RESEARCH ARTICLE

Synthetic Lethality Reveals Mechanisms of *Mycobacterium tuberculosis* Resistance to β-Lactams

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ABSTRACT **Most β-lactam antibiotics are ineffective against *Mycobacterium tuberculosis* due to the microbe's innate resistance. The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains has prompted interest to repurpose this class of drugs. To identify the genetic determinants of innate β-lactam resistance, we carried out a synthetic lethality screen on a transposon mutant library for susceptibility to imipenem, a carbapenem β-lactam antibiotic. Mutations in 74 unique genes demonstrated synthetic lethality. The majority of mutations were in genes associated with cell wall biosynthesis. A second quantitative real-time PCR (qPCR)-based synthetic lethality screen of randomly selected mutants confirmed the role of cell wall biosynthesis in β-lactam resistance. The global transcriptional response of the bacterium to β-lactams was investigated, and changes in levels of expression of cell wall biosynthetic genes were identified. Finally, we validated these screens *in vivo* using the *MT1616* transposon mutant, which lacks a functional acyl-transferase gene. Mice infected with the mutant responded to β-lactam treatment with a 100-fold decrease in bacillary lung burden over 4 weeks, while the numbers of organisms in the lungs of mice infected with wild-type bacilli proliferated. These findings reveal a road map of genes required for β-lactam resistance and validate synthetic lethality screening as a promising tool for repurposing existing classes of licensed, safe, well-characterized antimicrobials against tuberculosis.**

IMPORTANCE **The global emergence of multidrug-resistant and extensively drug-resistant *M. tuberculosis* strains has threatened public health worldwide, yet the pipeline of new tuberculosis drugs under development remains limited. One strategy to cope with the urgent need for new antituberculosis agents is to repurpose existing, approved antibiotics. The carbapenem class of β-lactam antibiotics has been proposed as one such class of drugs. Our study identifies molecular determinants of innate resistance to β-lactam drugs in *M. tuberculosis*, and we demonstrate that functional loss of one of these genes enables successful treatment of *M. tuberculosis* with β-lactams in the mouse model.**

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Despite more than a century of coordinated control efforts, tuberculosis (TB) continues to be one of the greatest infectious-disease threats to human health. Disease control has been complicated by the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* strains and further exacerbated by the difficulties of managing coinfection with *M. tuberculosis* and HIV (1). To confront these challenges, new and shorter courses of TB therapy must be developed. Consequently, identifying new drug targets and elucidating antibiotic resistance pathways of *M. tuberculosis* are critical.

Well-characterized transposon mutant libraries are a valuable tool for linking phenotypes and genotypes in *M. tuberculosis* (2, 3). Genetic interactions of *M. tuberculosis* have been elucidated by comparing the phenotypic differences among strains with single and double genetic mutations in such mutant collections (4). This is an example of gene-gene synthetic lethality (GGSL) wherein the genetic interaction of a combination of two separate nonlethal, null mutations results in lethality (5, 6). Similarly, inhibiting one pathway chemically and another with a mutation can lead to gene-compound synthetic lethality (GCSL).

β-Lactams are generally considered ineffective against *M. tuberculosis* (7, 8). This is in large part due to the presence of multiple *Mycobacterium*-encoded β-lactamases which degrade and inactivate the antibiotics (9, 10). Indeed, when combined with the commonly used β-lactamase inhibitor clavulanic acid, the carbapenem agent meropenem has potent *in vitro* bactericidal activity against both drug-susceptible and drug-resistant *M. tuberculosis* strains (11). Carbapenems (imipenem and meropenem) combined with clavulanic acid also significantly reduce the bacterial burden in macrophages and chronically infected mouse lungs (12). Furthermore, recent clinical studies have shown that

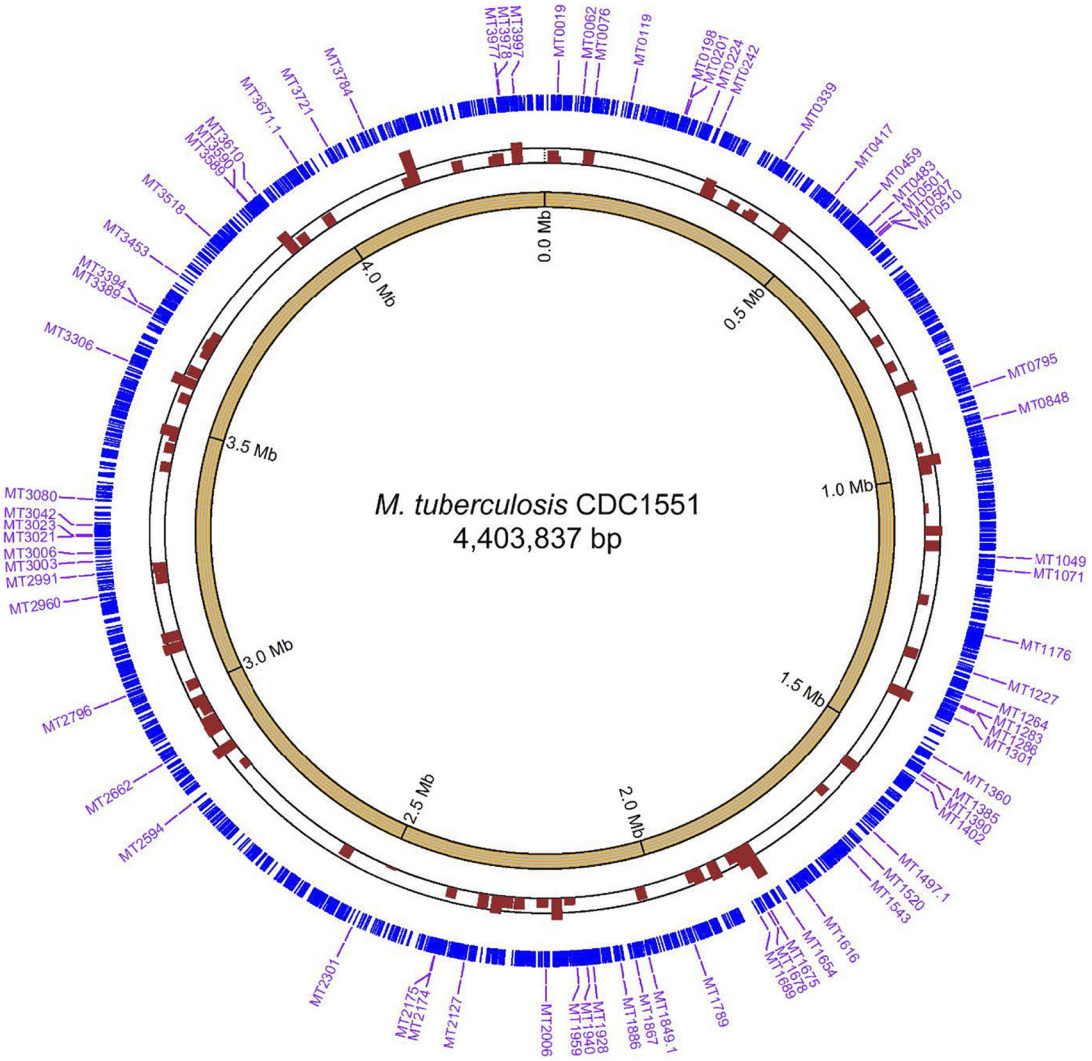

FIG 1 Circular plot of gene locations. From inside to outside, the first track represents the *M. tuberculosis* CDC1551 genome, with ticks indicating relative positions along the genome. The second track (the two black lines represent a fold change of 1) depicts the linear fold changes of the selected 73 gene mutations when treated with 100 μg/ml of penicillin. The third track indicates the transposon mutants (n = 1,712) screened in this study. The fourth track shows the gene locations of the 74 hits of the imipenem gene-compound synthetic lethality screen (Table S1). Data were plotted using RCircos (47).

meropenem-clavulanic acid combination therapy offers a potential benefit to certain patients with drug-resistant TB (13, 14). Mechanisms other than the abundance of β-lactamases, such as cell envelope permeability and variations in the peptidoglycan biosynthetic enzymes, also play a role in β-lactam resistance (8, 15). It has recently been demonstrated that *M. tuberculosis* possesses nonclassical transpeptidases which are innately resistant to early-generation β-lactam agents; however, studies that followed indicate that these nonclassical transpeptidases exhibit some susceptibility to certain carbapenems (15–17).

In this study, we sought to identify GCSL pairs by screening a well-characterized library of *M. tuberculosis* transposon mutants for β-lactam susceptibility. This was accomplished using high-throughput phenotypic screening (18, 19) in the presence of a subinhibitory concentration of imipenem. GCSL was verified by a second screen with penicillin using a pooled mutant competition assay and multiplex quantitative real-time PCR (qPCR) analysis. The genetic response networks to β-lactam treatment were also investigated using whole-transcriptome RNA-sequencing (RNA-Seq). Finally, we demonstrated that mice infected with a transposon mutant hit in our GCSL screen responded to β-lactam treatment, unlike mice infected with wild-type *M. tuberculosis*.

RESULTS

Imipenem gene-compound synthetic lethality screen. A 96-well plate format, high-throughput alamarBlue screening (HTS) system was established and validated as described in Text S1 in the supplemental material; the signal-to-noise ratio was 93.9, and the Z′ factor was 0.77 (Fig. S1). A total of 2,921 transposon mutants were screened (Fig. 1) in the presence of subinhibitory concentrations (0.5 or 1.0 μg/ml) of imipenem. This mutant library contained unique mutations in 1,712 genes which were disrupted by transposon insertion at points of insertion that had been previously defined by junctional sequencing (2). The imipenem working concentration was predetermined empirically and selected so that the percentage inhibition of wild-type *M. tuberculosis*

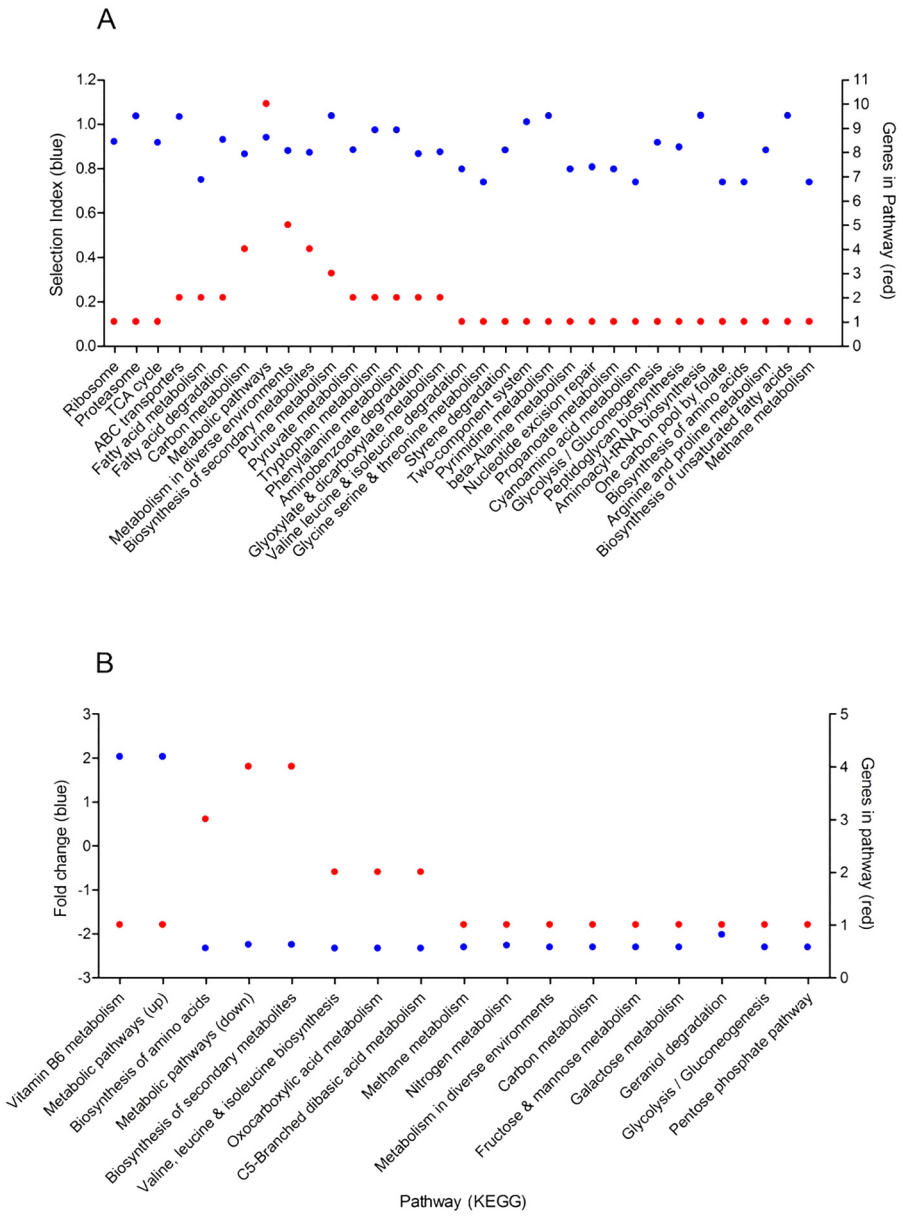

FIG 2 Pathway analysis. (A) GCSL screen hits were analyzed using the KEGG pathway search engine. The selection index (arithmetic mean, left *y* axis) and the number of genes identified in the pathway (right *y* axis) are indicated. (B) Differentially expressed genes in the RNA-Seq study were analyzed using the KEGG pathway search. Fold changes (arithmetic means, left *y* axis) and the numbers of genes identified in the pathway (right *y* axis) are indicated. KEGG, Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/); TCA, tricarboxylic acid.

CDC1551 growth was less than 35% upon exposure to imipenem. Results from the screen demonstrated a highly dynamic range of inhibition (raw inhibition as high as 100% and as low as 0%), suggesting the presence of different categories of GCSL mutations with various degrees of imipenem synergy. Hits were ranked by a selection index (percentage of net inhibition over raw inhibition), with an arbitrary cutoff set at 0.66. As the *in vitro* growth of transposon mutants in regular 7H9 media was not assessed in detail, we selected a stringent cutoff to ensure that any growth inhibition could be attributed to synergy with imipenem and not to an inherent growth defect. This produced a list of 76 mutants, representing mutations in 74 genes that exhibited synthetic lethality with imipenem (Fig. 1; Table S1).

GCSL with imipenem was observed in genes with diverse functional assignments. Among the top 76 hits were the L,D-transpeptidase LdtB (MT2594) and the putative lipoprotein LprQ (MT0501) genes, which were each hit twice, suggesting important roles in innate β-lactam resistance. The penicillin binding proteins (MT0019, MT3784), ABC transporters (MT1390, MT1789, MT1867, MT3006, MT3080), and fatty acid biosynthesis-, equilibrium-, integrity-, and metabolism-associated genes (n = 11, Fig. S2) were also identified in the top-hit list (Table S1). The GCSL screen also identified transcriptional regulators (n = 7), including the two-component system RegX3, a polyketide synthase (MT3003), and the putative acyltransferase (MT1616) among its top hits. Multiple genes playing roles in amino acid

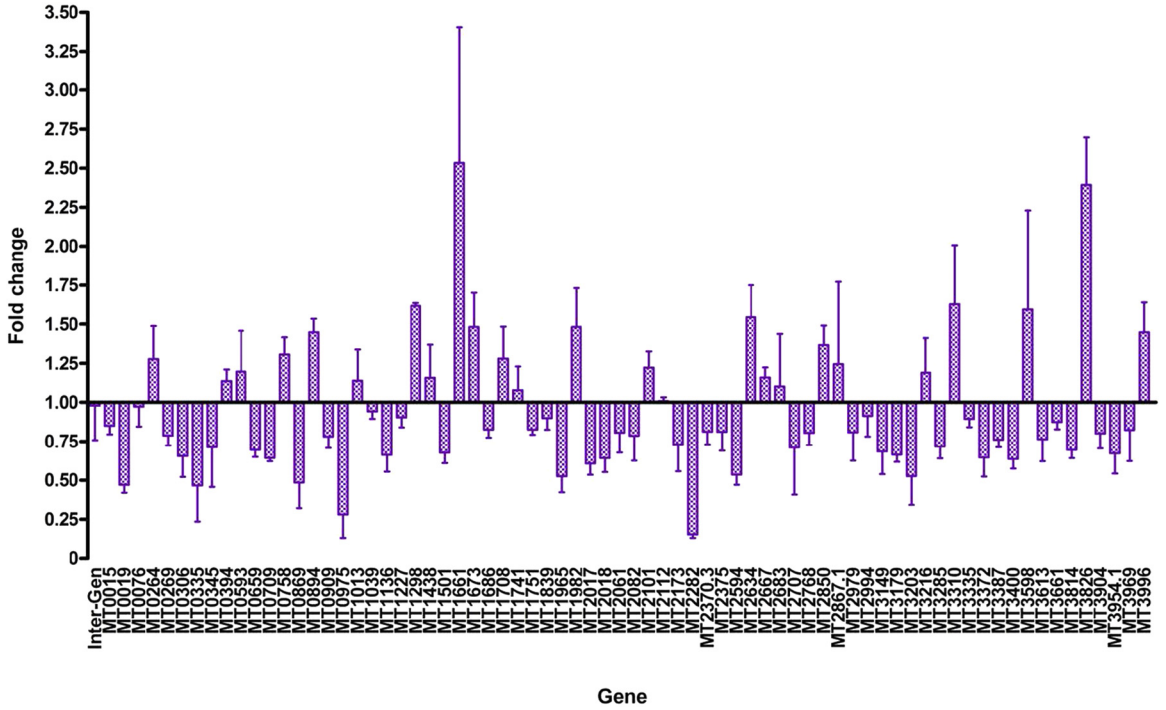

FIG 3 Bar graph of multiplex qPCR of 73 randomly selected transposon mutants. Fold changes are shown as means ± SD of results from three biological replicates for each gene mutation after penicillin treatment in competition pool studies. Data were normalized to those for *sigA*, and an intergenic mutant was included as a reference control.

biosynthesis and metabolism were also found in the top-hit list (Table S1). Finally, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis demonstrated that the 74 genes identified to play a role in β-lactam resistance by imipenem HTS were involved in 33 molecular pathways (Fig. 2A).

Penicillin gene-compound synthetic lethality by pooled-mutant competition assay and qPCR. To validate our high-throughput GCSL screen, we conducted a secondary validation screen using a pooled-mutant competition assay and qPCR. Seventy-three randomly selected transposon mutants were pooled into three overlapping groups and grown in either the presence or the absence of 100 μg/ml of penicillin. The relative abilities of individual mutants to survive in the presence of antibiotic were determined after 7 days of growth. This was determined by calculating the abundance of each mutant in the penicillin-exposed pool and comparing it to the abundance of the unexposed pool through qPCR for mutant-specific genomic DNA normalized to that of the housekeeping gene *sigA*. This revealed that the disruption of seven genes putatively involved in cell wall biosynthesis (*MT0019* [encoding a penicillin binding protein], *MT0335*, *MT1136*, *MT2017*, *MT2018*, *MT2282* [encoding a membrane-anchored esterase], and *MT2954* [encoding an L,D-transpeptidase]) resulted in reduced growth upon exposure to penicillin, with fold changes (FCs) of 0.47, 0.47, 0.66, 0.61, 0.64, 0.18, and 0.54, respectively (see Table S2 in the supplemental material). In addition, the chorismate mutase gene (*MT0975*) also demonstrated GCSL with penicillin (Table S2). Interestingly, some strains exhibited hyper-resistance to penicillin; these included the *MT1661* (FC = 2.54), *MT3826* (FC = 2.40), and *MT3310* (FC = 1.63) transposon mutants (Fig. 1 and 3; Table S2). Both the imipenem HTS and the penicillin pooled-mutant competition assay identified *MT0019*, *MT0076*, *MT1227*, and *MT2594* as genes involved in β-lactam susceptibility. This overlap validated the β-lactam GCSL phenomenon in *M. tuberculosis* (Tables S1 and S2).

Transcriptional response patterns of *M. tuberculosis* to meropenem. The transposon mutant library used in the GCSL screens is limited, as only nonessential genes can be investigated. In order to better define the roles of genes essential for the survival of *M. tuberculosis* in the presence of β-lactam drugs, we characterized the genetic response networks of *M. tuberculosis* H37Rv to one of these drugs, meropenem, using genome-wide RNA-Seq technology. The H37Rv strain was used for RNA sequencing studies because, first, it is better annotated and, second, it is better characterized with regard to antibiotic susceptibility studies than the CDC1551 strain. Both strains have similar virulence and antibiotic susceptibility profiles and are thus used interchangeably. Gene expression profiling was documented (Table S3), and differential expression was analyzed (Fig. 4; Table S4). In general, the gene expression profile fit a normal distribution in response to treatment with meropenem, with only a small number of genes displaying differential expression (Fig. S3). Data analysis revealed that 33 genes were up-regulated and 22 were down-regulated by at least 3 standard deviations (SD) from the median (Table S4). The major β-lactamase gene of *M. tuberculosis*, *blaC* (*Rv2068c*), was up-regulated 1.2-fold, with a difference from the median value of up to 1 times the standard deviation (Table S3).

We carried out gene ontology and KEGG molecular-pathway analysis on significantly differentially expressed genes. Gene ontology profiling revealed that meropenem treatment triggered up-regulation of multiple categories of genes, including extracellular factors (Ag85C [FbpC], Mpt70), stress response pathways (Hsp,

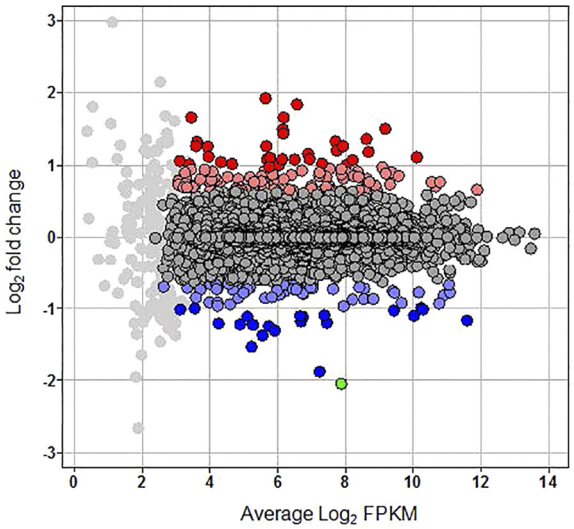

FIG 4 MA plot of gene expression after treatment with meropenem. Log_2 fold change (*y* axis) is plotted against the geometric mean of the log_2 FPKM values (*x* axis). Light-gray dots indicate genes which were excluded from analysis because of variable read numbers. Pink and red dots indicate genes with up-regulated expression with a difference from the median of 2 to 3 times or 3 to 6 times the SD, respectively. Light-blue, dark-blue, and green dots indicate genes with expression that was down-regulated from the median by 2 to 3 times, 3 to 6 times, or >6 times the SD, respectively. Values are MA:log ratios (log_2 fold changes) versus the mean average (average of log_2 FPKM values). FPKM, fragments per kilobase per million fragments mapped.

AhpC, AhpD), cell wall components (MmpS5, LprJ, Mpt83, Rv3675), a transcriptional regulator (SmtB), and conserved hypothetical proteins (Rv3354, Rv0678). Interestingly and not surprisingly, the multidrug transport integral membrane protein Mmr was also significantly up-regulated. Meropenem treatment also triggered down-regulation of multiple categories of genes, including the leucine biosynthetic process (LeuD, LeuC), electron carrier/transfer activity (NarX, FdxA), oxidoreductase activity (DesA3, Rv3131), PPE family protein (PPE19), and nitrate assimilation (NarK2) proteins. Worth mentioning is that some of the stress response genes, such as the TB31.7, Hrp1, and Rv2030c genes, were also down-regulated upon meropenem treatment. Though KEGG pathway information for *M. tuberculosis* genes remains limited, pathway analysis revealed that the vitamin B_6 metabolism pathway was up-regulated upon exposure to meropenem (Fig. 2B). In addition, meropenem exposure down-regulated 15 molecular pathways. These pathways include those for the biosynthesis of secondary metabolites or amino acids (FadE17, PfkB, LeuD, LeuC) and metabolic or carbon metabolism pathways (PfkB, LeuD, LeuC, DesA3). The pleiotropic protein PfkB was identified to be involved in multiple pathways, such as the glycolysis (gluconeogenesis), pentose phosphate, galactose, fructose, and methane metabolism pathways.

In vitro verification. Five mutants were selected from the imipenem and penicillin GCSL screens and the RNA-Seq analysis for further characterization *in vitro*. These strains contained mutations in the conserved outer membrane peptidase-like protein (*MT0335*), chorismate mutase (*MT0975*), putative acyltransferase (*MT1616*), putative carboxylesterase (*MT2282*), and L,D-transpeptidase (*MT2954*) genes. MICs of imipenem and clarithromycin were determined using the microplate alamarBlue assay, with isoniazid and rifampin serving as controls. All five mutant strains were more susceptible to imipenem than the wild-type strain. MICs were four to eight times lower in the mutant strains than in the parental strain, *M. tuberculosis* CDC1551 (Table 1). These results validated the GCSL determined by all methods and strongly suggested a role for these genes in β-lactam resistance. While all mutants showed the same susceptibility to rifampin as the wild type, we found that the *MT2282* (*Rv2224c*) mutant was more susceptible to isoniazid and clarithromycin, in addition to imipenem.

TABLE 1 In vitro MIC verification of selected transposon mutants[a]

Strain	MIC (μg/ml) of:			
	INH	RIF	IMI	CLA
CDC1551 (WT)	0.04	0.075	1.00	0.25
Δ*MT0335* (*Rv0320*) mutant	0.04	0.038	0.25	0.25
Δ*MT0975* (*Rv0948c*) mutant	0.02	0.075	0.25	1.00
Δ*MT1616* (*Rv1565c*) mutant	0.04	0.075	0.25	0.25
Δ*MT2282* (*Rv2224c*) mutant	0.02	0.075	0.125	0.125
Δ*MT2594* (*Rv2518c*) mutant	0.04	0.075	0.25	0.25

[a] INH, isoniazid; RIF, rifampin; IMI, imipenem; CLA, clarithromycin.

***In vivo* gene-compound synthetic lethality.** In order to determine whether our GCSL screens identified genes which were relevant to β-lactam resistance during infection, we selected one of the hits from our screens and evaluated its susceptibility to a β-lactam drug in the mouse model of TB. We chose to focus on the putative acyl-transferase *MT1616* (*Rv1565c*) gene mutant because its analogue was demonstrated to play a role in lipoarabinomannan (LAM) biosynthesis and biofilm formation (20, 21). BALB/c mice were aerosol infected with either wild-type *M. tuberculosis* CDC1551 or the isogenic *MT1616*::Tn mutant and were treated with 2.8, 14, or 70 mg of imipenem/kg of body weight twice daily to achieve daily doses of 5.6, 28, or 140 mg/kg. Imipenem monotherapy did not control the proliferation of the wild-type strain in mouse lungs, even at the highest doses (Fig. 5A). However, the 70-mg/kg imipenem treatment reduced the CFU burdens in the mouse lungs infected with the *MT1616*::Tn mutant by 100-fold over 28 days, indicating a significant *in vivo* synthetic lethality interaction between imipenem and the acyl-transferase gene (Fig. 5B). While dosing at 2.8 mg/kg had no obvious impact, treatment with 14 mg/kg of imipenem showed a bacteriostatic effect on the CFU burdens compared with those in the sham control, but without statistical significance (Fig. 5B; Table S5). The bioavailability of imipenem via intraperitoneal (i.p.) injection was verified by a single-dose pharmacokinetic study (Table S6).

DISCUSSION

Gene-compound synthetic lethality (GCSL) has been used to demonstrate key chemical biology interactions in *Saccharomyces cerevisiae* (22) and has been a powerful tool for cancer drug development (23). In the present study, we took advantage of a large, well-characterized mutant collection to seek GCSL pairs in *M. tuberculosis*. To date, β-lactams, while one of the most valuable antimicrobial classes in medicine, have not been deployed as anti-TB drugs despite the development of fifth-generation, ultra-broad-spectrum agents.

Peptidoglycan cross-linking enzymes are the targets of β-lactam antibiotics. Classical D,D cross-linking enzymes are the major targets for β-lactam antibiotics, but recently, nonclassical,

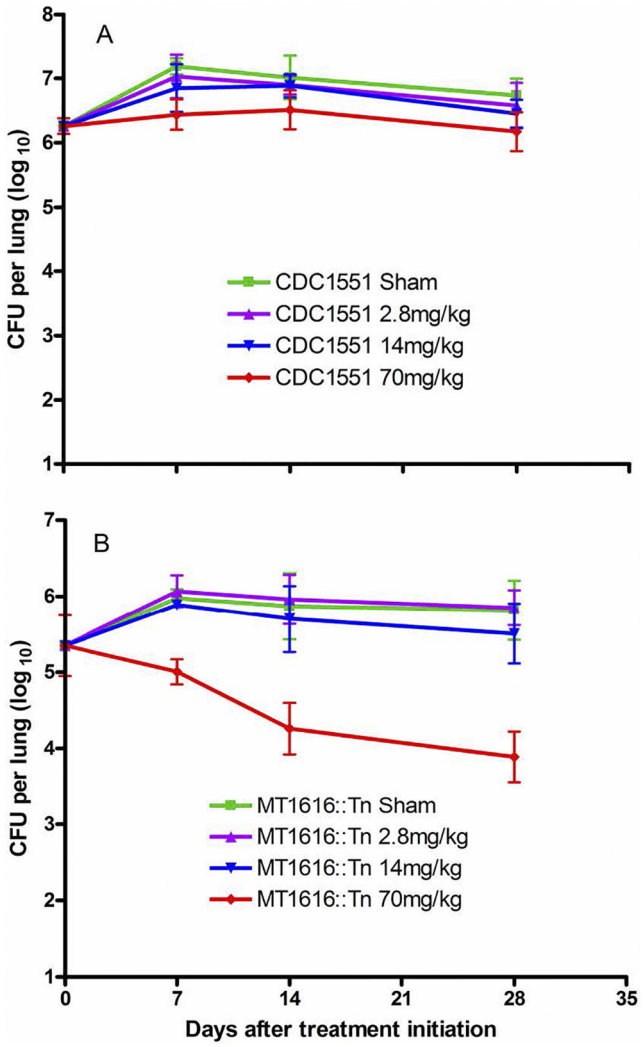

FIG 5 In vivo demonstration of β-lactam gene-compound synthetic lethality in whole animals. BALB/c mice were aerosol infected with wild-type *M. tuberculosis* CDC1551 (A) or the *M. tuberculosis* CDC1551 *MT1616* (Rv1565c) transposon mutant (strain JO0339) (B). Intraperitoneal dosing of imipenem was carried out at three dosing levels (2.8, 14, and 70 mg/kg twice daily [5.6, 28, and 140 mg/kg/day, respectively]), with biological saline as a sham control. Lung CFU burdens were determined at 7, 14, and 28 days after treatment.

L,D cross-linking enzymes have been identified in resistant Gram-positive organisms and *M. tuberculosis* (24). Because their peptide substrate specificity differs from those of their classical counterparts, L,D transpeptidases have innate resistance to β-lactam drugs and may use alternative active-site mechanisms (25). Indeed, genes involved in peptidoglycan biosynthesis were identified in our imipenem-directed GCSL screen. These include *Rv2518c* (LdtB, nonclassical, L,D-transpeptidase) and *Rv3682* (PonA2, penicillin binding protein, class A). LdtB of *M. tuberculosis* is a nontraditional transpeptidase that catalyzes the 3→3 cross-link in peptidoglycan biosynthesis and has been demonstrated to be synthetically lethal with amoxicillin (15). PonA2 is a penicillin binding protein which is also involved in peptidoglycan biosynthesis (26) and multidrug resistance (27).

Although peptidoglycan biosynthesis-related genes are major targets of β-lactam resistance, β-lactam resistance mechanisms and determinants in *M. tuberculosis* are complex. The ATP-binding cassette (ABC) superfamily transporters certainly play a role, as we identified five of them in our HTS (Rv2936, Rv1348, Rv1819c, Rv3000, and Rv1747). The ABC transporter encoded by *Rv0194* has been identified to play a role in multidrug resistance, including β-lactam resistance (28), and, interestingly, functions as an efflux pump with multiple substrates (28). In this study, we too identified an efflux pump (Rv3065) which was up-regulated after treatment with meropenem, suggesting that efflux pumps play an important role in β-lactam antibiotic resistance in *M. tuberculosis*. A study by Dinesh et al. identified that multiple efflux pumps play a role in β-lactam resistance (Rv0849, Rv1218c, and Rv1258c) and demonstrated that knocking out *Rv3065* rendered *M. tuberculosis* susceptible to multiple β-lactam antibiotics (29), indicating a high degree of agreement between RNA expression profiling and phenotype exhibition.

Recent studies indicate that from the resistance-nodulation-division (RND) family of transporters, MmpS5 and MmpL5 function as multidrug efflux pumps. This was demonstrated by resistance mechanism studies for azoles (30), bedaquiline (31), and clofazimine (32). Our study shows that both these transporters and their regulator, Rv0678 (33), are up-regulated upon exposure to meropenem. These results demonstrate that β-lactam antibiotics fall within the substrate spectrum of the regulator-MmpS5-MmpL5 efflux system, suggesting that this is an off-target β-lactam resistance mechanism. Mutations in *Rv0678*, along with the up-regulation of MmpL5, have been associated with cross-resistance between bedaquiline and clofazimine, and consequently, further cross-resistance with β-lactams should be considered in future studies (32).

The inhibition of cell wall biosynthesis by β-lactams will also trigger up-regulation of associated genes in a mode similar to that of a feedback control mechanism. This assumption was confirmed by the identification of *Rv3717*, which putatively encodes an *N*-acetylmuramoyl-L-alanine amidase that may be involved in the peptidoglycan catabolic process, as revealed by gene ontology analysis. This gene was identified by two independent global transcription profiling studies using two different methods and two different β-lactam antibiotics, namely, microarray analysis for ampicillin-resistant genes by Boshoff et al. (34) and RNA-Seq profiling for meropenem-resistant genes in our study. The finding that cell wall-associated proteins besides Rv3717, such as Rv1987, Rv1690, and Rv0129, overlapped in these two studies demonstrated the consistency of the two methodologies.

Our data indicate that both the high-throughput alamarBlue assay and the pooled-mutant qPCR analysis are suitable for GCSL screening of *M. tuberculosis*. The alamarBlue HTS system is a powerful genetic tool for investigating gene functions and genetic pathways. Ultimately, the HTS format can be scaled up so that mutant libraries may be screened against numerous compounds, hence enabling a comprehensive chemical-genomic portrait of *M. tuberculosis*. The use of two different β-lactams in these studies nevertheless produced highly consistent results between the two methods, suggesting that both are high-quality assays that are able to identify key β-lactam susceptibility genes. In addition to the core structure, members of the β-lactam class of antibiotics share many properties including binding proteins, inactivating enzymes, and potential targets. Meropenem and imipenem belong to the same subcategory as carbapenem in the β-lactam family. While we believed that there would be no difference in results

using either antibiotic, we used meropenem for the RNA-Seq experiment, as recent studies suggested a role for meropenem in treating drug-resistant tuberculosis (11) and data generated using meropenem can be referenced by other researchers in the community.

Genome-wide transcriptomic analysis by RNA-Seq is a powerful tool for global characterization of expression profiles or genes of interest, with or without variable biological/physiological conditions (35, 36). We expected the RNA-Seq study to identify genes different from those found by the GCSL screens because, first, GCSL screens are able to identify only individual nonessential genes and, second, RNA-Seq is able to identify multiple genes that coordinate a response in a pathway. As expected, this study reveals a complex picture of *M. tuberculosis* genetic responses following exposure to the β-lactam drug meropenem (Table S4). Multiple pathways were up- or down-regulated upon meropenem treatment. While the vitamin B_6 biosynthesis and metabolism pathway (Rv2607) was up-regulated, a significant number of biosynthesis and metabolism pathways for secondary metabolites, amino acids (valine, leucine, isoleucine), 2-oxocarboxylic acid, and C5-branched dibasic acid were down-regulated. When comparing the RNA-Seq data with the GCSL screen data, we found it interesting that *Rv0190*, the putative copper-responsive repressor (37), was significantly up-regulated in the RNA-Seq study. The immediate downstream gene, *Rv0191*, is predicted to encode an integral membrane protein possibly involved in drug transport across the membrane, and inactivation of this gene renders the strain synthetically lethal when combined with imipenem in the GCSL study (14th top hit; see Table S1 in the supplemental material).

Our data showed that all three methods were suitable for gene target identification, with some degree of overlap and distinction. Three genes were identified by both GCSL HTS and qPCR, namely, *MT2594*, *MT0019*, and *MT0076*. The first two genes belong to the peptidoglycan biosynthesis family, with *MT2594* also being up-regulated 1.3-fold (2 times the standard deviation) in the RNA-Seq profiling study. Furthermore, a comparison of the three methods revealed that cell wall-associated or cell process-associated genes were often related to β-lactam resistance. For example, the lipoprotein MT0501 had two hits in the GCSL HTS assay and was up-regulated 1.8-fold (3 times the standard deviation) in the RNA-Seq profiling study. In the case of *MT0795*, a putative 4-carboxymuconolactone decarboxylase, inactivation of the gene rendered the mutant susceptible to β-lactams in the GCSL HTS assay, although β-lactam exposure of wild-type *M. tuberculosis* suppressed its expression in the RNA-Seq study. This implies that this class of genes may not be required for immediate counteraction of the antibiotic but is required for long-term resistance. This may also imply an energy shift or preservation during antibiotic stress. A similar trend was observed between qPCR and RNA-Seq experiments for a putative, exported, cell wall-associated protein, MT0335, as it was underrepresented in the qPCR pool study but down-regulated in the RNA-Seq profiling study. Gene-compound specificity for this phenomenon remains to be uncovered.

In addition to the aforementioned genes, *MT2282* (*Rv2224c*) was shown to be synthetically lethal with penicillin by qPCR (fold change = 0.15) and exhibited GCSL with imipenem by HTS, although with a selection index lower than the cutoff percentage set for the HTS (0.52 versus 0.66). With MT2282's esterase activity and roles in virulence (38), innate immunity induction (39), and possibly multidrug resistance (27), including to β-lactam (imipenem) and macrolide antibiotics (clarithromycin) (Table 1), we hypothesize that the gene product of *MT2282* plays a role in cell wall biosynthesis and/or integrity. However, other types of acquired resistance do exist, for example, the production of drug-inactivating enzymes (esterases or kinases), the production of active ATP-dependent efflux proteins that transport the drug outside the cell, and mutation in the intrinsic macrolide-resistance *erm* gene (40). Consequently, further molecular mechanistic characterization for selected putative susceptible mutants is warranted.

An important finding of this study is that a gene-compound pair found to be synthetically lethal *in vitro* was also demonstrated to display synthetic lethality *in vivo*. Disruption of *MT1616* (*Rv1565c*) rendered the otherwise-isogenic strain susceptible to imipenem at the 70-mg/kg dose level. At this dose, the CFU reduction in mouse lungs was comparable to the result of treatment with isoniazid at the 10-mg/kg level (Table S5). While the *MT1616* gene is not well characterized, bioinformatic analysis predicts this gene to encode a transmembrane protein. Pfam and domain searches reveal only one functional domain, acyl-transferase 3, which also resides in the transmembrane portion. While the information regarding *MT1616* is limited, the *Mycobacterium marinum* orthologue (*MMAR_2380*) has been demonstrated to be essential for the biosynthesis of the mannose cap on lipoarabinomannan (LAM), and disruption of this gene results in cell aggregation in liquid media (20). In *Mycobacterium avium*, disruption of the *MT1616* orthologue results in a biofilm formation defect and colony morphology abnormality (21). This suggests that the *MT1616* gene product plays an important role in cell wall biosynthesis and in the organism's adaptive response to the environment. The *in vivo* GCSL effect of *MT1616* with imipenem was also consistent with *in vitro* findings, as the imipenem MIC for the transposon mutant was reduced 4-fold compared with that for the wild-type parental strain (Table 1). In this study, the disruption of *MT1616* appears to result in a slight reduction in virulence, as indicated by a lower CFU plateau in mouse lungs following infection (Fig. 5; Table S5). However, the impact of the functional loss of *MT1616* on the virulence and pathogenesis of *M. tuberculosis* requires further study.

Our study indicates that β-lactam drugs can kill *M. tuberculosis* when combined with gene inactivation. Since β-lactams have essentially no cross-resistance with the first-line anti-TB drugs (10), these observations imply that inhibitors of enzymes found to be synthetically lethal in this and other screens have the potential to enable the use of β-lactams against tuberculosis. Indeed, a recent case-control study of 37 MDR- or XDR-TB patients has already shown the effectiveness, safety, and tolerability of meropenem-clavulanate when added to linezolid-containing regimens in the treatment of drug-resistant TB (41). This same synthetic lethality strategy may have value as an approach that can be extended to other existing antibiotics that are not widely used to treat TB, such as the macrolides and tetracyclines.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *M. tuberculosis* CDC1551 wild-type and Himar I transposon mutant library were used in this study (2). The lab strain H37Rv was used for RNA-Seq. Regulator 7H9 broth was used for initial culturing. For GCSL screening and MIC determination, 7H9 broth without Tween 80 was used. Library growth was done in

a 24-well format with 1 ml of culture volume. All culturing was carried out at 37°C.

Mutant-library screening in the presence of imipenem. The high-throughput alamarBlue screening assay was verified as described previously (42), with the wild-type strain treated with 2.5 μg/ml amikacin as a positive control and the wild-type strain treated with 1% dimethyl sulfoxide (DMSO) as a negative control. Individual mutants were grown to mid-log phase in 7H9 without Tween 80 and monitored with a microplate reader (FLUOstar Optima; BMG Labtech) and diluted to an optical density at 600 nm (OD_{600}) of 0.01. A 96-well plate was set up with 160 μl of culture in each well, either with or without 0.5 to 1.0 μg/ml imipenem. After incubation for 5 days at 37°C, 12.5 μl of 20% Tween 80 and 20 μl of alamarBlue were added to each well. Plates were incubated for 16 h and read with a fluorescence microplate reader (BMG) at excitation and emission wavelengths of 544 and 590 nm, respectively. Growth inhibition was calculated with reference to positive and negative controls. A selection index was defined, and values were calculated as percentages of the net inhibition (raw inhibition minus wild-type inhibition) over the raw inhibition.

qPCR verifications. For multiplex quantitative real-time PCR analysis (qPCR), 73 randomly selected transposon mutants were grown to mid-log phase in 7H9 without Tween 80, diluted to an OD_{600} of 0.01, and pooled. Pools of 12, 23, and 42 mutants were generated, and two mutants were used as overlapping internal controls, which included an intergenic mutant as a negative control and an *MT2594* mutant as a positive control. All primers were optimized based on amplification of genes from genomic DNA to ensure limited variability based on differences in primer specificity. One milliliter of culture, in either the presence or the absence of 100 μg/ml penicillin, was incubated for 7 days in a 24-well plate. Genomic DNA was isolated, and qPCR was carried out using the transposon-specific primer paired with corresponding gene-specific primers. The abundance of individual mutants in the pool was estimated in reference to the total of all mutants in the pool, which is represented by the abundance of *sigA*.

RNA-Seq. Wild-type lab strain H37Rv was grown to mid-log phase (OD_{600} = 0.5). Cells were treated with meropenem at a final concentration of 4 μg/ml for 6 h. Parallel untreated cells were used as a control. Total RNA was isolated from treated and untreated cells by using a TRIzol (Invitrogen)/RNeasy (Qiagen) hybrid RNA extraction protocol. DNA contaminants were depleted with a Turbo DNA-free DNase kit (Ambion). Sample quality and quantity were monitored by a 2100 Bioanalyzer (Agilent Technologies) and a NanoDrop 2000c instrument (Thermo Scientific), respectively. Three and two biological replicates were carried out for the treatment and control, respectively. RNA samples were sequenced using the Illumina platform (Illumina), and sequencing reads were aligned to the published H37Rv genome sequence (43) using SOLiD BioScope software (Life Technologies Corp). Numbers of fragments per kilobase per million fragments mapped (FPKM) were determined using an in-house script which internally uses BEDTools (44). FPKM from meropenem-treated and control cells were first subjected to quantile normalization and then transformed into $\log_2$ notation with the Partek Genomics Suite platform (Partek Inc., St. Louis, MO, USA). The distribution of these resulting values was examined across all cell samples, and those genes for which the minimum $\log_2$ value was at least 2.0 were compared between meropenem-treated and control cells to determine the relative levels of mRNA expression of these two classes. Since the genes' relative expression values, expressed as $\log_2$(ratio) or $\log_2$(fold change), showed a normal distribution, they were binned by standard deviation from their median to provide cutoff thresholds for up- or down-regulation.

***In vitro* verifications.** MICs were determined using the microplate alamarBlue assay as described previously (45). Candidate mutants were selected based on the HTS GCSL, the qPCR verification, and the RNA-Seq experiment results. MICs of imipenem, clarithromycin, rifampin, and isoniazid for the selected mutants were identified. Three microplate alamarBlue assays were carried out, and results were verified by conical-tube broth dilution methods.

***In vivo* GCSL.** Four- to 6-week-old female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were aerosol infected with 10 ml of early-log-phase *M. tuberculosis* wild-type CDC1551 (OD_{600} = 0.2) or the *MT1616*::Tn mutant (OD_{600} = 0.3) strain using the inhalation exposure system (Glas-Col Inc., Terre Haute, IN). From 14 days after infection, groups of four mice were treated with 2.8, 14, and 70 mg of an imipenem equivalent/kg in the Primaxin i.v. formulation (imipenem and cilastatin for injection; Merck & Co., Inc.), 10 mg of isoniazid/kg as a positive control, or an equal volume (0.2 ml) of biological saline as a sham control, by intraperitoneal (i.p.) injection twice a day and 5 days a week, which resulted in a daily imipenem dose of 5.6, 28, and 140 mg/kg, respectively. At days 7, 14, and 28 after treatment initiation, 4 mice from each treatment group were sacrificed and the lungs removed. The lungs were bead beaten to homogenize them, diluted, and plated on 7H11 selective agar plates (BBL). Numbers of CFU per lung were determined. To verify imipenem bioavailability by i.p. injection, a single-dose pharmacokinetic study was carried out. Seven 20-gram female BALB/c mice were dosed at 70 mg of Primaxin/kg by i.p. injection. At 7, 15, 30, 60, 120, and 240 min after dosing, about 30 μl of whole blood was collected in triplicate by the retro-orbital bleeding technique. Immediately, 25 μl of blood was transferred into O-ring tubes containing 25 μl of 0.1 M EDTA and mixed well. Samples were kept at −80°C for future analysis. Imipenem concentrations in blood were analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS; AB SCIEX QTRAP 5500 system) (46), with detection of mass transitions of 300.0/142.0 and 300.0/98.0. Pharmacokinetic parameters were analyzed by using WinNonlin 6.3 (PharSight, Sunnyvale, CA) and noncompartmental modeling. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01767-14/-/DCSupplemental.

Text S1, DOCX file, 0.01 MB.
Figure S1, TIF file, 1 MB.
Figure S2, PDF file, 0.04 MB.
Figure S3, TIF file, 0.7 MB.
Table S1, XLSX file, 0.02 MB.
Table S2, XLSX file, 0.01 MB.
Table S3, XLSX file, 0.3 MB.
Table S4, XLSX file, 0.01 MB.
Table S5, DOCX file, 0.02 MB.
Table S6, DOCX file, 0.01 MB.

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