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**Mycobacterium tuberculosis** Requires the Outer Membrane Lipid Phthiocerol Dimycocerosate for Starvation-Induced Antibiotic Tolerance

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**ABSTRACT** Tolerance of *Mycobacterium tuberculosis* to antibiotics contributes to the long duration of tuberculosis (TB) treatment and the emergence of drug-resistant strains. *M. tuberculosis* drug tolerance is induced by nutrient restriction, but the genetic determinants that promote antibiotic tolerance triggered by nutrient limitation have not been comprehensively identified. Here, we show that *M. tuberculosis* requires production of the outer membrane lipid phthiocerol dimycocerosate (PDIM) to tolerate antibiotics under nutrient-limited conditions. We developed an arrayed transposon (Tn) mutant library in *M. tuberculosis* Erdman and used orthogonal pooling and transposon sequencing (Tn-seq) to map the locations of individual mutants in the library. We screened a subset of the library (~1,000 mutants) by Tn-seq and identified 32 and 102 Tn mutants with altered tolerance to antibiotics under stationary-phase and phosphate-starved conditions, respectively. Two mutants recovered from the arrayed library, *ppgK::Tn* and *clpS::Tn*, showed increased susceptibility to two different drug combinations under both nutrient-limited conditions, but their phenotypes were not complemented by the Tn-disrupted gene. Whole-genome sequencing revealed single nucleotide polymorphisms in both the *ppgK::Tn* and *clpS::Tn* mutants that prevented PDIM production. Complementation of the *clpS::Tn* *ppsD Q291* mutant with *ppsD* restored PDIM production and antibiotic tolerance, demonstrating that loss of PDIM sensitized *M. tuberculosis* to antibiotics. Our data suggest that drugs targeting production of PDIM, a critical *M. tuberculosis* virulence determinant, have the potential to enhance the efficacy of existing antibiotics, thereby shortening TB treatment and limiting development of drug resistance.

**IMPORTANCE** *Mycobacterium tuberculosis* causes 10 million cases of active TB disease and over 1 million deaths worldwide each year. TB treatment is complex, requiring at least 6 months of therapy with a combination of antibiotics. One factor that contributes to the length of TB treatment is *M. tuberculosis* phenotypic antibiotic tolerance, which allows the bacteria to survive prolonged drug exposure even in the absence of genetic mutations causing drug resistance. Here, we report a genetic screen to identify *M. tuberculosis* genes that promote drug tolerance during nutrient starvation. Our study revealed the outer membrane lipid phthiocerol dimycocerosate (PDIM) as a key determinant of *M. tuberculosis* antibiotic tolerance triggered by nutrient starvation. Our study implicates PDIM synthesis as a potential target for development of new TB drugs that would sensitize *M. tuberculosis* to existing antibiotics to shorten TB treatment.

**KEYWORDS** PDIM, *Mycobacterium tuberculosis*, Tn-seq, antibiotic resistance, drug tolerance, membrane permeability, nutrient limitation

Tuberculosis (TB) infections caused by *Mycobacterium tuberculosis* are notoriously difficult to treat, requiring a lengthy 6- to 9-month course of combination antibiotic therapy to achieve cure (1). This is due, in part, to *M. tuberculosis* phenotypic antibiotic
tolerance that prolongs survival during exposure to drugs. Phenotypic tolerance contributes to the emergence of drug resistance during treatment of other bacterial infections (2, 3) and increases the frequency of drug-resistant M. tuberculosis mutants in vitro (4). Multidrug-resistant (MDR) M. tuberculosis, which is resistant to the two most effective first-line agents, rifampicin (RIF) and isoniazid (INH), accounts for 3 to 4% of the 10 million newly diagnosed active TB cases annually (5). MDR-TB is more challenging to treat, requiring up to 2 years of therapy with less effective second-line agents (6). Identifying the molecular mechanisms driving M. tuberculosis drug tolerance will be critical to shorten TB treatment and limit emergence of new drug-resistant mutant strains.

Drug efficacy can be reduced by heritable drug resistance mutations, which allow bacteria to grow in the presence of the antibiotic, or by phenotypic drug tolerance and persistence, which increase the time required for an antibiotic to kill the bacterial population (7). Tolerance is defined as increased recalcitrance of the entire bacterial population to the drug, while antibiotic persisters are a subpopulation of bacteria that survive drug exposure and are typically revealed by biphasic killing in time-based antibiotic kill curve assays (7). The distinction between tolerance and persistence is blurry, but environmental triggers that affect the entire bacterial population (e.g., nutrient restriction, stress, or antibiotic treatment) were proposed to increase tolerance by driving bacteria into a slower-growing state (8). In M. tuberculosis, drug tolerance can arise through any mechanism that decreases target vulnerability or antibiotic interaction with its target (9), including slow growth (10, 11), reduced metabolism (12), decreased intracellular drug concentration (13), or slow prodrug activation (14). Drug-tolerant M. tuberculosis has been observed within infected mouse lungs (15), in interferon gamma (IFN-γ)-activated macrophages (16), in the hypoxic caseum of necrotic granulomas (17), and in human sputum (18, 19).

Some molecular mechanisms underlying M. tuberculosis drug tolerance have been revealed by genetic approaches, including identification of genes expressed in the drug-tolerant population (20, 21) and screens for strains exhibiting altered drug tolerance in vitro (22, 23) or during infection of macrophages or mice (24–27). Specific metabolic pathways and toxins of toxin-antitoxin (TA) systems induce M. tuberculosis drug tolerance by slowing growth. For example, loss-of-function mutations in the glycerol kinase gene glpK, which is required for growth on glycerol, which is a primary carbon source in the standard Middlebrook culture medium, increase drug tolerance in vitro and during infection (22, 25, 28). Many TA systems are upregulated in the drug-tolerant subpopulation (20, 21), and the toxins generally act to inhibit bacterial growth (29). The VapC12 toxin, an RNase that targets proT tRNA, was specifically implicated in drug tolerance of M. tuberculosis grown on cholesterol by slowing bacterial replication (30).

Nutrient starvation is one signal that triggers M. tuberculosis drug tolerance (31), but the molecular mechanisms underlying this process remain poorly characterized. M. tuberculosis growth arrest and tolerance to INH during nutrient starvation require production of the stringent response alarmone (pppGpp) by Rel (12), but whether Rel activity is required for tolerance to other antibiotics has not been explored. Nutrient-starved M. tuberculosis exhibits lower intracellular concentrations of RIF and fluoroquinolone antibiotics, which may be due to reduced drug uptake, as drug efflux pump inhibitors did not reverse the drug tolerance induced by nutrient restriction (32). However, the genetic determinants that promote M. tuberculosis drug tolerance triggered by nutrient limitation have not been comprehensively identified.

Here, we describe a genetic screen using transposon (Tn) sequencing (Tn-seq) to identify and characterize M. tuberculosis factors that influence antibiotic tolerance triggered by nutrient limitation. We identify over 100 M. tuberculosis Tn mutants with altered drug tolerance under either phosphate-starved or stationary-phase conditions. We confirm decreased antibiotic tolerance phenotypes of individual Tn mutants but show that for two of these mutants the phenotypes are unlinked to the Tn-disrupted gene. Instead, we find that secondary mutations preventing production of the outer membrane lipid phthiocerol dimycolates (PDIM) cause increased susceptibility to antibiotics. As PDIM is also a critical
virulence determinant (33), our findings suggest that PDIM synthesis is an attractive target for development of new drugs that would both decrease virulence and sensitize *M. tuberculosis* to existing antibiotics.

**RESULTS**

**Construction of an arrayed and sequence-mapped *M. tuberculosis* Erdman transposon mutant library.** To identify *M. tuberculosis* determinants of drug tolerance in nutrient starvation, we planned to screen transposon (Tn) mutants for those with defects surviving drug exposure. Since a large percentage of the population is killed by antibiotic treatment, we expected our screen to have an inherent bottleneck that would cause stochastic loss of individual Tn mutant strains. To overcome this bottleneck, we screened defined pools of Tn mutants, which we created as an arrayed library. To enable recovery of auxotrophs, Tn mutants were selected on a nutrient-rich medium, MtbYM, that contains additional carbon and nitrogen sources, vitamins, and cofactors compared to the standard Middlebrook 7H9 medium (34). Approximately 8,000 *M. tuberculosis* Erdman Tn mutants were arrayed in 80 racks, each with 96 barcoded tubes. Tn mutant pools for experiments were created by combining all ~96 Tn mutants in a rack.

To facilitate recovery of Tn mutants of interest, we used orthogonal pooling and Tn-seq to map the location of Tn mutants in the library. The library was divided into two sets of 40 racks. For each set of 40 racks, pools were created of all mutants in each row (rows A to H, 8 pools, each with 12 × 40 = 480 mutants), all mutants in each column (columns 1 to 12, 12 pools, each with 8 × 40 = 320 mutants), and all mutants in each rack (racks 1 to 40 or racks 41 to 80, 40 pools, each with 96 mutants) to generate 60 pooled samples per set of racks for Tn-seq. For Tn mutants with no sibling clones in the set of racks, sequence reads corresponding to the Tn insertion site appear in equal abundance in one rack pool, one column pool, and one row pool. For racks 1 to 40, we used two mapping methods: the heuristic Straight Three strategy (35) and the probabilistic Knockout Sudoku algorithm (36). We found good agreement between these mapping methods, with a larger percentage of Tn mutants mapped by Knockout Sudoku (see Table S1 in the supplemental material). Mutants in racks 41 to 80 were mapped only by Knockout Sudoku (see Table S1 in the supplemental material). Our arrayed library contains 11,189 total Tn insertions at 6,842 unique locations in the *M. tuberculosis* Erdman genome. These include 1,323 unique insertions in intergenic regions and 5,519 unique insertions within 2,328 of the 4,302 annotated *M. tuberculosis* Erdman open reading frames (ORFs) (~54% coverage). The library contains Tn insertions in 1,975 of the 3,102 ORFs previously described as being nonessential for growth of *M. tuberculosis* H37Rv in MtbYM rich medium (34). Tn insertions were distributed evenly throughout the *M. tuberculosis* Erdman genome (Fig. S1A). We confidently mapped the location of 6,917 Tn mutants (61.8%) by Knockout Sudoku (Table S1), which is comparable to the ~68% mapping confidence reported for a similar *Enterococcus faecalis* Tn mutant library (35). Most tubes (3,879) have a single Tn insertion mapped (Fig. S1B).

**Tn-seq identifies starvation-induced drug tolerance determinants.** To identify nutrient-limited conditions that reproducibly increase *M. tuberculosis* antibiotic tolerance, we either grew cultures to stationary phase (a general nutrient limitation) or starved the cultures of inorganic phosphate (P<sub>n</sub>, a defined nutrient limitation). We used combinations of two drugs, each with different modes of action, to prevent emergence of drug-resistant mutants: ciprofloxacin and isoniazid (CIP+INH) or rifampicin and isoniazid (RIF+INH). Each combination included a bactericidal drug (RIF or CIP) and INH at a bacteriostatic low dose to promote isolation of persister variants (37). We compared wild-type (WT) *M. tuberculosis* Erdman drug tolerance under these conditions between Middlebrook 7H9 and MtbYM rich media. The rate at which *M. tuberculosis* was killed by antibiotics was decreased in MtbYM compared to 7H9 medium under both stationary-phase and P<sub>n</sub>-starved conditions and with both the CIP+INH and RIF+INH drug combinations (Fig. 1A and B). These data suggest that the additional nutrient sources in MtbYM induce higher *M. tuberculosis* drug tolerance.

To identify *M. tuberculosis* determinants of starvation-induced drug tolerance, we
screened low-complexity pools of Tn mutants in P_{i}-starved or stationary-phase MtbYM cultures treated with CIP or INH and identified Tn mutants with altered fitness by Tn-seq. We note that our screen cannot distinguish between mutations that alter intrinsic drug resistance, drug tolerance, or formation of persister variants, any of which would alter Tn mutant fitness upon drug exposure. Briefly, Tn mutant pools were grown to stationary phase (7 days in MtbYM) or P_{i} starved (72 h in P_{i}-free MtbYM). Each starved culture was plated on MtbYM agar prior to drug exposure as an input control and then split into triplicate no-drug control, CIP or INH-treated, or RIF or INH-treated experimental groups. Cultures were incubated for 9 days before plating on MtbYM agar to recover surviving Tn mutants. Tn mutant abundance under each experimental condition (input, no-drug output, CIP or INH output, RIF or INH output) was determined by Tn-seq. In preliminary experiments, we determined that we could screen ~500 Tn mutants (pools of 5 racks) simultaneously without stochastic loss of mutants in individual biological replicates.

We screened two pools, each with ~500 Tn mutants (racks 6 to 10 and racks 16 to 20), and obtained similar numbers of Tn-seq reads mapped to the \textit{M. tuberculosis} genome under all experimental conditions (Table S2). To determine the fitness of Tn mutants, we used TnseqDiff, a parametric method that identifies conditionally essential genes between conditions based on Tn insertion-level data and that is compatible with low-density Tn libraries (38). We compared the normalized frequency of sequence reads at each Tn insertion site between the experimental conditions and the input (CIP or INH/input, RIF or INH/input, or no-drug control/input) or between drug-treated conditions and the no-drug control (CIP or INH/control or RIF or INH/control) with TnseqDiff (38). Complete TnseqDiff analyses are available in Table S3. Using statistical significance cutoffs of \( \pm 2 \log_{2} \) fold change and an adjusted \( P \) value of \(<0.025\), we identified 122 Tn insertions that exhibited differential fitness in one or more comparisons corresponding to 17 intergenic insertions and 92 unique ORFs disrupted (Table S4).

Under the P_{i} starvation condition, we identified 102 Tn mutants with significantly altered fitness (Fig. 2; Table S4). Of the 86 Tn mutants with significantly reduced fitness (negative fold change), 11 showed phenotypes in the no-drug control/input comparison (Fig. 2C), suggesting that these gene products are required for survival of P_{i} starvation. These included Tn insertions in genes putatively involved in nucleotide metabolism or transport (\textit{purN}, \textit{pyrR}, \textit{mkl}), central metabolism (\textit{pckA}), cell division (\textit{ftsX}), and stress responses (\textit{htpX}, \textit{uvrB}) (Fig. 2C; Table S4). We identified 76 mutants that showed significantly reduced fitness in the CIP or INH/input comparison (Fig. 2A) and 11 mutants with significantly reduced fitness in the RIF or INH/input comparison (Fig. 2B). Of these, five Tn mutants (three of which were in ORFs) exhibited reduced fitness under both drug treatment conditions, but not the no-drug control (Fig. 2D; Table 1). Similar TnseqDiff analyses were done comparing relative Tn mutant abundance under each
drug treatment condition to the no-drug control at day 9. Only four Tn mutants were identified that met our statistical significance criteria in these comparisons (Table S4; Fig. S2).

Under the Pi starvation condition, we observed several genes or pathways with multiple independent Tn insertions that exhibited significantly altered fitness upon drug treatment (Table 1). Mutants significantly impaired for survival of CIP+INH treatment during Pi starvation included two with Tn insertions in genes required for production of the outer membrane lipid phthiocerol dimycocerosate (PDIM; ppsC, fadD28) (Table 1). PDIM limits permeability of the Mycobacterium tuberculosis outer membrane to small hydrophilic nutrients, including glucose and glycerol (39, 40), and may also restrict diffusion of certain antibiotics, such as the glycopeptide vancomycin (41). We also identified two independent Tn insertions each in Erdman_0220 (rv0194), which encodes an ATP-binding cassette (ABC)-type efflux pump previously implicated in intrinsic resistance of Mycobacterium bovis BCG to multiple drugs, including ampicillin (42), and Erdman_3338 (rv3049), which encodes a putative monooxygenase (Fig. 2A; Table 1).

In Pi starvation, we also identified 16 mutants with significantly increased fitness (positive fold change). These included five independent Tn insertions in phoPR, which encodes a two-component system that responds to acid stress (43–45). Although the phoPR mutants exhibited increased fitness in both drug combinations (Fig. 2A and B), they were also more abundant in the no-drug control/input comparison (Fig. 2C), suggesting that PhoPR normally impairs survival of Pi limitation, rather than specifically altering antibiotic susceptibility.

**FIG 2** Mutants with altered fitness upon drug treatment in Pi-limited MtbYM medium. (A to C) Volcano plots of TnseqDiff statistical analysis of Tn-seq data for Pi-starved Tn mutant pools treated with CIP+INH (A) or RIF+INH (B) or no-drug control (C) compared to input. Dashed lines indicate cutoffs for statistical significance of ≥ 2 log2 fold change and adjusted P value of < 0.025. Tn mutants meeting significance are colored. (D and E) Venn diagrams displaying the number of Tn mutants with significant negative (D) or positive (E) fold changes in relative fitness.
To validate the phenotypes observed in our screen, we determined the sensitivity of individual Tn mutants to antibiotics in monoculture. We selected only Tn mutants that exhibited significantly altered fitness in antibiotic-treated Pi-starved cultures.

We identified eight additional Tn mutants with increased abundance in both the CIP + INH/INH/input and RIF + INH/input comparisons (Fig. 2A, B, and E). These included four independent Tn insertions in the mce1 locus (Fig. 2A and B; Table 1), which encodes a fatty acid transporter (46), suggesting Mce1 contributes to drug susceptibility.

Under the stationary-phase condition, we identified 32 Tn mutants with significantly altered fitness. In comparisons with the input control, three Tn mutants decreased in relative abundance (negative fold change) and 25 Tn mutants increased in relative abundance (positive fold change) (Fig. 3; Table S4). Only two Tn mutants (in the same gene or pathway for which significant phenotypes were observed in the same culture condition or for which significant phenotypes were observed under both drug treatment conditions but not the no-drug control).

We retested confir...
analyses to characterize pathways that, when inhibited, would sensitize *M. tuberculosis* to existing antibiotics. We focused on genes that were not previously implicated in mycobacterial drug tolerance. The three Tn mutants we selected (rv0457c::Tn, ppgK::Tn, and clpS::Tn) had relatively severe phenotypes based on the TnseqDiff fold change, had the Tn insertion in the middle of the ORF, and were identified only in the Pi starvation screen (Table 3). Each of these genes was identified by only a single Tn insertion (Table S4). rv0457c::Tn and ppgK::Tn were also the only two mutants that exhibited significantly reduced fitness in the RIF+INH-treated versus no-drug control comparison (Fig. S2; Table S4). Each mutant was recovered from the arrayed library, and the Tn insertion site was confirmed by PCR and sequencing.

The rv0457c::Tn mutant exhibited specific susceptibility to RIF+INH in the Pi starvation screen (Table 3). rv0457c encodes a prolyl oligopeptidase (53) and is located immediately 5’ of the mazE1-mazF1 operon that encodes a TA system. MazF toxins were previously implicated in *M. tuberculosis* drug tolerance (54). The rv0457c::Tn mutant displayed a subtle but statistically significant increase in sensitivity to RIF+INH, but not CIP+INH, under Pi starvation conditions (Fig. 4A and B). As the rv0457c::Tn mutant did not exhibit strong phenotypes upon retesting, it was not pursued further.

The ppgK::Tn mutant exhibited the highest sensitivity to RIF+INH under Pi-starved conditions in our screen (Fig. 2B) and was also susceptible to the CIP+INH combination, though it did not reach our statistical significance cutoffs (Table 3). ppgK encodes the dominant glucokinase in *M. tuberculosis* (55), catalyzing phosphorylation of glucose with a preference for polyphosphate as the phosphodonor (56). The ppgK::Tn mutant was significantly

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**FIG 3** Mutants with altered fitness upon drug treatment during stationary phase in MtbYM medium. (A to C) Volcano plots of TnseqDiff statistical analysis of Tn-seq data for Tn mutant pools grown to stationary phase in MtbYM and treated with CIP+INH (A) or RIF+INH (B) or no-drug control (C) compared to input. Dashed lines indicate cutoffs for statistical significance of ≥2 log₂ fold change and adjusted P value of <0.025. Tn mutants meeting significance are colored. (D and E) Venn diagrams displaying the number of Tn mutants with significant negative (D) or positive (E) fold changes in relative fitness.

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M. tuberculosis inhibits degradation of SsrA-tagged proteins derived from translationally stalled degradation of proteins with destabilizing N-terminal residues (the N-end rule) and adaptor for the ClpC1P1P2 (ClpCP) protease. ClpS promotes ClpCP and enhances drug tolerance because it degrades antitoxins from several classes of TA systems (58). Loss of the ClpS complementation was polar on expression of downstream genes. A cosmid covering the com-

TABLE 2 Subset of Tn mutants identified by TnseqDiff as having significantly altered fitness in antibiotic-treated stationary-phase cultures

<table>
<thead>
<tr>
<th>Position</th>
<th>Locus tag</th>
<th>Gene</th>
<th>Gene product function</th>
<th>Log(fl) change (adjusted P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>203040</td>
<td>Erdman_0197</td>
<td>mce1D</td>
<td>Fatty acid transport</td>
<td>-0.26 (0.20)</td>
</tr>
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<td>202444</td>
<td>Erdman_0197</td>
<td>mce1D</td>
<td>Fatty acid transport</td>
<td>-0.10 (0.38)</td>
</tr>
<tr>
<td>205440</td>
<td>Erdman_0199</td>
<td>mce1F</td>
<td>Fatty acid transport</td>
<td>-0.29 (0.22)</td>
</tr>
<tr>
<td>1833015</td>
<td>Erdman_1805</td>
<td>lysX</td>
<td>Lysinylation of phosphatidylglycerol</td>
<td>1.03 (0.007)</td>
</tr>
<tr>
<td>2041171</td>
<td>Erdman_1997</td>
<td>PPE32</td>
<td>Unknown PPE family protein</td>
<td>0.23 (0.03)</td>
</tr>
<tr>
<td>2041579</td>
<td>Erdman_1997</td>
<td>PPE32</td>
<td>Unknown PPE family protein</td>
<td>1.62 (0.004)</td>
</tr>
<tr>
<td>2041961</td>
<td>Erdman_1997</td>
<td>PPE32</td>
<td>Unknown PPE family protein</td>
<td>1.74 (0.003)</td>
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<tr>
<td>2194032</td>
<td>Erdman_2155</td>
<td>rv1957</td>
<td>TA chaperone</td>
<td>0.27 (0.23)</td>
</tr>
<tr>
<td>2194075</td>
<td>Erdman_2155</td>
<td>rv1957</td>
<td>TA chaperone</td>
<td>0.51 (0.10)</td>
</tr>
<tr>
<td>2364484</td>
<td>Erdman_2328</td>
<td>dop</td>
<td>Deamidase of Pup</td>
<td>1.38 (0.005)</td>
</tr>
<tr>
<td>2367722</td>
<td>Erdman_2331</td>
<td>mpa</td>
<td>Proteasome ATPase</td>
<td>0.56 (0.35)</td>
</tr>
<tr>
<td>2369014</td>
<td>Erdman_2331</td>
<td>mpa</td>
<td>Proteasome ATPase</td>
<td>0.48 (0.39)</td>
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<tr>
<td>3009590</td>
<td>Erdman_2972</td>
<td>sigB</td>
<td>Sigma factor SigB</td>
<td>0.87 (0.13)</td>
</tr>
<tr>
<td>3009923</td>
<td>Erdman_2972</td>
<td>sigB</td>
<td>Sigma factor SigB</td>
<td>0.39 (0.59)</td>
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<tr>
<td>3511066</td>
<td>Erdman_3458</td>
<td>nuoM</td>
<td>NADH dehydrogenase I</td>
<td>0.23 (0.42)</td>
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<tr>
<td>3513262</td>
<td>Erdman_3459</td>
<td>nuoN</td>
<td>NADH dehydrogenase I</td>
<td>0.85 (0.02)</td>
</tr>
</tbody>
</table>

The table includes only Tn insertions within open reading frames either for which either multiple independent Tn insertions with significant phenotypes were observed in the same gene or pathway or for which significant phenotypes were observed under both drug treatment conditions but not the no-drug control.

The MIC90 for RIF was reduced 4- to 8-fold, suggesting that the clpS::Tn mutant met the statistical significance cutoffs of > ±2 log, fold change and adjusted P value of < 0.025.

First nucleotide position of the Tn insertion site in the Erdman ATCC 35801 genome AP012340.1.

more susceptible to both CIP + INH and RIF + INH in Pi-free MtbYM medium (Fig. 4C and D). However, the MICs (MIC90) for all three drugs were similar between the ppgK::Tn and WT strains, suggesting that the ppgK::Tn mutant has altered antibiotic tolerance (Table 4). We attempted to complement these phenotypes by providing ppgK in trans using a construct similar to that previously reported to complement a ΔppgK mutant (55). Quantitative reverse transcription-PCR (qRT-PCR) confirmed ppgK transcription from the pMV-ppgK vector (data not shown), but complementation did not increase the tolerance of the ppgK::Tn mutant to either drug combination (Fig. 4C and D). These data suggest that the ppgK::Tn mutant harbors a secondary mutation, unlinked to the Tn, that causes increased susceptibility to antibiotics.

The clpS::Tn (rv1331::Tn) mutant exhibited high susceptibility to CIP + INH, but not RIF + INH, under Pi-starved conditions in our screen (Table 3). clpS encodes an adaptor for the M. tuberculosis ClpC1P1P2 (ClpCP) protease. ClpS promotes ClpCP degradation of proteins with destabilizing N-terminal residues (the N-end rule) and inhibits degradation of SsrA-tagged proteins derived from translationally stalled ribosomes (57, 58). M. tuberculosis ClpCP was implicated in drug tolerance because it degrades antitoxins from several classes of TA systems (58). Loss of the ClpS adaptor may therefore alter the stability of certain ClpCP protease substrates that influence drug tolerance. When tested in monoculture, the clpS::Tn mutant was highly susceptible to both CIP + INH and RIF + INH in Pi-free MtbYM medium (Fig. 4E and F). While the clpS::Tn mutant had a similar MIC90 for both CIP and INH as that of the WT control, the MIC90 for RIF was reduced 4- to 8-fold, suggesting that the clpS::Tn mutant has reduced intrinsic resistance to RIF (Table 4). We attempted to complement the clpS::Tn mutant by providing clpS in trans. We observed clpS transcript from pMV-clpS by qRT-PCR (data not shown), but the complemented strain remained susceptible to both drug combinations (Fig. 4E and F). As clpS is carried at the 5’ end of a putative operon, we considered the possibility that the Tn insertion was polar on expression of downstream genes. A cosmid covering the complete clpS region also failed to complement the clpS::Tn mutant phenotype (Fig. 4E and F). These data suggest that the clpS::Tn mutant also has a secondary mutation that increases susceptibility to antibiotics.
**TABLE 3** Tn mutants with significant antibiotic tolerance defects in Pi starvation selected for individual retesting

<table>
<thead>
<tr>
<th>Position&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Locus tag</th>
<th>Gene</th>
<th>Gene product function</th>
<th>Control/input</th>
<th>CIP+INH/input</th>
<th>RIF+INH/input</th>
</tr>
</thead>
<tbody>
<tr>
<td>551129</td>
<td>Erdman_0502</td>
<td>rv0457c</td>
<td>Probable peptidase</td>
<td>0.30 (0.06)</td>
<td>–0.36 (0.12)</td>
<td>–2.31 (0.001)</td>
</tr>
<tr>
<td>201967</td>
<td>Erdman_1491</td>
<td>clpS</td>
<td>ClpCP protease adaptor</td>
<td>–1.89 (0.0017)</td>
<td><strong>–2.46 (0.0083)</strong></td>
<td>–1.55 (0.0072)</td>
</tr>
<tr>
<td>3004201</td>
<td>Erdman_2965</td>
<td>ppgK</td>
<td>Polyphosphate glucokinase</td>
<td>–1.84 (0.002)</td>
<td>–1.99 (0.002)</td>
<td><strong>–3.98 (0.0005)</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup>Log<sub>2</sub> fold change and adjusted <i>P</i> values were determined using TnseqDiff relative to the input control. Boldface indicates comparisons for which the Tn mutant met the statistical significance cutoffs of >±2 log<sub>2</sub> fold change and adjusted <i>P</i> value of <0.025.

<sup>b</sup>First nucleotide position of the Tn insertion site in the <i>M. tuberculosis</i> Erdman ATCC 35801 genome AP012340.1.

Tn mutants with defects in drug tolerance harbor secondary mutations that disrupt production of PDIM and cause decreased drug tolerance. Since neither the <i>ppgK</i>:Tn nor <i>clpS</i>:Tn mutant phenotype could be complemented, we sought to identify secondary mutations responsible for their drug susceptibility phenotypes. We conducted whole-genome resequencing on <i>rv0457c</i>:Tn, <i>ppgK</i>:Tn, and <i>clpS</i>:Tn mutants and our WT <i>M. tuberculosis</i> Erdman strain. This sequencing confirmed the predicted Tn insertion sites in each strain and demonstrated that each strain harbored a single Tn, ruling out the possibility that a secondary Tn insertion was responsible for their phenotypes (Fig. S4A to C). In both the <i>ppgK</i>:Tn and <i>clpS</i>:Tn mutants, we identified nonsynonymous single nucleotide polymorphisms (SNPs) in genes required for production of PDIM.

**FIG 4** Individual Tn mutants identified in the Pi starvation Tn-seq screen exhibit reduced fitness during drug treatment. <i>M. tuberculosis</i> strains were Pi starved for 72 h in Pi-free MtbyM medium before addition of the drug combinations ciprofloxacin (CIP, 8 μg/mL) plus isoniazid (INH, 0.2 μg/mL) (A, C, and E) or rifampicin (RIF, 0.1 μg/mL) plus INH (0.2 μg/mL) (B, D, and F). Surviving bacteria were enumerated by plating serial dilutions on 7H10 agar. Data represent the average ± standard error of the mean from at least two independent experiments. Asterisks indicate statistically significant differences between Tn mutant and WT: *, <i>P</i> < 0.05; **, <i>P</i> < 0.005.
of the lipid phthiocerol dimycocerosate (PDIM). No mutations in genes required for PDIM biosynthesis were identified in the WT Erdman or rv0457c::Tn strain. The ppgK::Tn strain had a G-to-C mutation at position 2360 in ppsE (Fig. S4D), which encodes a polyketide synthase required for production of the phthiocerol chain of PDIM (59). This SNP is predicted to cause a W787S amino acid substitution in PpsE that may alter PpsE activity and PDIM production. The clpS::Tn strain had a C-to-T mutation at position 655 in ppsD (Fig. S4E), which is predicted to introduce a premature amber stop codon at position 219 in PpsD. As ppsD also encodes a polyketide synthase required for production of the phthiocerol component of PDIM (59), the ppsDQ219* mutation is predicted to completely block PDIM production. Excluding highly repetitive sequences, such as the pe and ppe genes, which are difficult to resolve by short-read sequencing, these were the only nonsynonymous SNPs identified in the ppgK::Tn and clpS::Tn strains.

To directly test whether the ppsD Q219* or ppsE W787S mutation blocks PDIM production by the clpS::Tn or ppgK::Tn strain, respectively, we analyzed PDIM production by the clpS::Tn or ppgK::Tn strain, specifically, we analyzed PDIM production by an established radiolabeling method. Bacteria were labeled with [14C]propionate, which is selectively incorporated into PDIM, and the PDIM (DIM A) and phthiodiolone dimycocerosate precursor (DIM B) were detected in apolar lipid extracts by thin-layer chromatography (TLC) (33, 60). As expected, the clpS::Tn ppsD Q219* mutant did not produce any detectable PDIM (Fig. 5A, lane 2). The ppgK::Tn ppsE W787S mutant exhibited an intermediate PDIM production phenotype, with a 2.3-fold reduction in both DIM A and DIM B compared to the WT control (Fig. 5A, lane 4). These results suggest that the antibiotic susceptibility phenotypes of both mutants could be due to reduced PDIM production rather than the Tn insertion. These data also suggest that the intermediate drug susceptibility phenotypes of the ppgK::Tn ppsE W787S mutant could be caused by its intermediate level of PDIM production.

To determine if PDIM deficiency caused increased susceptibility of the clpS::Tn ppsD Q219* mutant to antibiotics, we complemented the ppsD Q219* mutation with ppsD on a plasmid. Complementation with ppsD fully restored PDIM production (Fig. 5A, lane 3).
We tested the sensitivity of the ppsD complemented strain to both CIP+INH and RIF+INH under Pi starvation conditions and observed similar resistance to both drug combinations as that with the WT control (Fig. 5B and C). These data demonstrate that loss of PDIM production, rather than loss of ClpS function, causes increased drug susceptibility of the clpS::Tn ppsD Q219* mutant. Since two of the Tn mutants that we identified in our Pi starvation screen exhibited reduced fitness upon antibiotic treatment due to spontaneous mutations in the PDIM biosynthesis locus, we cannot exclude the possibility that other Tn mutants with reduced antibiotic tolerance harbor similar secondary mutations responsible for their phenotypes.

**PDIM-deficient mutants are hypersusceptible to antibiotics in stationary- and exponential-phase MtbYM cultures.** *M. tuberculosis* strains were grown to early stationary phase (SP) (A, B, and D) or exponential phase (Exp) (C and E) in MtbYM medium before adding ciprofloxacin (CIP, 8 μg/mL) plus isoniazid (INH, 0.2 μg/mL) or rifampicin (RIF, 0.1 μg/mL) plus INH (0.2 μg/mL), as indicated. Data represent the average ± standard error of the mean from at least two independent experiments (A to C) or the average ± standard error of the mean from biological triplicate cultures (D and E). Asterisks indicate statistically significant differences: *, P < 0.05; **, P < 0.005. ns, not significant.

![Graph A](#)  
**A** Log CFU/ml vs. Day for SP CIP+INH.  
![Graph B](#)  
**B** Log CFU/ml vs. Day for SP RIF+INH.  
![Graph C](#)  
**C** Log CFU/ml vs. Day for Exp CIP+INH.  
![Graph D](#)  
**D** Log CFU/ml for Stationary phase.  
![Graph E](#)  
**E** Log CFU/ml for Exponential phase.
stationary phase, though this was not statistically significant (Fig. 6B). These data suggest that some Tn mutants with antibiotic tolerance phenotypes were not uncovered by our stationary-phase Tn-seq screen, perhaps due to the stringent statistical significance cutoffs we used. The decreased antibiotic tolerance of the \textit{clpS::Tn ppsD Q219*} mutant in stationary phase was fully complemented by \textit{ppsD} (Fig. 6D), confirming that PDIM deficiency also increases susceptibility to antibiotics in stationary phase. The \textit{clpS::Tn ppsD Q219*} mutant also exhibited a modest, but statistically significant, decrease in antibiotic tolerance in exponential phase for the \textit{CIP+INH} drug combination, which was partially complemented by \textit{ppsD} (Fig. 6C and E). These data suggest that the \textit{clpS::Tn ppsD Q219*} mutant also produces fewer stochastic persister variants due to loss of PDIM production.

**DISCUSSION**

Molecular mechanisms driving \textit{M. tuberculosis} recalcitrance to antibiotics under nutrient starvation are poorly characterized. Here, using a Tn-seq screen, we identify PDIM production as a critical determinant of \textit{M. tuberculosis} drug tolerance under nutrient-limited conditions. We identified two Tn mutants, \textit{clpS::Tn} and \textit{ppgK::Tn}, that were hypersusceptible to antibiotics. Both mutants harbored secondary mutations, unlinked to the Tn, that disrupted PDIM production. We restored PDIM production to the \textit{clpS::Tn} strain by complementing the \textit{ppsD Q291*} mutation and showed that this restored normal drug tolerance, directly demonstrating that \textit{M. tuberculosis} requires PDIM to tolerate antibiotic exposure. Loss of PDIM caused a decrease in the MIC\textsubscript{90} for RIF, demonstrating that PDIM contributes to intrinsic RIF resistance. However, PDIM-deficient strains also exhibited increased susceptibility to the \textit{CIP+INH} combination despite no change in the MIC\textsubscript{90} for these drugs, indicating that PDIM also promotes antibiotic tolerance. The \textit{ppgK::Tn ppsE W787S} mutant exhibited an intermediate drug tolerance phenotype that was associated with reduced, but not absent, PDIM production, suggesting that even partial inhibition of PDIM synthesis can sensitize \textit{M. tuberculosis} to antibiotics.

There are at least two mechanisms by which PDIM could increase \textit{M. tuberculosis} drug tolerance: decreasing the intracellular concentration of antibiotics by decreasing permeability of the outer membrane or altering the intracellular concentrations of central metabolites by functioning as a metabolic sink for propionate. PDIM decreases permeability of the \textit{M. tuberculosis} outer membrane to small molecules, including glucose and glycerol (39, 40), and may also restrict diffusion of some antibiotics. Indeed, PDIM is required for intrinsic resistance to vancomycin, likely by decreasing vancomycin access to its peptidoglycan target (41). PDIM has previously been implicated in drug tolerance in other mycobacteria. PDIM-deficient \textit{Mycobacterium bovis} BCG exhibited increased susceptibility to RIF, with a 4-fold decrease in MIC, but there was no change in susceptibility to INH or CIP (41). PDIM also increases antibiotic tolerance of \textit{Mycobacterium marinum}, which was correlated with reduced outer membrane permeability (61, 62). We observed that PDIM enhances \textit{M. tuberculosis} drug tolerance particularly under nutrient-limited conditions that limit accumulation of RIF and fluoroquinolone antibiotics (32). Since efflux pump inhibitors did not reverse the drug tolerance triggered by nutrient starvation (32), it is tempting to speculate that PDIM decreases drug uptake under nutrient-limited conditions by limiting import of antibiotics through the outer membrane.

Alternatively, PDIM could alter drug tolerance by effects on central metabolism. Synthesis of the long-chain branched fatty acids in PDIM requires the metabolite methylmalonyl coenzyme A (methylmalonyl-CoA), which is derived from propionate (59). During infection, \textit{M. tuberculosis} catabolizes fatty acids and cholesterol, which serve as primary carbon sources, to propionate (63, 64). Excess propionate stimulates increased production of PDIM with longer mycocerosic acid chain lengths both \textit{in vitro} and during infection of macrophages or mice (63, 65). PDIM was therefore proposed to act as a sink for propionyl-CoA, which can be toxic at high concentrations (63, 66). PDIM-deficient strains may be more susceptible to antibiotics, particularly under growth...
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conditions with fatty acids or cholesterol as carbon sources, due to the combined effects of the antibiotic and accumulation of toxic central metabolites. Growth in medium with either propionate or cholesterol as a carbon source increases the intrinsic resistance of M. tuberculosis to RIF (67). This increased RIF resistance was correlated with increased production and chain length of sulfolipid-1 (SL-1) (67), another branched-chain outer membrane lipid synthesized from propionate (65). PDIM production and chain length also increase with propionate as a carbon source, and PDIM is much more abundant than SL-1 (65), suggesting that PDIM could be primarily responsible for the carbon source-dependent increase in RIF resistance.

Connections between M. tuberculosis central metabolism and antibiotic tolerance have previously been reported. Genomic analysis of drug-resistant M. tuberculosis clinical isolates identified mutations in prpR, which encodes a transcriptional activator of PrpDC that catabolizes propionate through the methyl citrate cycle (68). Strains harboring prpR mutations exhibited increased drug tolerance specifically in medium with propionate as the sole carbon source (68). The authors of that study proposed that propionate accumulation in the prpR mutants limits antibiotic efficacy, but the propionate-dependent drug tolerance of prpR mutants could also simply be due to their slow growth with propionate as the sole carbon source (68). M. tuberculosis isocitrate lyase (ICL) is required for catabolism of both even- and odd-chain fatty acids and for tolerance to several different classes of antibiotics (69). The increased susceptibility of mutants lacking ICL activity was correlated with accumulation of tricarboxylic acid (TCA) cycle intermediates and with increased endogenous oxidative stress (69). However, ICL is also required for propionate catabolism (66), suggesting that accumulation of toxic propionate metabolites could also cause the hypersusceptibility of icl mutants to antibiotics.

Our results contrast with a previous study, in which selection for M. tuberculosis mutants with higher antibiotic persistence revealed multiple strains harboring spontaneous mutations in genes required for PDIM production (22). These PDIM-deficient mutants exhibited increased tolerance to multiple classes of antibiotics in exponential phase in the standard Middlebrook 7H9 medium, which contains glucose and glycerol as primary carbon sources (22). We may have observed decreased antibiotic tolerance of PDIM-deficient strains either because we used stationary-phase or P₁-starved cultures or because we used MtBYM rich medium. MtBYM rich medium contains additional carbon sources, including branched-chain amino acids and pyruvate, which are catabolized to propionate, and vitamin B₁₂, which activates production of methylmalonyl-CoA that is used for PDIM synthesis (70, 71). It is unclear which in vitro growth medium more closely reflects the conditions M. tuberculosis experiences in the host or whether loss of PDIM would enhance drug susceptibility during lung infection. This question will be challenging to address because PDIM is also a critical M. tuberculosis virulence determinant that is required for resistance to innate immunity (33, 60, 72). We intend to explore the role of PDIM in antibiotic tolerance during lung infection in our future studies.

Our screen identified over 100 unique M. tuberculosis Tn insertion mutants with altered drug tolerance phenotypes, including several genes or pathways with multiple independent Tn insertions. Our results point to the importance of regulated protein degradation in M. tuberculosis drug tolerance. Loss of Rv1957, a chaperone of the HigA1 antitoxin, or loss of proteasome components (Mpa or Paf) caused increased drug tolerance, possibly due to stabilization of toxins that inhibit bacterial replication. Mutations in genes encoding the Mce1 system, which is required for uptake of fatty acids (46), also increased antibiotic tolerance. Mce1 may also function in uptake of antibiotics, such that loss of Mce1 reduces antibiotic import. Alternatively, loss of Mce1 function may reduce accumulation of fatty acid-derived metabolites that synergize with antibiotics by reducing fatty acid uptake. We identified two independent Tn insertions in sigB that increased drug tolerance. SigB is an alternative sigma factor that was reported to be required for mycobacterial tolerance to RIF and INH (73, 74). Our results contrast with these studies, possibly due to our use of
different growth media, and suggest that SigB can under certain conditions limit *M. tuberculosis* drug tolerance.

Our results highlight several advantages of screening low-complexity Tn mutant pools made from an arrayed library. These include identification of Tn mutants with robust phenotypes from selection conditions with strict bottlenecks, efficient recovery of individual Tn mutants, and reproducibility of mutant phenotypes upon individual retesting. However, our results also uncovered one drawback of this method: the potential for recovery of Tn mutants with secondary mutations that alter the phenotype of interest. In standard Tn-seq screens that use high-complexity Tn mutant libraries, secondary mutations are less likely to influence identification of genes that significantly impact fitness due to the presence of multiple independent Tn insertions in each gene. Our screen identified numerous Tn mutants with decreased drug tolerance, but it is possible that many of these strains harbor spontaneous secondary mutations causing loss of PDIM production, similar to the *clpS* : Tn and *ppgK* : Tn mutants. Distinguishing whether the decreased drug tolerance of these mutants is due to the Tn insertion or to loss of PDIM will require recovery and individual retesting of these Tn mutants, which can be efficiently done from our arrayed Tn mutant library.

Overall, our results demonstrate that *M. tuberculosis* requires PDIM for drug tolerance under nutrient starvation conditions in vitro. As PDIM is also a critical virulence determinant that is required to counteract host immune pressures, our results suggest that inhibitors of PDIM production could synergize with both host-imposed stress and existing antibiotics to kill *M. tuberculosis* more efficiently. This could dramatically shorten TB treatment times and prevent emergence of new drug-resistant strains. PDIM biosynthesis is a complex process, requiring multiple polyketide syntheses, fatty acyl ligases, and thioesterases, several of which have already been explored as potential drug targets (59, 75, 76). It will be critical to determine whether PDIM deficiency also increases *M. tuberculosis* antibiotic susceptibility during infection to further support development of new inhibitors targeting PDIM production, which we intend to pursue in our future studies.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Bacterial strains used in this study are listed in Table S5 in the supplemental material. For routine culture, *M. tuberculosis* Erdman wild-type and derivative strains were grown aerobically at 37°C in Middlebrook 7H9 (Difco) liquid medium supplemented with 10% albumin-dextrose-saline (ADS), 0.5% glycerol, and 0.1% Tween 80 or on Middlebrook 7H10 (Difco) agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; BD Biosciences) and 0.5% glycerol. Frozen stocks of *M. tuberculosis* strains were made from mid-exponential phase cultures by adding glycerol to a 15% final concentration and storing them at −80°C. All experiments used MtbYM rich liquid medium (MtbYM), pH 6.6 (34), supplemented with 10% OADC and 0.05% tyloxapol. For P. starvation experiments, bacteria were grown in P-free MtbYM [made by replacing Na2HPO4 and KH2PO4 with NaCl and KCl and buffering with 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 6.6]. Antibiotics were used at the following concentrations unless otherwise noted: kanamycin (Kan), 25 μg/mL for agar or 15 μg/mL for liquid; hygromycin (Hyg), 50 μg/mL; ciprofloxacin (CIP), 8 μg/mL; rifampicin (RIF), 0.1 μg/mL; and isoliazid (INH), 0.2 μg/mL.

**Creation and mapping of an *M. tuberculosis* Erdman arrayed transposon mutant library.** Transposon (Tn) mutagenesis of wild-type *M. tuberculosis* Erdman was performed by transduction with the mycobacteriophage phAE159 carrying the Himar1 Tn as previously described (77). Wild-type bacteria were grown to mid-exponential phase (optical density at 600 nm [OD600] of 0.4 to 0.6) in 7H9 broth, washed and resuspended in MP buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 10 mM MgCl2, 2 mM CaCl2), and then transduced with mycobacteriophage at a multiplicity of infection (MOI) of at least 20:1 for 3 h at 40°C. Phage adsorption was stopped with stop buffer (MP buffer with 60 mM sodium citrate and 0.6% Tween 80), and transduced cells were plated on MtbYM agar (pH 6.6) with Kan at a density of 100 to 200 colonies per plate. Plates were incubated at 37°C with 5% CO2 for at least 3 weeks. Approximately 8,000 individual Tn mutant colonies were picked from plates into 600 μL of MtbYM broth in 1-mL V-bottom Matrix screw-cap tubes in a 96-well rack (Thermo Scientific) and incubated with shaking at 37°C for 2 weeks, until turbid.

Tn mutants were orthogonally pooled using the Straight Three strategy and sequenced, as previously described (35). Briefly, the Tn library was pooled in 2 groups of 40 racks each tracks 1 to 40 and racks 41 to 80. For each rack, a small volume of culture was removed from each tube and combined appropriately to form 8 row pools (rows A to H), 12 column pools (columns 1 to 12), and a rack pool. Each individual rack pool was aliquoted into one sample for sequencing and nine 1-mL aliquots for experimental use, which were stored at −80°C with glycerol at a 15% final concentration. After pooling, glycerol was added at a 15% final concentration to each Tn mutant culture and racks were stored at

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– 80°C. For each group of 40 racks, analogous row and column pools were pooled from all 40 plates to generate 8 row and 12 column pools. These were multiplexed with the 40 individual rack pools for a total of 60 samples for Tn-seq. Genomic DNA (gDNA) was extracted from each row, column, and rack pool using the cetyltrimethylammonium bromide (CTAB)-lysozyme method (78) and submitted to the University of Minnesota Genomics Center (UMGC) for library creation and Tn-seq as described below. Tn mutants associated with the reads were traced back to their rack location in the arrayed Tn library using two approaches: Straight Three (35) and Knockout Sudoku (36).

**Drug tolerance Tn-seq screen in stationary phase and P. stearns.** Five frozen rack pools (1 mL each) generated during orthogonal pooling were inoculated in 250 mL MtbYM broth with Kan and grown at 37°C with aeration to mid-exponential phase (OD$_{600}$ of 0.4 to 0.6). A portion of the culture was removed to start a 250-mL P. stearns-starved culture. Bacteria were washed twice in P. free MtbYM broth, inoculated in P. free MtbYM broth with Kan at an OD$_{600}$ of 0.1, and incubated at 37°C with aeration for 72 h. The remaining MtbYM culture was grown at 37°C with aeration for a total of 7 days to reach early stationary phase. We experimentally determined that at least 10$^6$ CFU of WT Erdman is recovered from a 12-mL culture after 9 days of drug treatment under either P. free or stationary-phase conditions (Fig. 1). Therefore, as input controls, the P. free or stationary-phase cultures were serially diluted and plated on MtbYM agar at a density of −10$^3$ CFU/plate before addition of antibiotics. Cultures then split into triplicate 12-mL antibiotic-treated (CIP + INH or RIF + INH) or untreated-control cultures and incubated with aeration at 37°C for 9 days. Antibiotic-treated bacteria were collected by centrifugation (3,720 × g, 10 min), washed twice with an equal volume of PBS-T (Gibco phosphate-buffered saline [PBS], pH 7.4, with 0.05% Tween 80) to remove antibiotics, concentrated 100-fold in PBS-T, and plated on YM agar with Kan to recover at least 10$^6$ CFU. Untreated control cultures were serially diluted and plated at a density of −10$^6$ CFU/plate. Plates were incubated at 37°C with 5% CO$_2$ until the biomass on the agar was confluent, up to 2 weeks. Confluent plates were flooded with 2 mL of GTE buffer (78) and gently scraped with a plastic 10-µL loop to loosen the biomass. Bacteria were collected by centrifugation (3,720 × g, 10 min), and gDNA was extracted from cell pellets by the CTAB-lysozyme method (78) and cleaned using the genomic DNA Clean and Concentrator kit (Zymo) before submission to UMGC for Tn-seq library preparation and Illumina sequencing.

**Tn-seq and data analysis.** Transposon sequencing (Tn-seq) was performed as previously described (34). M. tuberculosis genomic DNA was fragmented with a Covaris S220 ultrasonicator, and a whole-genome library was prepared using the TruSeq Nano library preparation kit (Illumina). Library fragments containing Tn junctions were PCR amplified from the whole-genome library using the Tn-specific primer Mariner_1R_TnSeq_noMm and Illumina p7 primer (Table S6). The amplified products were uniquely indexed to allow sample pooling and multiplexed sequencing. Resulting Tn-seq libraries were sequenced on an Illumina 2500 high-output instrument in 125-bp paired-end output mode using v4 chemistry (Illumina). Sequencing reads were filtered to remove reads without the Tn sequence GAGCCTATAGCCAACCGTG. The 5’ Illumina adaptor sequences were trimmed using BBMap (https://sourceforge.net/projects/bbmap/). Each trimmed read was cut to 30 bases, and sequences not starting with TA were removed. Remaining reads were mapped to the M. tuberculosis Erdman genome (NC_002559.1) using HISAT2. Mapped reads were counted at each TA insertion site in the M. tuberculosis Erdman genome to generate read count tables for TnseqDiff analysis. TnseqDiff normalized the read counts using the default trimmed mean of M values (TMM) normalization method (79, 80) and then determined conditional essentiality for each TA insertion site between experimental conditions (control/input, CIP + INH/input, RIF + INH/input, CIP + INH/control, RIF + INH/control). TnseqDiff calculated the fold change and corresponding two-sided P value for each TA insertion site (38). All P values were adjusted for multiple testing using the Benjamini-Hochberg procedure in TnseqDiff. The cutoff values for statistical significance were set at a fold change of > ±2 log$_2$ and an adjusted P value of < 0.025.

**Recovery of Tn mutants from the arrayed Tn mutant library.** Each Tn mutant individually retested was isolated from the tube corresponding to the Tn mutant location in the arrayed library by streaking for individual colonies on MtbYM agar containing Kan. Plates were incubated for at least 3 weeks at 37°C. Up to four individual colonies were picked and grown in 10 mL of MtbYM broth with Kan at 37°C with aeration until turbid. The Tn insertion site was confirmed by PCR using a gene-specific primer 5’ or 3’ of the TA site and a primer specific to the Tn Kan resistance cassette (Table S6) followed by Sanger sequencing.

**Tn mutant complementation.** Vectors for complementation of Tn mutants were made in the integrating plasmid pMV306hyg (Table S5). We generated pMV306hyg by replacing the aph Kan resistance marker in pMV306 (81) with a Hyg resistance cassette. pMV306 without aph was PCR amplified with primers pMV306_F and pMV306_R (Table S6), digested with SfiI and AffI, and ligated to a Hyg resistance cassette that was removed from pJT6a (82) by restriction with SfiI and AffI. Each gene was PCR amplified along with ~ 300 bases 5’ of the translation start site to include the native promoter (Table S6), cloned in pcR2.1 TOPO (Invitrogen) and sequenced, and then removed from pcR2.1 by restriction with XbaI and HindIII and ligated to XbaI- and HindIII-digested pMV306hyg. Cosmid BC3.1 containing the genomic region rv1317 to rv1345 surrounding cp5 was obtained from the lab of William R. Jacobs. The pMV361-hyg-ppsD vector was generated by replacing the Kan resistance cassette in an existing pMV361-ppsD vector (60) with a Hyg resistance cassette by Gibson assembly. Primers pMV361_FOR/pMV361_REV and hyg_fwd/rev were used to PCR amplify pMV361-ppsD without the Kan resistance cassette and the Hyg resistance cassette from pMV306hyg, respectively (Table S6). PCR products were assembled with New England Biolabs (NEB) HiFi Assembly master mix (New England Biolabs) following by sequencing with ppsD and the Hyg resistance cassette. Tn mutants were electroporated with the corresponding complementation vector or cosmid as described previously (83). Transformants were selected on
Middlebrook 7H10 agar containing Kan and Hyg. The presence of the complementing plasmid or cosmid was confirmed by PCR (Table S6).

**Individual retesting of Tn mutant antibiotic tolerance.** Bacteria were grown from frozen stocks to mid-exponential phase (OD\text{\text{600}} of 0.4 to 0.7) in 7H9 complete medium. For \text{P}\text{,}\text{F}\text{,} free experiments, starter cultures were washed twice with \text{P}\text{,}\text{F}\text{-free MtbYM broth, resuspended at an OD\text{\text{600}} of 0.1 in P\text{,}\text{F}-free MtbYM broth, and grown for 72 h. For stationary-phase experiments, starter cultures were diluted to an OD\text{\text{600}} of 0.05 in MtbYM broth and grown for 7 days. For exponential-phase experiments, starter cultures were diluted to an OD\text{\text{600}} of 0.025 in MtbYM medium, incubated for 3 days to reach mid-exponential phase (OD\text{\text{600}} of 0.4 to 0.7), and then diluted to an OD\text{\text{600}} of 0.2. Cultures were serially diluted and plated on 7H10 agar to enumerate the input CFU per milliliter. Cultures were then split into CIF + INH treated or RIF + INH treated and aliquoted to create 12-mL single-use cultures for each time point. This method enables formation of stable drug-tolerant populations by reducing production of toxic reactive oxygen species that are generated due to changes in oxygen saturation upon repeated sampling of a culture (37). Cultures were washed with PBS-T, serially diluted, and plated on 7H10 agar to determine surviving CFU per milliliter as previously described (84). Plates were incubated at 37°C with 5% CO\text{\text{2}} for at least 4 weeks prior to enumerating surviving CFU.

**MIC assay.** Bacteria were grown from frozen stocks to mid-exponential phase (OD\text{\text{600}} of ~0.5) in MtbYM broth and diluted to an OD\text{\text{600}} of 0.01 in 5 mL fresh MtbYM broth. Antibiotics were added at 2-fold-increasing concentrations. Cultures without antibiotics were included as controls. Cultures were incubated at 37°C with aeration. The OD\text{\text{600}} of each culture was measured on day 7 for INH or day 14 for RIF and CIP. The MIC\text{min} was defined as the minimum concentration of antibiotic required to inhibit at least 90% of growth relative to the no-antibiotic control.

**Whole-genome sequencing of \text{M. tuberculosis} wild-type Erdman and Tn mutants.** Genomic DNA (gDNA) was extracted from the wild-type Erdman parental strain and \text{nov}py \text{::Tn, ppg} \text{::Tn, and cfp} \text{::Tn mutants grown to late exponential phase in 7H9 broth by the CTAB-lysozyme method (78). The gDNA was cleaned with the genomic DNA Concentrator and Cleanup kit 25 (Zymo) and then sheared to ~300 bp by ultrasonication (Covaris S220). Sizing postshearing was done with an Agilent Bioanalyzer. Libraries were prepared from sheared gDNA using the NEBNext Ultra II DNA library prep kit for Illumina (New England Biolabs). Briefly, the ends of the fragmented DNA were repaired by 5’ phosphorylation and 3’ tailing, followed by Illumina adaptor ligation. Adaptor-ligated DNA was size selected for a 350-bp insert using AMPure beads (Beckman Coulter). Adaptor-ligated DNA was then PCR amplified, uniquely barcoded, and cleaned with AMPure beads. Library quality control (QC), pooling, and Illumina sequencing were done at the UMGC. Samples were sequenced on an Illumina iSeq 100 with 150-bp paired-end output. To generate a consensus sequence for each strain, paired reads were mapped to the \text{M. tuberculosis} Erdman reference genome (NC_020559.1) using the "map to reference" function in Geneious 2020 software (Biomatters, Ltd.) with the following settings: mapper – Geneious; sensitivity – medium sensitivity/fast; fine tuning – iterate up to 5 times.

To identify single nucleotide polymorphisms (SNPs) in Tn mutant genomes, the WT Erdman and Tn mutant consensus sequences were aligned in Geneious using the "Align Whole Genomes" function with the default Mauve Genome parameters (Alignment algorithm – progressiveMauve algorithm; automatically calculate seed weight; compute Locally Colinear Blocks [LCBs]; automatically calculate minimum LCB score; full alignment). Regions containing PE-PGRS genes that were poorly mapped in either consensus sequence were excluded from SNP analysis. SNPs identified in genes required for PDIM synthesis were confirmed by PCR amplification and Sanger sequencing (Table S6). To confirm Tn insertion sites in the Tn mutants, reads were mapped to the \text{Himar1} Tn sequence in Geneious as described above. Sequences adjacent to the Tn were compared to the \text{M. tuberculosis} Erdman reference genome to identify the Tn insertion site.

**PDIM labeling and detection.** To detect phthiocerol dimycocerosate (PDIM) production, we used a radiolabeling and thin-layer chromatography (TLC) method (60). Briefly, \text{M. tuberculosis} cultures grown to mid-logarithmic phase in 10 mL of 7H9 broth were labeled for 48 h with 10 μCi of [1-\text{14}C]propionic acid, sodium salt (American Radiolabeled Chemicals, Inc.; specific activity, 50 to 60 mCi/mmol). Labeled bacteria were collected by centrifugation (2,500 × g, 10 min), Apolar lipids were extracted twice in 2 mL 10:1 (vol/vol) methanol-0.3% NaCl and 2 mL petroleum ether by vortexing for 4 min and collecting the upper petroleum ether layer after phase separation by centrifugation (750 × g, 10 min). Combined petroleum ether fractions were inactivated for 1 h with an equal volume of chloroform and then evaporated overnight to reduce the extract to ~4 mL. Extracts (30 μL) were spotted on a silica gel 60 F\text{254} TLC plate (5 by 10 cm; Supelco). The TLC plate was developed in 9:1 (vol/vol) petroleum ether-diethyl ether, 10:1 (vol/vol) methanol-0.3% NaCl and 2 mL petroleum ether by vortexing for 4 min and collecting the upper petroleum ether layer after phase separation by centrifugation (750 × g, 10 min). Combined petroleum ether fractions were inactivated for 1 h with an equal volume of chloroform and then evaporated overnight to reduce the extract to ~4 mL. Extracts (30 μL) were spotted on a silica gel 60 F\text{254} TLC plate (5 by 10 cm; Supelco). The TLC plate was developed in 9:1 (vol/vol) petroleum ether-diethyl ether, air dried, and exposed to a phosphor storage screen (Amersham) for 3 days. Radioactive bands were detected using a Typhoon FLA 9500 Imager (GE Healthcare). Intensity of the radioactive signal in each PDIM spot was quantified with ImageJ.

**Custom scripts and code.** All custom scripts and R code used for Illumina sequence read processing and TnseqDiff data analysis are available at https://github.com/bloc078/umn-fischer-tnseq.

**Statistical analysis.** Student’s unpaired t test (two-tailed) was used for pairwise comparisons between WT, mutant, and complemented strains. P values were calculated using GraphPad Prism 8.0 (GraphPad Software, Inc.). P values of <0.05 were considered significant.

**Data availability.** Raw sequencing data are publicly available in FASTA format at the Data Repository for the University of Minnesota. Tn-seq data for Tn library mapping are at https://hdl.handle.net/11299/234209. Tn-seq data from drug tolerance experiments and whole-genome sequencing data are at https://hdl.handle.net/11299/231054. All raw sequencing data are also available from the NCBI Sequence Read Archive (accession no. PRJNA894209).
SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, EPS file, 0.2 MB.
FIG S2, EPS file, 0.8 MB.
FIG S3, EPS file, 0.8 MB.
FIG S4, EPS file, 0.6 MB.

TABLE S1, XLSX file, 0.9 MB.
TABLE S2, XLSX file, 0.2 MB.
TABLE S3, XLSX file, 1 MB.
TABLE S4, XLSX file, 0.05 MB.
TABLE S5, XLSX file, 0.01 MB.
TABLE S6, XLSX file, 0.01 MB.

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