High Persister Mutants in *Mycobacterium tuberculosis*

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**Abstract**

*Mycobacterium tuberculosis* forms drug-tolerant persister cells that are the probable cause of its recalcitrance to antibiotic therapy. While genetically identical to the rest of the population, persisters are dormant, which protects them from killing by bactericidal antibiotics. The mechanism of persister formation in *M. tuberculosis* is not well understood. In this study, we selected for high persister (*hip*) mutants and characterized them by whole genome sequencing and transcriptome analysis. In parallel, we identified and characterized clinical isolates that naturally produce high levels of persisters. We compared the *hip* mutants obtained *in vitro* with clinical isolates to identify candidate persister genes. Genes involved in lipid biosynthesis, carbon metabolism, toxin-antitoxin systems, and transcriptional regulators were among those identified. We also found that clinical *hip* isolates exhibited greater ex vivo survival than the low persister isolates. Our data suggest that *M. tuberculosis* persister formation involves multiple pathways, and *hip* mutants may contribute to the recalcitrance of the infection.

**Introduction**

Over one third of the global human population is infected with *Mycobacterium tuberculosis*, the etiological agent of tuberculosis [1]. While up to 90% of tuberculosis infections are latent, with no clinical symptoms, they remain a concern due to their potential for reactivation [2], [3]. Treatment of active tuberculosis requires at least six months of antibiotic therapy. This lengthy treatment as well as the vast reservoir of latent infection is likely due to the presence of persister cells [4].

Persisters form a subpopulation of phenotypically drug tolerant cells. Unlike resistant mutants, persisters do not grow in the presence of antibiotics. Upon regrowth, persisters reestablish a population that retains the same susceptibility as the original population [5]. Persisters are produced by all bacterial species studied to date, but much of what we know about them is based on studies of *Escherichia coli*. Persisters are non-growing cells [6] whose gene expression profile [7] and low levels of translation [8] indicate that they are in a dormant state. The target
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pathways of antibiotics are inactive in dormant cells, which accounts for their tolerance to drug exposure [8]. In *E. coli*, pathways leading to dormancy are highly redundant and largely depend on the action of toxin-antitoxin (TA) modules [7], [9]. For example, protein synthesis inhibition by the HipA toxin [10], [11], a kinase [12] and by at least 10 different mRNA endonucleases such as RelE, MazF and YafQ [7], [9], [13], [14] leads to dormancy. In addition, damage of DNA induces the SOS response and expression of the TisB toxin [15], an endogenous antimicrobial peptide [16] that causes persister formation by opening an ion channel that decreases the proton motive force and ATP levels leading to a dormant, drug-tolerant state [17]. Typically overexpression of a persister gene leads to shutdown of an important cellular function, which results in drug tolerant persisters. Apart from TA modules, additional genes have been implicated in persister formation in *E. coli* including those involved in glycerol and nucleotide metabolism as well as some global regulators [18], [19], [20].

Transcriptome analysis of *M. tuberculosis* persister cells highlighted a metabolic downshift and upregulation of TA modules that is consistent with that observed in *E. coli* [7], [21]. In *M. tuberculosis*, there are close to 80 TA modules identified so far [22], [23], indicating a potential for an extremely high level of redundancy in mechanisms governing dormancy. Direct evidence of TA systems playing a role in drug tolerance of *M. tuberculosis* has been reported. Ectopic overexpression of three individual *E. coli* *relE* homologues (Rv1246c, Rv2866, Rv3358) was found to increase drug tolerance [24]. Interestingly, *relE* overexpression effects are drug and isoform specific, which suggests that more than one subpopulation of persisters exist. Other mechanisms responsible for differential antibiotic susceptibility of mycobacteria include asymmetrical growth during cell division [25], stochastic expression of genes affecting antimicrobial action such as catalase-peroxidase *katG*, which activates the prodrug isoniazid and affects drug tolerance in a subset of cells [26], and changes that affect storage lipid accumulation [27] such as shifts in carbon flux through the TCA cycle [28]. The latter points to alterations in metabolism as a key factor influencing drug tolerance in *M. tuberculosis*.

While a persister cell is in a temporary state, the proportion of persisters within a population is relatively stable. Typically, the frequency of persister formation increases with cell density and reaches about 1% in stationary phase [5], [21], [29]. The frequency of persister formation can also change with mutations. For example in *E. coli*, a gain of function mutation in the HipA toxin, obtained through *in vitro* mutagenesis, increases the level of persister production 1,000-fold [30]. In *Salmonella typhimurium*, a high persister (*hip*) mutation in the RelB toxin also increases persister production over 1,000-fold [31]. Selection for *hip* mutants can also occur in a clinical setting. Antibiotic treatment has been shown to select for *hip* mutants in patients with *Candida albicans* biofilms [32] or with *Pseudomonas aeruginosa* infection [33]. In *E. coli*, gain of function HipA mutants were recently discovered in clinical isolates and shown to have increased levels of persisters in an ex *vivo* model of infection [34]. These findings link persisters to clinical manifestation of disease. To date no *hip* mutants of *M. tuberculosis* have been reported. Here we report identification of *hip* mutants of *M. tuberculosis* obtained from an *in vitro* selection, and from a screen of clinical isolates.

**Results**

**Characterization of *hip* mutants obtained *in vitro***

**Hip phenotype selection.** To identify genes responsible for persister formation, we mutagenized *M. tuberculosis* mc²6020, an auxotroph of H37Rv [35], and selected for mutants surviving treatment with a lethal dose of streptomycin and rifampicin. With each round of selection, the level of persisters surviving antibiotic treatment increased in both exponential and stationary phase (Fig 1A and 1B). Three representative mutant strains were characterized
Fig 1. Characterization of hip mutants obtained in vitro. Persister assays, performed by antibiotic treatment with streptomycin (10 μg/ml) and rifampicin (1 μg/ml) for 14 days, reveal the number of drug tolerant persister cells based on CFU counts. Exponential (A) and stationary phase (B) treatment of mutagenized strain mc²6020 at each stage of the hip mutant selection process. Time-dependent persister assays in exponential (C) and stationary phase (D) with independent mutants KL2801, KL2825, KL2849, and wild type strain (mc²6020). Late exponential phase cultures were treated with various concentrations of streptomycin.
further. The strains produced 100- to 1,000-fold more persisters than the parental wild type strain (mc²6020) in time-dependent (Fig 1C and 1D) and concentration-dependent antibiotic treatment (Fig 1E and 1F). In addition, the mutants consistently produced significantly more (p-value < 0.05) persisters than the wild type (mc²6020) when treated with antibiotics belonging to different classes that were not used in the selection process (Fig 1G), and when grown on various carbon sources (glycerol, butyrate, and propionate) (Fig 1H). For each mutant, neither minimum inhibitory concentration (MIC) (S4 Table) nor growth rate (S5 Table) differed significantly from the wild type (mc²6020) (p-value < 0.05). These selection criteria ruled out the possibility that increased drug resistance or growth defects were responsible for increased survival. Taken together, these results show that the hip mutants selected in vitro are multidrug tolerant and that their phenotype is independent of antibiotic class, antibiotic concentration, growth phase, or carbon source.

Whole genome sequencing and transcriptome analysis to identify candidate genes. The genomes of 18 hip mutants derived from 12 independent mutageneses were sequenced by Illumina technology and found to carry between one and 16 non-synonymous mutations (Fig 2 and Table 1). In one case, three mutants derived from the same mutagenesis (KL2849, KL2850, KL2851) contained identical non-synonymous mutations while strains derived from independent mutageneses contained mostly unique mutations. Since gene expression information could help elucidate the potential involvement of a candidate gene in the hip phenotype, transcriptome analysis of stationary phase hip mutants was carried out (S6 Table). Interestingly, transcriptome analysis showed high similarity in gene expression in the three hip mutants (KL2801, KL2825, KL2849) (S7 Table) even though there was no overlap in non-synonymous mutations (Table 1). This suggested that they each contained mutations in a common pathway. In KL2849, the single non-synonymous mutation in fatty-acid-CoA ligase fadD26 (G74/C3) was an interesting candidate. FadD26 is involved in biosynthesis of phthiocerol dimycocerosate (PDIM), an important virulence lipid. Subsequent total lipid analysis confirmed an absence of PDIM among the three strains (S1 Fig). With KL2801 and KL2825, while there was no mutation in the ORFs of its biosynthetic pathway, it is possible that a mutation in a promoter or regulatory region is responsible for the PDIM-null phenotype. Sequence analysis revealed multiple mutations and single non-synonymous mutations in genes associated with PDIM biosynthesis in several other hip mutants (Table 1).

To test whether the loss of PDIM is associated with the hip phenotype, we compared the growth-persister pattern of the fadD26 (G74̅) PDIM-null hip mutant (KL2849) and the parental wild type strain (mc²6020) with that of a previously published fadD26 transposon (Tn) null mutant and its parental wild type strain (Erdman) [36]. While the backgrounds of the fadD26::Tn and hip mutant differ (H37Rv derivative versus Erdman), comparison is possible since a common role for PDIM has been reported in these two strains [36], [37]. In both cases, the PDIM mutant produced more persisters than its parental strain (Fig 3A and 3B). Interestingly, a PDIM-transport protein drrA (Rv2936) H37Rv mutant [38], which produces PDIM but does not correctly localize it to the cell wall, did not have a hip phenotype (S2 Fig). This suggests that it is the biosynthesis of PDIM, rather than the lipid itself, that is responsible for the difference in drug tolerance. We noticed that the fadD26::Tn mutant exhibited a faster growth rate than the wild type (Erdman) (Fig 3B). While there was no significant difference in growth rate
Fig 2. Genetic analysis of hip mutants obtained in vitro. Representative antibiotic survival plots of 18 hip mutant strains, obtained from 12 independent mutageneses, are presented along with lists of genes containing non-synonymous mutations within each strain.

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Table 1. Non-synonymous mutations identified in individual in vitro hip mutant strains by whole genome sequencing.

| Strain     | Gene          | Annotation                        | Location | Reference | Mutant | Change |
|------------|---------------|-----------------------------------|----------|-----------|--------|--------|
| KL2801     | Rv1387        | PPE family protein                | 1563341  | C         | T      | P525S  |
| KL2801     | Rv1395        | transcriptional regulator          | 1571669  | C         | T      | P208L  |
| KL2801     | Rv2071c       | precorrin-4 c11-methyltransferase cobM | 2328463  | C         | T      | A172V  |
| KL2801     | Rv2249c       | glycerol-3-phosphate dehydrogenase gldP1 | 2523381  | C         | T      | R471W  |
| KL2801     | Rv2423        | hypothetical protein               | 2719708  | G         | A      | V38M   |
| KL2802     | Rv0351        | chaperone grpE                     | 422212   | G         | A      | M168I  |
| KL2802     | Rv1656        | ornithine carbamoyltransferase argF | 1870565  | C         | T      | A215V  |
| KL2802     | Rv1702c       | conserved hypothetical protein     | 1927776  | G         | A      | G267D  |
| KL2802     | Rv2379c       | peptide synthetase mbtF            | 2661260  | T         | C      | F276L  |
| KL2802     | Rv2933        | phenolthiocerol synthesis type-I polyketide synthase ppsC | 3261616  | T         | C      | W1978R |
| KL2803     | Rv1564c       | maltoligosyltrehalose synthase treX | 1769662  | C         | T      | A647V  |
| KL2803     | Rv1576c       | phiRv1 phage protein               | 1781577  | C         | T      | T163I  |
| KL2825     | Rv0254c       | bifunctional cobalamin biosynthesis protein cobU | 305878   | G         | A      | A158T  |
| KL2825     | Rv3560c       | acyl-CoA dehydrogenase fadE30      | 4000449  | C         | T      | R381W  |
| KL2826     | Rv1227c       | transmembrane protein              | 1370665  | G         | A      | W54*   |
| KL2826     | Rv2307c       | conserved hypothetical protein      | 2578588  | C         | T      | P37S   |
| KL2826     | Rv2368c       | phosphate starvation-inducible protein phoH1 | 2649391  | C         | T      | T195M  |
| KL2826     | Rv2383c       | phenoxazoline synthase mbtB        | 2671835  | C         | T      | P135S  |
| KL2826     | Rv2389c       | resuscitation-promoting factor rpfD | 2683423  | C         | T      | A97V   |
| KL2826     | Rv2428        | alkyl hydroperoxide reductase C protein ahpC | 2726541  | G         | A      | E117K  |
| KL2826     | Rv2442c       | 50S ribosomal protein L21 rplU      | 2740088  | C         | T      | R92C   |
| KL2826     | Rv2454c       | oxidoreductase beta subunit         | 2754530  | C         | T      | P73S   |
| KL2826     | Rv2462c       | trigger factor protein tg           | 2764088  | C         | T      | P402S  |
| KL2826     | Rv2482c       | glycerol-3-phosphate acyltransferase pslB2 | 2788623  | C         | T      | L211F  |
| KL2826     | Rv2508c       | conserved alanine and leucine rich membrane protein | 2824394  | C         | T      | A67V   |
| KL2826     | Rv2516c       | hypothetical protein                | 2832738  | C         | T      | A259V  |
| KL2826     | Rv2543        | lipoprotein lppA                    | 2866837  | G         | A      | V124L  |
| KL2826     | Rv2557c       | protein-export membrane protein secD | 2915001  | C         | T      | P246S  |
| KL2826     | Rv2930        | fatty-acid-CoA ligase fadD26        | 3243916  | G         | T      | G74*   |
| KL2827     | Rv2940c       | multifunctional mycocerosic acid synthase membrane-associated mas | 3280534  | T         | C      | S728P  |
| KL2849     | Rv2930        | fatty-acid-CoA ligase fadD26        | 3243916  | G         | T      | G74*   |
| KL2850     | Rv2930        | fatty-acid-CoA ligase fadD26        | 3243916  | G         | T      | G74*   |
| KL2851     | Rv2930        | fatty-acid-CoA ligase fadD26        | 3243916  | G         | T      | G74*   |
| KL1090     | Rv2324        | transcriptional regulator, asnC-family | 2596356  | A         | G      | D6G    |
| KL1090     | Rv2933        | phenolthiocerol synthesis type-I polyketide synthase ppsC | 3257052  | AC        | AGACGAATGC... |
| KL1105     | Rv2082        | conserved hypothetical protein      | 342139   | G         | T      | V4L    |
| KL1105     | Rv2281        | phosphate-transport permease pitB   | 2554762  | G         | A      | W530*  |
| KL1105     | Rv2324        | transcriptional regulator, asnC-family | 2596559  | G         | A      | G76S   |
| KL1105     | Rv2362c       | conserved hypothetical protein      | 2643744  | C         | T      | T172I  |
| KL1105     | Rv2391        | ferredoxin-dependent nitrite reductase nirA | 2685163  | G         | A      | G162E  |
| KL1105     | Rv2402        | conserved hypothetical protein      | 2699244  | G         | A      | W239*  |
| KL1105     | Rv2425c       | conserved hypothetical protein      | 2722000  | C         | T      | L437F  |
| KL1116     | Rv2324        | transcriptional regulator, asnC-family | 2596492  | C         | A      | F52L   |
| KL1117     | Rv3696c       | glycerol kinase glpK               | 4139183  | A         | AC     | G335E  |
| KL1117     | Rv1131        | citrate synthase I gltA1           | 1257138  | G         | A      | G335E  |

(Continued)
of KL2849 compared to wild type (mc^2 6020), the hip mutant had a slightly shorter generation time during early exponential growth (S5 Table). Taken together, these results suggest that the absence of PDIM production gives PDIM-null mutants a growth advantage. Since persister formation is growth stage-dependent, the changes in PDIM production may indirectly affect antibiotic tolerance by changing the timing of transition from exponential to stationary phase.

Genes and pathways that have previously been associated with persister formation were also of interest when analyzing the hip mutants. In E. coli, glycerol metabolism has been linked to persister formation [18], [20]. In the hip mutants, two confirmed E. coli persister gene candidates, glycerol-3-phosphate dehydrogenase glpD1 (Rv2249c) and glycerol-3-phosphate acyltransferase plsB2 (Rv2484c), were found to contain non-synonymous mutations. We tested whether overexpression of either glpD1 or plsB2, confirmed by immunoblot, had an effect on persister formation but the results were negative (S3 Fig). There are several possible explanations for the lack of an effect on persisters by overexpression of these two genes. First, there are two copies of each of these genes in M. tuberculosis, which may provide functional genetic redundancy. Second, downregulation rather than overexpression may be required as in the case of plsB in E. coli. Both genes were significantly downregulated in various hip mutants, supporting this possibility (S6 Table). In addition to glpD1 and plsB2, we found several mutations in glycerol kinase glpK (Rv3696c). In E. coli, a glpK-null mutant had no persister phenotype [18] therefore we did not pursue it further as a candidate. Genes involved in the TCA cycle have also been implicated in drug tolerance in M. tuberculosis [28], [39]. In the three

### Table 1. (Continued)

| Strain   | Gene           | Annotation                                | Location | Reference | Mutant | Change  |
|----------|----------------|-------------------------------------------|----------|-----------|--------|---------|
| KL1120   | Rv2324         | transcriptionsal regulator, asnC-family   | 2596619  | G         | T      | A95S    |
|          | Rv2930         | fatty-acid-CoA ligase fadD26               | 3244737  | CA        | C      |         |
| KL1130   | Rv2323c        | conserved hypothetical protein             | 2596217  | G         | A      | R17H    |
|          | Rv1521         | fatty-acid-CoA ligase fadD25               | 1711950  | G         | A      | G401R   |
|          | Rv1536         | isoleucyl-tRNA synthetase ileS            | 1738703  | G         | A      | E728K   |
|          | Rv2184c        | conserved hypothetical protein             | 2464234  | C         | T      | A237V   |
|          | Rv2211c        | aminomethyltransferase gcvT                | 2477679  | C         | T      | P128S   |
|          | Rv2245         | 3-oxoacyl-[acyl-carrier protein] synthase 1| 2519289  | G         | A      | G391D   |
|          | Rv2263         | oxiredutase                               | 2535777  | G         | A      | R45H    |
|          | Rv2308         | conserved hypothetical protein             | 2580606  | G         | A      | G62D    |
|          | Rv2327         | conserved hypothetical protein             | 2600309  | G         | A      | E107K   |
|          | Rv2376c        | low molecular weight KL protein antigen cfp2| 2655937 | C         | T      | A59V    |
|          | Rv2934         | phenolpiocerol synthesis type-I polyketide synthase ppsD | 3264484 | C         | A      | A745E   |
| KL1137   | Rv2019c        | conserved hypothetical protein             | 223958   | C         | T      | P131L   |
|          | Rv2931         | phenolpiocerol synthesis type-I polyketide synthase ppsA | 3247786 | G         | A      | G780D   |
|          | Rv3696c        | glycerol kinase glpK                      | 4138776  | G         | A      | G326D   |
|          | Rv3919c        | glucose-inhibited division protein B gid   | 4407977  | G         | A      | G75S    |
| KL1170   | Rv3696c        | glycerol kinase glpK                      | 4138299  | G         | A      | R485Q   |

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 Hip Mutants in Mycobacterium tuberculosis

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representative *hip* mutants, isocitrate lyase *icl* (Rv0467) was significantly upregulated in stationary phase (*S7 Table*) while citrate synthase *gltA1* (Rv1131) contained a non-synonymous SNP and was upregulated in KL1117 (*S6 Table*).

We noted global changes in metabolism of the *hip* mutants by transcriptome analysis showing significant upregulation of genes associated with energy production, such as a possible oxidoreductase (Rv3742c), inorganic ion metabolism, such as rubredoxin *rubA* and *rubB* (Rv3250c-Rv3251c), and lactate dehydrogenase (Rv1872c) (*S8 Table*). Downregulation of two transcriptional regulators (Rv0273 and Rv3066) is of interest as they may control of the expression of multiple genes (*S8 Table*). Another candidate is probable asparagine synthase C *asnC* family transcriptional regulator (Rv2324). As a feast/famine regulatory protein, the *asnC*
transcriptional regulator controls the expression of a large number of genes in response to stress or changes in the environment [40]. While there was no clear trend in differential expression of Rv2324 (S6 Table), multiple independent mutations occurred in the hip mutants in this gene as well as in the hypothetical protein (Rv2323c) directly upstream of it, which suggests an association with the hip phenotype.

Characterization of clinical isolates with a hip phenotype

**Longitudinal clinical isolate analysis.** Repeated exposure of a mutagenized population to high doses of antibiotics enabled us to select for hip mutants of *M. tuberculosis* in vitro. We reasoned that in a clinical setting repeated treatment of patients with high doses of antibiotics might also select for hip mutants. To examine this possibility, we assessed persister levels of matched pairs of isolates from four longitudinal clinical tuberculosis cases. In each case, due to relapse or treatment failure, the patient had been treated for an extended period (S9 Table). The early and late isolates were collected at least 24 months apart and the pairs were matched based on identical high-resolution (24-loci) mycobacterial interspersed repetitive unit—variable number tandem repeat (MIRU-VNTR) genotyping patterns. Importantly, while some of the isolates were drug resistant (S9 Table), there was no change in MIC of the antibiotic used in the persister assay (S10 Table). To control for variation in growth rates among isolates, we performed the assay in stationary phase. When treated with kanamycin (125 μg/ml), one longitudinal pair (Case 3) exhibited a 10-fold increase in the level of persister formation from early to late (Fig 4A). This suggested that hip mutant selection could occur in clinical isolates of *M. tuberculosis*.

**Screen of independent, drug-sensitive clinical isolates.** In the longitudinal study, we observed large variation in persister levels among independent isolates (Fig 4A), which further supported the possibility that hip strains exist among clinical isolates. To examine this possibility, we screened a large panel of 39 independent, drug-sensitive clinical isolates (S9 Table). The isolates were all equally susceptible to the three antibiotics used in the persister assay (S11 Table). When treated in stationary phase with kanamycin (125 μg/ml), the level of persisters varied up to 10,000-fold among individual isolates (Fig 4B). Large variations in persister levels were also observed when the isolates were treated with moxifloxacin (20 μg/ml) or rifampicin (10 μg/ml) (S4 Fig). There was high correlation in isolate-specific persister level between kanamycin and moxifloxacin (r = 0.75, p-value < 0.01) but less correlation between kanamycin and rifampicin (r = 0.32, p-value < 0.05). To elucidate underlying genetic differences of strains that produce more persisters, we selected four high and low persister clinical isolates for further characterization.

**Whole genome sequencing analysis of clinical isolates.** Whole genome sequencing showed that the total number of non-synonymous SNPs between the clinical isolates and the reference strain (H37Rv) ranged from 156 to 854 with a mean of 709 per isolate (S12 Table). For the longitudinal pair of Case 3, we filtered the number of SNPs analyzed by eliminating any that occurred in Case 1, where there was no change in persister level (Fig 4C and S13 Table). Of the 28 non-synonymous SNPs identified, 23 occurred in proline-glutamate (PE) or proline-proline-glutamate (PPE) genes. Since PE/PPE genes are known to be highly variable in *M. tuberculosis*, we excluded them from further consideration as candidate genes involved in the hip phenotype. The remaining five included a glutamate transport transmembrane protein (Rv0072), a phosphate ABC transporter (Rv0933), and three hypothetical proteins (Rv0095c, Rv1879, and Rv2819c). For analysis of the panel of drug sensitive isolates, similar to our assessment of the hip mutants obtained in vitro, we looked for overlapping mutations. There were four identical non-synonymous SNPs that were unique to the hip isolates (Fig 4D and S14 Table). A possible
Fig 4. Characterization of clinical isolates. Longitudinal pairs of isolates from four cases of recalcitrant tuberculosis infection (A) or individual drug sensitive clinical isolates (B) were grown to stationary phase and treated with kanamycin (125 μg/ml) for 14 days and survival monitored by CFU counts. Genes containing non-synonymous SNPs differentiating the early and late isolate from Case 3 (C), unique to the four hip isolates (D), or unique the four low persister isolates (E) are presented. The values are an average of three biological replicates, error bars represent standard deviation, and stars represent significant difference (p-value < 0.001).

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maturase protein (Rv0071c) was the only non-PE/PPE gene that contained a common SNP in all four hip clinical isolates. There were no non-synonymous SNPs unique to all four low persister isolates, although one mutation in a PE/PPE gene (Rv0279c) occurred in three of them (Fig 4E and S14 Table). Next, we compared the high and low persister clinical isolates with the hip mutants obtained in vitro. Half of the 60 genes that possessed non-synonymous mutations in the in vitro hip mutants also contained mutations in the clinical isolates (S15 Table). Of those 30 genes, 12 contained non-synonymous mutations that were specific to at least one hip clinical isolate including a transcriptional regulator (Rv1395), plsB2 (Rv2482c), phenolphthiocerol synthesis type-1 polyketide synthase ppsD (Rv2933), and mycoserosic acid synthase mas (Rv2940c). This comprehensive genomic comparison of in vitro mutants and clinical isolates points to several novel candidate genes that may play a role in persister formation.

Transcriptome analysis of persister cells from clinical hip isolates. To further elucidate the differences between the clinical hip and low persister isolates, we compared their transcriptome profiles. We note that the in vitro transcriptome of clinical isolates could be different from their transcriptome during infection. At the same time, we reasoned that the genetic changes leading to a higher probability for producing persisters and that are observed in vitro, will similarly manifest themselves in vivo. The most straightforward way to isolate persisters in vitro is by lysing a growing culture with a cell wall synthesis inhibitor and collecting surviving persisters by centrifugation [7]. D-cycloserine is an effective cell-lysing antibiotic of M. tuberculosis [41] and has been successfully used to isolate persisters [21]. Importantly, there was no difference in the D-cycloserine MIC among the eight clinical isolates (S11 Table). The hip isolates, treated in mid-exponential phase (OD_{600} 0.5) with D-cycloserine, produced more survivors than the low persister isolates (Fig 5A). The greatest difference between the hip and low persister isolates occurred at day 7 of treatment. We therefore collected samples at days 0 and 7 for transcriptome analysis. Comparative analysis between sample pairs of each isolate on day 0 and day 7 revealed that 13 genes were upregulated >4-fold in all four hip clinical isolates (S16 Table). Three of these genes, including a transcriptional regulator (Rv2989), a hypothetical protein (Rv1291c), and an amino acid cysteine synthase (Rv0848), were uniquely upregulated in the hip isolates while the other 10 were upregulated >4-fold in all of the low persister isolates (S17 Table). Upregulated genes in both groups include a heat shock protein (Rv0251c), catalase-peroxidase-peroxynitritase T katG (Rv1908c), gltA1 (Rv1131), clpC2 (Rv2667), and glpD1 (Rv2249c). There were 16 genes downregulated >4-fold in all four hip isolates (S18 Table). The majority of these showed significantly higher expression in the hip isolates compared to the low persister isolates at day 0 (representing the bulk population), prior to antibiotic treatment. In the low persister isolates, genes that were downregulated >4-fold included many of those identified in the hip isolates including two resuscitation-promoting factors, rpfC (Rv1884c) and rpfD (Rv2389c) (S19 Table). Comparative analysis of hip versus low persister isolates at day 0 and day 7 revealed genes that were upregulated >4-fold including triacylglycerol synthase tgs1 (Rv3130c), the devRS two-component system (Rv3132c-Rv3133c) and cation-transporting ATPase (Rv3743c) (S20 Table). Interestingly, Rv3743c is involved in energy metabolism and is directly adjacent to the most upregulated gene identified in the in vitro hip mutants, Rv3742c (S8 Table).

TA modules have previously been associated with antibiotic tolerance. We therefore assessed the clinical isolate persister transcriptomes for each of the 79 TA modules identified to date in M. tuberculosis [23]. There were over 34 TA modules upregulated in both the hip and low persister transcriptomes and 15 that were uniquely upregulated in hip isolates (S21 Table). Among the upregulated TA modules were the 10 identified in the previous M. tuberculosis H37Rv persister transcriptome [21], including E. coli relE homologue (Rv2866) that is known for its involvement in M. tuberculosis drug tolerance [24].
Fig 5. Antibiotic tolerance of hip and low persister clinical isolates. Eight individual clinical isolates were grown exponential phase and treated with D-cycloserine (125 μg/ml) for 21 days and survival monitored by CFU counts (A). Macrophages were infected with either a hip or a low persister clinical isolate for 12 hrs and then treated with D-cycloserine (125 μg/ml) for 5 to 6 days and bacterial survival was determined by lysing the macrophages and plating for CFU (B). The values are an average of three biological replicates for each sample and the error bars represent standard deviation.

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Of the genes identified from the longitudinal study, phosphate-transport ATP-binding protein ABC transporter \textit{pstB} (Rv0933) was downregulated in all strains and glutamine-transport transmembrane protein ABC transporter (Rv0072) was dramatically downregulated (>400-fold) in the \textit{hip} isolates compared to the low persister isolates \cite{S22}. Of the clinical \textit{hip} isolate mutations, the possible maturase gene (Rv0071) was also significantly downregulated in the \textit{hip} compared to the low persister isolates \cite{S22}. Of the \textit{in vitro} \textit{hip} mutant candidate genes identified by whole genome sequencing analysis, genes highly upregulated in both the \textit{hip} and low persister isolates included \textit{gltA1} (Rv1131), \textit{glpD1} (Rv2249c), hypothetical protein (Rv2323c), and transcriptional regulator \textit{AsnC} family (Rv2324) \cite{S22}. Of the PDIM biosynthesis genes, \textit{fadD26}, \textit{ppsA} (Rv2931), and \textit{mas} were significantly downregulated in both \textit{hip} and low persister isolates while other PDIM genes showed variable expression \cite{S23}. Of the 15 genes significantly differentially expressed in the \textit{hip} mutants obtained \textit{in vitro}, eight showed similar differential expression in the clinical \textit{hip} compared to low persister isolates at day 0, prior to antibiotic treatment \cite{S24}. Eight out of 15 genes highly expressed in H37Rv persister transcriptome \cite{21} were upregulated in both \textit{hip} and low persister isolate transcriptomes \cite{S25}.

Clincial \textit{hip} isolates in an \textit{ex vivo} model of \textit{M. tuberculosis} infection

With reduced killing of the \textit{hip} clinical isolates by bactericidal antibiotics in the \textit{in vitro} persister assays, we next sought to determine whether these isolates would exhibit a survival advantage in an \textit{ex vivo} model of tuberculosis infection. Murine macrophages were infected with either a \textit{hip} or a low persister clinical isolate. Once the infection was established, cells were treated with two separate antibiotics. To reduce variability between the \textit{in vitro} and \textit{ex vivo} assays, kanamycin and D-cycloserine were used. Even with moderate intracellular killing activity \cite{42}, these two antibiotics killed the low persister isolates to a greater extent than the \textit{hip} isolates. Survival of the \textit{hip} isolates was significantly higher (\textit{p-value} < 0.05) than the low persister isolates at the later time points with D-cycloserine treatment \cite{Fig 5B}. Similar data were obtained using a two-fold higher concentration of antibiotic \cite{S5}. These results point to a link between the \textit{hip} phenotype and increased \textit{ex vivo} drug tolerance.

Discussion

Persisters are thought to be responsible for the lengthy therapy of acute tuberculosis, and for the existence of the latent disease. In this study, we aimed to identify potential persister genes by studying high persister (\textit{hip}) mutants \cite{Fig 6}. Repeated selection for cells surviving antibiotic treatment \textit{in vitro} resulted in the formation of up to 1,000 times more persister cells by the \textit{hip} mutants than by the parental wild type strain. Antibiotic therapy similarly exposes patients to periodic treatment with high levels of bactericidal drugs. Importantly, we also found \textit{hip} mutants among human isolates, which links persisters to the clinical manifestation of disease.

Whole genome sequencing of \textit{hip} mutants obtained \textit{in vitro} identified several candidate persister genes. Multiple independent mutations were found within the PDIM biosynthetic operon. PDIM is an important cell wall lipid and virulence factor whose synthesis involves a large locus consisting of 13 genes. Repeated culture was reported to lead to a loss of PDIM; it is expensive to produce, and not required \textit{in vitro} \cite{37}, \cite{43}, \cite{44}. Indeed, PDIM-null mutants have a growth advantage \cite{37}. We also find that PDIM mutants grow to a higher density. Since the frequency of persister formation is density dependent, the higher cell concentration of PDIM mutants could be responsible for their \textit{hip} phenotype. Mutations in PDIM biosynthetic genes were also identified by whole genome sequencing of clinical isolates. While the \textit{ppsD} (Rv2934) mutation in the \textit{hip} clinical isolate 82 (S1132R) and \textit{mas} (Rv2940c) mutation in \textit{hip} isolate 102
(A195V) did not seem to be directly associated with any change in expression, transcriptome analysis showed significant downregulation of \textit{fadD26}, \textit{ppsA}, \textit{mas}, \textit{fadD28} (Rv2941), and transmembrane transport protein \textit{mmpL7} (Rv2942) in various clinical isolates. The decreased expression of PDIM may allow \textit{M. tuberculosis} to retain its virulence and at the same time increase the production of drug-tolerant persisters. The mechanism underlying the decreased expression of PDIM genes in \textit{hip} mutants remains to be established.

Phospholipid biosynthesis has been associated with persister formation in \textit{E. coli}. Mutations in \textit{plsB2} (Rv2482c) and cdph-diacylglycerol pyrophosphatase \textit{cdh} (Rv2289), both involved in phospholipid biosynthesis were identified in \textit{hip} mutants obtained \textit{in vitro} and in clinical isolates. Mutations in \textit{plsB2} were identified in \textit{hip} mutant KL2826 (L221F), obtained \textit{in vitro}, as well as in a \textit{hip} clinical isolate 82 (R159H and D617H) and in a low persister clinical isolate 70 (R179Q and D328G). There was no obvious trend in differential expression of \textit{plsB2} in the persister fraction from the clinical isolates, however, this gene was significantly downregulated in stationary phase compared to wild type (mc\textsuperscript{2}6020) in several of the \textit{hip} mutants obtained \textit{in vitro} (KL2801, KL2849, KL1116, KL1130). Mutations in \textit{cdh} were identified in \textit{hip} mutant KL1120 (A122V) obtained \textit{in vitro} as well as a \textit{hip} clinical isolate 82 (K215I) and a low persister clinical isolate 76 (L233R). This gene was significantly downregulated in the persister fraction.
of the clinical isolates. Together this data further suggests that a decrease in phospholipid biosynthesis may be associated with persister formation in *M. tuberculosis*.

Besides phospholipid biosynthesis, alterations in other metabolic pathways are known to affect persister formation. For example, genes that inhibit lipid catabolism have been linked to drug tolerance [28]. Mutations in acyl-CoA dehydrogenase *fadE30* (Rv3560c), a gene involved in lipid degradation, were identified in a *hip* mutant KL2825 (R381W) obtained *in vitro* and in *hip* clinical isolate 82 (E34A) where it was found to be downregulated in the persister fraction. Changes in amino acid metabolism are also known to affect persistence [45]. Among the mutant genes differentiating the late isolate of our longitudinal study, a phosphate ABC transporter (Rv0933) and a glutamate ABC transporter (Rv0072) involved in amino acid metabolism, were identified. Importantly, the expression of Rv0072 was more than 400-fold lower in the *hip* isolates compared to the low persister isolates. Mutations in isoleucyl-tRNA synthetase *ileS* (Rv1536) were identified in *hip* mutant KL1134 (E728K) obtained *in vitro* and *hip* clinical isolate 82 (R39H). Also involved in amino acid biosynthesis and reported to be upregulated during Mg\(^{2+}\) starvation [46], this gene was upregulated in the persister fraction of clinical isolate 82. We also observed a mutation in *gltA1* (Rv1131) and its upregulation in a *hip* mutant obtained *in vitro* (KL1117). While there were no mutations in *gltA1* in the clinical isolates, this gene was significantly upregulated in the persister fraction of all isolates. Upregulation of *gltA1* has been linked to a metabolic shift in carbon away from the TCA cycle, directly affecting antibiotic tolerance [28].

Pathways not previously associated with persister formation were also identified in our study. A single non-synonymous mutation in maturase-like protein (Rv0071) was unique to all four *hip* clinical isolates (A118V). This gene was significantly downregulated in the *hip* clinical isolates. Little is known about RNA maturation enzymes in bacteria. They function like group II introns and may play a role in RNA editing [47]. In addition, mutations in the probable transcriptional regulator of the AsnC family (Rv2324) were identified in several *hip* mutants obtained *in vitro* (KL1090, KL1105, KL1116, KL1117). AsnC regulatory proteins are known as the ‘feast or famine’ proteins in bacteria and are involved in direct regulation of multiple genes in response to environmental signals [40]. While no mutations were found in the clinical isolates, this transcriptional regulator was significantly upregulated over 16-fold in the persister fraction of three *hip* clinical isolates (82, 98, 102). Another candidate identified was transcriptional regulator Rv1395, known to induce the expression of cytochrome P450 gene Rv1394 [48]. Mutations in Rv1395 were identified in *hip* mutant KL2801 (P208L) obtained *in vitro* and in *hip* clinical isolates 65 (I105M) and 82 (R220C). This gene was significantly upregulated in the persister fraction of all eight clinical isolates and as such it is an interesting candidate for further analysis.

Transcriptome analysis revealed an upregulation in TA module expression in the persister fraction of *M. tuberculosis* clinical isolates. Various TA modules induce drug tolerance in *M. smegmatis, M. tuberculosis*, and *E. coli* [7], [21], [23]. One of the most upregulated TA modules identified in our study was *HigBA1* (Rv1955-Rv1957). This is a tripartite module comprised of toxin-antitoxin-chaperone (TAC) where activation of the toxin likely requires unavailability of the chaperone and/or degradation of the antitoxin [23]. Interestingly, the antitoxin *HigA1* (Rv1956) of this TAC is a putative regulator of the fatty acid metabolism gene cluster Rv2930-Rv2939, comprising PDIM biosynthetic genes including *fadD26* [49]. It is also capable of interacting with the stress response gene cluster Rv3249-Rv3252, including *rubA* and *rubB*, which were upregulated in the *hip* mutants obtained *in vitro*. While only one mutation was identified in this TAC module in our study (clinical isolate 73; Rv1957, D83N), the notable transcriptional profile and association with other persister gene candidates provides some evidence for a putative role in the *hip* phenotype. Moreover, we found that, just as with H37Rv
[21], clpC2 (Rv2667) was upregulated in the persister fraction of clinical isolates. Since proteases typically degrade antitoxins leading to toxin-mediated drug tolerance, this further supports a role for TA modules in *M. tuberculosis* persister formation.

The identification of *hip* mutants in longitudinal isolates of *C. albicans* [32] and *P. aeruginosa* [33] suggests that *hip* mutants may be a general feature of recalcitrant infectious diseases. Our identification of *hip* clinical isolates of *M. tuberculosis* supports this possibility. Interestingly, infection with the *hip* clinical isolate 67 resulted in relapse whereas infection with none of the low persister isolates did (S9 Table). Note that *hip* isolate 102 was isolated from a patient with a history of four repeated episodes of tuberculosis. The *hip* clinical isolates showed increased drug tolerance both in *vitro* and in an *ex vivo* model of tuberculosis. These findings are in agreement with a previous report of delayed killing in *vitro* of *M. tuberculosis* isolates from patients with relapse [50].

The results of this study reveal an overlap in genes and pathways we identified by transcriptome analysis of persisters and genomic analysis of *hip* mutants with those reported to be associated with persister formation, such as TA modules and carbon and amino acid metabolism. Our study also points to novel genes that may be unique to *M. tuberculosis* persisters, such as lipid biosynthesis and feast or famine regulation. Knowledge of *M. tuberculosis* persister cells will facilitate development of effective therapies for their eradication.

### Materials and Methods

#### Strains, media and culture conditions

Bacterial strains used in this study are shown in Table 2. *M. tuberculosis* H37Rv double auxotrophic strain mc²6020 [35] was used as the parental strain of the *in vitro* *hip* mutants. Clinical isolates included in this study were obtained from new tuberculosis cases and retreatment cases of subjects enrolled in a prospective longitudinal cohort study (ClinicalTrials.gov identifier, NCT00341601) at the National Masan Hospital (NMH) in the Republic of Korea. The institutional review boards of National Institute of Allergy and Infectious Diseases (NIAID) and NMH approved the study and all subjects gave written informed consent. H37Rv (American Type Culture Collection [ATCC] No. 27294) was used as the wild type strain in the clinical isolate experiments. PDIM mutant strains mc²3105 [36] and *drrA*::Tn [38] were kindly provided. Strains were grown in Middlebrook 7H9 Broth or on 7H10 Agar (Difco) medium supplemented with 0.05% Tween-80 (*in vitro* *hip* mutant broth experiments) or 0.05% tyloxapol (*in vitro* *hip* mutant broth experiments), and required supplements for the double auxotroph mc²6020 [35] including 0.2% casamino acids (Amresco), pantothenic acid (24 μg/ml), and lysine (80 μg/ml). Freezer stocks were diluted 1:100 into 7H9 broth medium and grown in a shaking incubator at 37°C at 100 rpm. Minimal media was prepared as previously described [51] with 0.5% glycerol (vol/vol), 0.1% butyrate (wt/vol), or 0.1% propionate (wt/vol), 0.05% tyloxapol (vol/vol), pantothenic acid (24 μg/ml), and lysine (80 μg/ml). The clinical isolates were grown first on Lowenstein-Jensen slants and then transferred to 7H9 media prior to freezing stocks. To limit the introduction of genetic mutations due to *in vitro* growth conditions, the number of passages of strains from the original stock was limited to a maximum of five in accordance with ATCC recommendations. All chemicals were obtained from Sigma-Aldrich unless otherwise indicated. The antibiotics and their concentrations used in this study were streptomycin (10 μg/ml), rifampicin (1 μg/ml), kanamycin (50 μg/ml), ofloxacin (10 μg/ml), hygromycin (50 μg/ml), D-cycloserine (125 μg/ml), and moxifloxacin (20 μg/ml) (Waterstone Technology). Antibiotic stocks were prepared as recommended [52] and subsequent dilutions were made in 7H9 broth medium.
| Strain Name       | SRA Accession Number | Description                  | Parent Strain Name | Reference |
|------------------|----------------------|------------------------------|--------------------|-----------|
| mc²6020          |                      | H37Rv ΔlysA ΔpanCD           | H37Rv              | [35]      |
| KL2801           | SRX014112            | Hip mutant KL2801            | mc²6020            | This study|
| KL2802           | SRX016225            | Hip mutant KL2802            | mc²6020            | This study|
| KL2803           | SRX014110            | Hip mutant KL2803            | mc²6020            | This study|
| KL2825           | SRX016226            | Hip mutant KL2825            | mc²6020            | This study|
| KL2826           | SRX016227            | Hip mutant KL2826            | mc²6020            | This study|
| KL2827           | SRX016228            | Hip mutant KL2827            | mc²6020            | This study|
| KL2849           | SRX014107            | Hip mutant KL2849            | mc²6020            | This study|
| KL2850           | SRX014911            | Hip mutant KL2850            | mc²6020            | This study|
| KL2851           | SRX014909            | Hip mutant KL2851            | mc²6020            | This study|
| KL1090           | SRX005187            | Hip mutant HTS1090           | mc²6020            | This study|
| KL1105           | SRX000677            | Hip mutant HTS1105           | mc²6020            | This study|
| KL1116           | SRX081440            | Hip mutant HT1116            | mc²6020            | This study|
| KL1117           | SRX081849            | Hip mutant HT1117            | mc²6020            | This study|
| KL1120           | SRX081404            | Hip mutant HT1120            | mc²6020            | This study|
| KL1130           | SRX081415            | Hip mutant HT1130            | mc²6020            | This study|
| KL1134           | SRX081412            | Hip mutant HT1134            | mc²6020            | This study|
| KL1137           | SRX081421            | Hip mutant HT1137            | mc²6020            | This study|
| KL1170           | SRX081442            | Hip mutant HT1170            | mc²6020            | This study|
| p-glPD1          |                      | mc²6020 carrying pTETglPD1, KanR | mc²6020            | This study|
| p-glPD1-flag     |                      | mc²6020 carrying pTETglPD1-flag, KanR | mc²6020            | This study|
| p-plsB2          |                      | mc²6020 carrying pTETplsB2, KanR | mc²6020            | This study|
| p-plsB2-flag     |                      | mc²6020 carrying pTETplsB2-flag, KanR | mc²6020            | This study|
| fadD26::Tn (Erdman) | mc²3105, Erdman fadD26::Tn5370, HygR | Erdman | [36] |
| Wild type (Erdman) | Wild type             | Erdman                       | [36]               |
| Wild type (H37Rv) | H37Rv carrying JEB403, KanR | H37Rv              | [38]              |
| drrA::Tn (H37Rv) | H37Rv drrA::Tn.1 carrying JEB403, HygR, KanR | H37Rv              | [38]              |
| drrA::Tn + complement (H37Rv) | H37Rv drrA::Tn.1 carrying JEBdrrA, HygR, KanR | H37Rv              | [38]              |
| H37Rv 21         | Clinical isolate     | ATCC                         | ClinicalTrials.gov identifier, NCT00341601 |
| 24               | Clinical isolate     | ClinicalTrials.gov identifier, NCT00341601 |
| 25               | Clinical isolate     | ClinicalTrials.gov identifier, NCT00341601 |
| 26               | Clinical isolate     | ClinicalTrials.gov identifier, NCT00341601 |
| 57               | Clinical isolate     | ClinicalTrials.gov identifier, NCT00341601 |
| 60               | Clinical isolate     | ClinicalTrials.gov identifier, NCT00341601 |
| 61               | Clinical isolate     | ClinicalTrials.gov identifier, NCT00341601 |

(Continued)
| Strain Name | SRA Accession Number | Description | Parent Strain Name | Reference |
|-------------|----------------------|-------------|--------------------|-----------|
| 62          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 63          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 66          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 67          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 68          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 69          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 70          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 71          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 72          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 73          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 76          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 79          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 81          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 82          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 83          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 94          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 96          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 97          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 98          |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 101         |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 102         |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 104         |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 107         |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 115         |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 118         |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |
| 120         |                      | Clinical isolate | ClinicalTrials.gov identifier, NCT00341601 |           |

(Continued)
Mutant strains and plasmid construction

Chemical mutagenesis was performed as previously described [53] with modifications. Strain mc²6020 was grown to exponential phase with an optical density at 600 nm (OD₆₀₀) of 0.8 and washed two times with equal volumes of minimal A buffer (K₂HPO₄ 10.5 mg/ml, KH₂PO₄ 4.5 mg/ml, [NH₄]₂SO₄ 1 mg/ml, [C₆H₅Na₃O₇]₂H₂O 0.5 mg/ml). Bacterial cells were resuspended in an equal volume of minimal A buffer. The cultures were split into 20 ml aliquots, 300 μl of ethyl methanesulfonate was added, and cultures were incubated on a shaker (100 rpm) at 37°C for 60 min. Mutagenized cells were washed two times with equal volumes of minimal A buffer and resuspended in 220 ml of 7H9 medium in 1,300 ml roller bottles (Corning). The roller bottles were incubated on a roller platform (6 rpm) at 37°C for one week until the culture reached stationary phase. Cultures were then diluted 1:100 into 40 ml of 7H9 medium, grown to exponential phase (OD₆₀₀ 0.8) and then treated with a combination of streptomycin (10 μg/ml) and rifampicin (1 μg/ml) for one week. Cells were washed once with an equal volume of phosphate buffered saline (PBS) and resuspended in 40 ml of 7H9 medium. Before and after the antibiotic challenges, samples were taken for bacterial cell enumeration. The enrichment procedure was repeated. The washed cells were grown again to stationary phase, diluted 1:100, grown to exponential phase, and treated for one week using the same antibiotic combination. After the fourth round of enrichment individual strains were separated from the mutagenized populations by isolating individual colonies for further characterization.
Overexpression plasmids were constructed by cloning candidate genes using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) with the primers listed in S1 Table. Cloned products were ligated into the Gateway® Cloning system (Invitrogen) and then transferred to an expression vector (pTET) containing a tetracycline inducible mycobacterial promoter [54]. Purified plasmids were transformed into *M. tuberculosis* mc²6020 and selected for using kanamycin (50 μg/ml). Overexpression strains were induced 72 hrs prior to antibiotic challenge by addition of anhydrous tetracycline (aTc) (100 ng/ml) to the media.

**Persister assay**

To determine the level of persister formation in various strains and under various conditions, antibiotic treatments were performed in biological triplicate as previously described [21]. For stationary phase treatments, the initial two-week-old cultures were diluted 1:100 and grown for an additional two weeks prior to antibiotic treatment. For persister assays with the clinical isolates, freezer stocks were diluted 1:10 into broth media and grown for three weeks to stationary phase. Clinical isolate cultures were then diluted 1:100 into fresh broth media and grown for four to five days for exponential phase challenge or for three weeks for stationary phase challenge. Samples were plated at selected time points and bacterial survival was monitored by enumeration of colony forming units (CFU). The Student’s T-test was used to determine significant differences and Spearman’s Rank-order test was used to assess correlation.

**Growth rate determination**

*Hip* mutant and wild type (mc²6020) strains were grown in triplicate in standard 7H9 liquid media and samples were taken at the indicated time points for cell enumeration by plating for CFU from which the generation time was calculated [55]. The data represent an average of three biological replicates plus or minus the standard deviation.

**Drug resistance assays**

The drug sensitivity testing (DST) of clinical isolates was performed on solid media with absolute concentrations of first- and second-line anti-tuberculosis drugs. The anti-tuberculosis drugs that were tested included isoniazid (INH/H), rifampicin (RIF/R), streptomycin (SM/S), ethambutol (EMB/E), kanamycin (KM/K), capreomycin (CPM), prothionamide (PTH/T), cycloserine (CS/C), para-aminosalicylic acid (PAS/P), ofloxacin (OFX/O), moxifloxacin (MOX), amikacin (AMK), levofloxacin (LEV/Lf), rifabutin (RBU), and pyrazinamide (PZA/Z). The testing was carried out at the NMTH and the NIH according to standard guidelines for DST.

The MIC testing was liquid-based using the micro-plate based Alamar Blue assay as described [56] with minor modifications. Cultures was grown to early exponential phase (OD₆₀₀ 0.3) and diluted to 1:100 for the assay. Antibiotics were serially diluted in 96-well plates and equal volumes of diluted bacterial cultures were added. The plates were sealed with Breathe-Easy® (3M Company) and incubated at 37°C. After four days, 20 μl of Alamar Blue (Serotec) was added to each well and the plates were resealed and incubated for 24 to 48 hrs. The colors of all wells were recorded after visual inspection and absorbance measurement using the Synergy HT microplate reader (BioTek) with excitation and emission wavelengths of 530 nm and 590 nm, respectively.

**Total lipid analysis by thin layer chromatography**

Total lipids were extracted by centrifugation of bacterial cells (4,000 rpm, 15 min) at room temperature. Pellets were resuspended in 5 ml of 2:1 CHCl₃:MeOH and transferred to a clean glass
centrifuge tube with a Teflon-lined cap. To the glass tube, 25 ml of CHCl₃:MeOH (2:1) was added and lipids were extracted for at least 1 hr on an orbital rocker. Tubes were centrifuged (2,000 rpm, 15 min) at room temperature and supernatants were transferred to clean glass tubes. Pellets were resuspended in 30 mL of CHCl₃:MeOH (1:1) and lipids extracted as above. Pellets were resuspended for a final time in 30 mL of CHCl₃:MeOH (1:2). The organic phases were combined and evaporated using a rotovap. Extracted lipids were dissolved in CHCl₃:MeOH (1:1) and 150 μg of each sample were spotted onto a 20 x 20 cm silica glass plate (84101, Scientific Adsorbents). Petroleum ether:diethyl ether (90:10) was used as the solvent and the revelation solution consisted of 8% H₃PO₄ (vol/vol) and 3% Cu Acetate (wt/vol) in water. Plates were sprayed and heated in an oven to 160°C.

Genomic DNA extraction and whole genome sequencing

Genomic DNA was isolated as previously described [57]. The concentration of genomic DNA was measured using a NanoDrop spectrophotometer (Thermo Scientific) and stored at -20°C. The Illumina paired-end reads design was used for whole genome sequencing of genomic DNA from the hip mutant and wild type (mc²6020) strains. All sequencing was performed at the Broad Institute (Cambridge, MA). Illumina fragment libraries were generated as previously described [58] with the following modifications. For each sample, 100 ng of genomic DNA was sheared to 200 bp in size using a Covaris LE220 instrument (Covaris, MA) with the following parameters: temperature: 7–9°C; duty cycle: 20%; intensity: 5; cycles per burst: 200; time: 90 sec; shearing tubes: Crimp Cap microTUBES with AFA fibers (Covaris, MA). DNA fragments were end repaired, 3’ adenylated, ligated with indexed Illumina sequencing adapter, and PCR enriched, as previously described [59]. The resulting Illumina fragment sequencing libraries were normalized and were size selected to contain inserts of 180 bp ± 3% in length using a Pippen Prep system (Sage Science, MA) following the manufacturer’s recommendations. Sequencing coverage is presented (S2 Table) and the data is available at the National Center for Biotechnology Information (NCBI) sequence read archive (SRA) under BioProject PRJNA38649. Sequenced strains were aligned to the fully assembled H37Rv library. The library name was Solexa-16183, source was genomic, selection was random, and layout was paired with a 5’3’-3’5’ orientation, a nominal length of 166, and a nominal standard deviation of 34.33. Coding single nucleotide polymorphisms (SNPs) as well as insertions and deletions that were identified between the hip mutants and the H37Rv reference assembly and that did not occur in the parental wild type strain (mc²6020) were classified as mutations. For analysis of the clinical isolates, we considered any coding SNP identified between each isolate and the H37Rv reference strain as a mutation.

Transcriptome analysis

In collaboration with the Broad Institute (Cambridge, MA), Affymetrix microarray technology was used for conducting the transcriptome analysis of the in vitro generated hip mutant strains. Total RNA was extracted as previously described [21] from stationary phase cultures that were grown for 14 days from a 1:100 inoculum. Quality control of microarray data in our study was measured with RNA degradation and box plots. RNA degradation starts from the 5’ end to 3’ end so 5’ end probes show lower intensities than the 3’ end probes. RNA degradation plots measure this trend and a high slope indicates degradation. In this study RNA degradation was minimal. A boxplot is a tool to summarize intensity distributions and indicate if there are any sample outliers. Differences in amplification or labeling tend to cause these outliers. Our study did not show any outliers. Statistical analysis of the data was performed using the Bioconductor program and oneChannelGUI package [60] available through the R Project for Statistical
Computing (www.r-project.org). Data is presented as log2 fold change. The microarray data have been deposited in NCBI’s Gene Expression Omnibus (GEO) [61] and are accessible through GEO Series accession number GSE55647.

For transcriptome analysis of the clinical isolates, cultures were grown from a 1:100 inoculum to mid-exponential phase (OD600 0.5) and then treated with D-cycloserine (125 μg/ml). Culture samples were collected at Day 0 (2 ml) and Day 7 (40 ml) of treatment for RNA isolation as previously described [21]. Strand-specific RNAseq libraries were created by the Broad Technology Labs specialized service facility as described [62]. Briefly, 48 RNA samples were fragmented, and RNA 3’ ends were tagged with a DNA oligonucleotide containing a sample barcode and a partial 5’ Illumina adapter. Resulting barcoded RNAs were then pooled and subjected to rRNA depletion with RiboZero (Epicentre), cDNA synthesis and ligation to a second oligonucleotide containing a partial Illumina 3’ adapter. A second barcode specific to this pool was then added by amplification with full-length barcoded Illumina adapter primers, yielding a single strand-specific sequence-ready pooled RNA-seq library. The pool of 48 Illumina RNA-seq libraries was quantified using qPCR (KAPA Biosystems) and sequenced with 76 base paired-end reads across 4 lanes using an Illumina HiSeq 2000 sequencer (Illumina) running v3 SBS chemistry. Sequence data were processed and demultiplexed using the Picard analysis pipeline (http://picard.sourceforge.net). Reads were aligned to the genome of M. tuberculosis H37Rv (RefSeq NC_000962) using BWA [63] version 5.9. Gene annotations were obtained from RefSeq and Rfam [64]. The overall fragment coverage of genomic regions corresponding to features such as ORFs and tRNAs was conducted as described [65]. RNAseq coverage metrics are presented (S3 Table). Differential expression analysis was conducted using DESeq [66]. Data is presented as log2 fold change. The RNAseq data are accessible through GEO Series accession number GSE62025.

Immunoblot analysis

Flag-tagged proteins were visualized by immunoblot analysis. Proteins were extracted from bacterial cells by centrifugation (11,000 rpm, 15 min), followed by resuspension of the pellet in 250 μl of Bacterial Protein Extraction Regent (Thermo Scientific Pierce). Samples were placed in a bead beater tube with Lysing matrix B (MP Biochemicals), and disrupted by bead beating using FastPrep-24 (MP Biochemicals) for 45 sec at the power setting of 6. Proteins were separated on NuPAGE Novex 4–12% Bis-Tris Gels (Invitrogen) and transferred to nitrocellulose membranes. Monoclonal ANTI-FLAG® M1 antibody produced in mouse (F30401; Sigma) was used at a dilution of 1:1,000. Membranes treated with the WesternBreeze® Chemiluminescent Kit, anti-mouse (Invitrogen) according to the manufacturer’s instructions. Blots were developed with an X-ray developing system.

Macrophage infections

The murine leukemic monocyte macrophage cell line RAW264.7 (ATCC) was used for all macrophage infections. Macrophages were propagated in Dulbecco’s Modification of Eagle’s Media with high glucose (Invitrogen) with 10% Fetal Bovine Serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 1% penicillin/streptomycin (optional) at 37°C with 5% CO2. Cells were grown to confluence and then seeded in 6-well plates at a concentration of 1 x 10⁶ cells per well and allowed to adhere to the surface of the plate overnight at 37°C with 5% CO2. Bacteria were grown to early exponential phase (OD600 0.3) in 40 ml of 7H9 broth and collected by centrifugation (4,000 rpm, 10 min). Cells were resuspended in 5 ml of macrophage media (without penicillin/streptomycin), sonicated three times (5 sec) at amplitude 4 on the Misonix Ultrasonic Liquid Processor S-4000 (Qsonica), and vortexed three times (5 sec). The
volume was brought to 40 ml with macrophage media (without penicillin/streptomycin) and the remaining clumps were allowed to settle for 15 min after which the top 35 ml was transferred to a new tube. The OD<sub>600</sub> was measured and adjusted to 0.08 (~1 x 10<sup>7</sup> CFU/ml). Bacteria were deposited in the 6-well plates containing macrophages at a multiplicity of infection of 10: 1 (bacteria: macrophages) for 12 hrs after which the extracellular bacteria were washed away five times and fresh media with or without antibiotics was added. At selected time points macrophages were lysed with 0.5% Triton-100 in PBS, serially diluted in PBS, and plated on 7H10 media for bacterial cell enumeration.

**Supporting Information**

**S1 Fig. Thin layer chromatography of total lipid extracts.** Total lipid extract (150 μg) from each strain was spotted on the plate along with a DIM-A standard and run in petroleum ether / diethyl ether (90/10) solvent. Spots were visualized by treating the plate with H₃PO₄ (8% v/v), Cu Acetate (3% v/v) and heat (140–160°C). DIM-A is present in the wild type strain (mc<sup>2</sup>6020) and hip mutant KL1116 but absent in all of the other hip mutant strains.

(TIF)

**S2 Fig. Persister assay of drrA::Tn PDIM mutant.** The drrA::Tn mutant and complemented strain along with wild type (H37Rv) were grown to either exponential or stationary phase and treated with streptomycin (10 μg/ml) and rifampicin (1 μg/ml) for 14 days. Survival was monitored by CFU counts. The values are an average of three biological replicates and error bars represent standard deviation.

(TIF)

**S3 Fig. Overexpression of candidate persister genes glpD1 and plsB2.** Overexpression strains were induced with aTc (100 ng/μl) 72 hrs prior to antibiotic treatment. Induced overexpression of glpD1 (A) and plsB2 (B) resulted in no change in level of persister formation in either exponential or stationary phase. Immunoblot analysis confirms overexpression (right). The values are an average of three biological replicates and error bars represent standard deviation.

(TIF)

**S4 Fig. Characterization of persister level in clinical isolates.** Individual drug sensitive clinical isolates were treated in stationary phase with moxifloxacin (20 μg/ml) (A) or rifampicin (10 μg/ml) (B) for 14 days and bacterial survival was determined plating for CFU. The values are an average of three biological replicates and error bars represent standard deviation.

(TIF)

**S5 Fig. Antibiotic tolerance of hip and low persister clinical isolates during macrophage infection.** Murine macrophages were infected with either a hip or a low persister clinical isolate for 12 hrs and then treated with either kanamycin (250 μg/ml) or D-cycloserine (250 μg/ml) for up to 6 days. Bacterial survival was determined by plating for CFU after lysing the macrophages. The values are an average of three biological replicates for each sample and the error bars represent standard deviation.

(TIF)

**S1 Table. Oligonucleotides used in this study.**

(TIF)

**S2 Table. Whole genome sequencing coverage metrics.**

(TIF)
S3 Table. RNAseq coverage metrics.
(TIF)

S4 Table. Minimum inhibitory concentration of antibiotics for wild type (mc²6020) and *in vitro hip* mutant strains.
(TIF)

S5 Table. Generation time (hrs) of wild type (mc²6020) and *in vitro hip* mutant strains under normal growth conditions.
(TIF)

S6 Table. Stationary phase gene expression of *hip* mutant versus wild type (mc²6020) of genes identified by whole genome sequencing of the 12 independent *in vitro hip* mutants.
(TIF)

S7 Table. Differential expression of top upregulated genes of three independent *in vitro hip* mutants (KL2801, KL2925, KL2849) versus wild type (mc²6020) in stationary phase.
(TIF)

S8 Table. Differential expression of 12 independent *in vitro hip* mutants versus wild type (mc²6020) in stationary phase.
(TIF)

S9 Table. Clinical isolate treatment history and drug resistance profile.
(TIF)

S10 Table. Minimum inhibitory concentration of kanamycin for longitudinal clinical isolates and H37Rv.
(TIF)

S11 Table. Minimum inhibitory concentration of antibiotics for clinical isolates and H37Rv.
(TIF)

S12 Table. Total number of non-synonymous SNPs in clinical isolates compared to reference strain H37Rv.
(TIF)

S13 Table. Non-synonymous SNPs differences between longitudinal isolates of Case 3 (94 and 96) compared to Case 1 (127 and 130).
(TIF)

S14 Table. Non-synonymous mutations unique to *hip* or low persister clinical isolates.
(TIF)

S15 Table. Non-synonymous SNPs in the clinical isolates that occur in the same genes as mutations in the *in vitro hip* mutants.
(TIF)

S16 Table. Genes upregulated (>4-fold) in all four *hip* clinical isolates.
(TIF)

S17 Table. Genes upregulated (>4-fold) in all four low persister clinical isolates.
(TIF)

S18 Table. Genes downregulated (>4-fold) in all four *hip* clinical isolates.
(TIF)
S19 Table. Genes downregulated (>4-fold) in all four low persister clinical isolates.

(TIF)

S20 Table. Genes upregulated (>4-fold) in hip versus low persister clinical isolates.

(TIF)

S21 Table. Clinical isolate transcriptome analysis of M. tuberculosis TA module genes.

(TIF)

S22 Table. Clinical isolate transcriptome analysis of candidate genes identified through whole genome sequencing.

(TIF)

S23 Table. Clinical isolate transcriptome analysis of PDIM biosynthetic operon genes.

(TIF)

S24 Table. Clinical isolate transcriptome analysis of genes differentially expressed in stationary phase in vitro hip mutants.

(TIF)

S25 Table. Clinical isolate transcriptome analysis of genes upregulated (>4-fold) in H37Rv persister cells.

(TIF)

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Author Contributions

Conceived and designed the experiments: HLT IK KL. Performed the experiments: HLT. Analyzed the data: HLT IK KL. Contributed reagents/materials/analysis tools: LEV JSL KL. Wrote the paper: HLT KL.

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