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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated
☐	☒ Clearly defined error bars *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection	Genomes were sequenced on the Illumina GAIIx or HiSeq 2500 platforms, generating single end reads of 72 bp or 101 bp in length.
Data analysis	The code used to analyze the data in this study include commercially available and custom softwares as described in detail in the methods. We have described it briefly below. For each sample duplicate reads were removed by custom Perl scripts. Further quality control was conducted using the NGSQCToolkit with a cutoff of Q20. Valid reads were then aligned to the reference genome sequence of H37Rv (GenBank accession NC_000962) using the Burrows-Wheeler algorithm as implemented in BWA. SNPs were identified with a minimum depth of 10X and a consensus quality score of 50 using SAMtools. SNPs located within repetitive regions, including transposases, PE/PPE genes, prophages and exact sequence repeats of the H37Rv genome previously proposed or identified by RepeatMasker (http://www.repeatmasker.org/) or PhageFinder 40, were excluded. Mixed base calls were considered valid only if the numbers of the most abundant (n1) and the second most abundant (n2) nucleotides at each SNP in each strain satisfied the criteria n1/n2≧5. The detailed workflow of the SNP-calling procedure of datasets for follow-up analysis is given in Supplementary Figure 7. The refined SNP set was used to construct the maximum-likelihood phylogeny using RAxML under the GTRgamma substitution model. The reliability of each node was tested via a bootstrap analysis on 100 resampled datasets. The iTOL server and MEGA5 software were employed for the manipulation and presentation of the phylogenetic trees. Genomic associations were performed using a method similar to that of Farhat et al which we termed phyOverlap. We performed

maximum parsimony ancestral sequence reconstruction to determine the state of each SNP residue at the common ancestor of our strain collection using the Mesquite software package (http://mesquiteproject.org/). In cases where the ancestral sequence could not be unambiguously determined, we excluded the site from further analysis. Each isolate was then scored as ancestral or derived for a given SNP locus. Isolates with ambiguous base calls were considered ancestral for this calculation to minimize the potential of error driving associations with drug resistance. The overlap with drug resistance was scored by dividing the number of isolates containing a derived allele which also were resistant to isoniazid by the total number of isolates with a derived allele at a given SNP locus. To generate a gene-wide score, we excluded synonymous SNPs and averaged the individual SNP scores, weighting the scores by the number of times derived alleles evolved across the phylogenetic tree. The number of times a site mutated across the tree was calculated as the parsimony score at each SNP position using the Fitch algorithm as implemented in the R package phangorn. This was performed with mixed bases treated as missing data so that low quality base-calls would not appear highly convergent.
Significance testing was performed by redistributing the mutation events in each SNP in each gene randomly across the phylogenetic tree, with the probability of a mutation occurring on any branch proportional to the branch length and all offspring of a branch being assigned the derived allele for calculation of the permuted overlap score. The gene-wide score was then recalculated and compared with the actual value. This process was repeated 50,000 times to derive an empirical p-value. For all statistical calculations, associations which achieved a p-value of 0 were considered <2x10-5. False discovery rate q-values were then calculated to account for multiple hypothesis testing using the Benjamini-Hochberg procedure.

Scripts for this method are available online at: https://github.com/nathan-d-hicks/phyOverlap

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Sequencing reads have been submitted to the NCBI Sequence Read Archive (SRA) under accession PRJNA268900. A complete list of the new TB strains analyzed in this study together with phenotypic and sequencing information is given in Supplementary Table 1. The accession numbers for previously sequenced strains used in Figures 1B, 1D, and Table 1 are available at in Supplementary Table 2 and Supplementary Table 8.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size calculations are relevant for experiments shown in Figures 2-5. The growth curves and expression data presented in the manuscript consist of three biological replicates (Figure 2). We used three biologically independent replicates for in vitro killing assays (Figures 3, 4, 5) which our preliminary data indicated would be sufficient to measure 2-fold or greater changes in bacterial numbers. Mouse experiments included 5 mice per group which was sufficient to identify roughly 10-fold changes in in vivo abundance of mutants in previously published experiments.
Data exclusions	We excluded three datapoints in the entire in the entire study as described below. Figure 3D: A single datapoint was excluded each from the ofloxacin acetate data on day 2 and propionate day 2 due to contamination of the CFU plate. A single datapoint was excluded from the high-dose INH Day 6 propionate in figure 3D where the prpR WT complement had substantially lower reads than in the two other replicates (while true WT H37Rv was unaffected). This exclusion weakened the statistical significance reported at this timepoint, rather than increasing the magnitude of the effect. Supplemental Figure 5: RIF Day 57 only has 4 mice because one spleen was contaminated during extraction.
Replication	We have indicated the number of times experiment was independently performed as described below and in the figure legends. The growth curves shown in figure 2 were performed three times, each with three replicates, and yielded similar results. The MIC assays presented in Figure 3 a-c were performed 3 times for the prpR complement and deletion strain and in all cases we did not see prpR dependent antibiotic protection. The library format antibiotic killing in figure 3D was performed once, and then the propionate mediated protection of strains was confirmed in the single strain CFU assays presented in figures 3e-g, which were repeated >3 times. In all cases the prpR mutants displayed

increased antibiotic tolerance. We assessed prpR mutant antibiotic susceptibility in primary macrophages in two independent experiments and confirmed these findings in THP1 cells in two independent experiments. The mouse experiment was performed once. B12 mediated reversal of antibiotic protection in vitro shown in Figure 5 was performed twice and in both cases demonstrated re-sensitization of prpR mutants. The THP-1 infection B12 reversal of protection was performed once.

Randomization: There was no randomization in this study because there were no features for which randomization was deemed appropriate.

Blinding: Similarly blinding was not performed for any of the in vitro or in vivo experimentation. The measurements of optical density, CFU, RNA expression, and representation of strains in competition assays do not require researcher-based judgments and therefore we deemed blinding not necessary.

Materials & experimental systems

Policy information about availability of materials

n/a	Involved in the study
☐	☒ Unique materials
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☐	☒ Research animals
☐	☒ Human research participants

Unique materials

Obtaining unique materials: All bacterial strains generated in this study will be made available by the investigators upon request.

Research animals

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Animals/animal-derived materials: 6-8 week old female C57BL/6J mice were obtained from Jackson Laboratories.

Human research participants

Policy information about studies involving human research participants

Population characteristics: The population characteristics of the Chinese CDC cohort are described in detail in: Zhao et al, NEJM 2012. The sub-selection of strains for sequencing is described in detail in the methods.

Human research participants were used to acquire blood components for the macrophage experiments. Human blood components (buffy coats) for macrophage experiments were procured through Blood Transfusion Services (BTS) at Massachusetts General Hospital (MGH) for in vitro research by Investigators at the Ragon Institute of MGH, MIT and Harvard. These specimens were collected from donations for clinical use and only excess blood or blood products that are not needed are then processed for research purposes. The specimens supplied for these purposes are not identifiable. Donors in the Blood Transfusion Services at MGH sign a Donor Consent within a Registration Form that includes the following sentence, "I give permission for my blood to be used for transfusion to patients or for research."

Method-specific reporting

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ Magnetic resonance imaging