/H for all peptides across all PZA concentrations and compressed to their respective amino acid substitutions. Any amino acid substitution enriched or depleted after either in vitro and/or mice selection, were cataloged as PZA resistant or susceptible, respectively.

**Clinical isolate comparison.** PZA DST in clinical isolates was obtained from Miotto et al.15 and Walker et al.16 PZA resistant non-synonymous SNPs were extracted based on sequencing of pncA and associated drug-susceptibility to PZA as determined in each study. Indels, multi-nucleotide polymorphisms, and discrepant (both resistant and susceptible) PZA drug-susceptibility calls were removed.

**PncA protein structure modeling.** PncA amino acids (29) to which no substitution resulted in PZA resistance were mapped to the previously reported Mtb PncA crystal structure46.

**Statistical analysis.** Sample sizes were chosen as the minimum number of replicates (based on limited resources and time) for statistical analysis and are indicated for each experiment. Unless otherwise indicated, no samples were preferentially excluded from any analysis and all data are presumed to meet assumptions of each statistical test with variance between groups similar.

Data availability. The raw Illumina sequencings reads generated in this study can be accessed at https://www.ncbi.nlm.nih.gov/ using the SRA accession number SRP093213. The authors declare that all other data supporting the findings of this study are available within the article and its Supplementary Information files, or are available from the authors upon request.

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