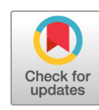
RESEARCH ARTICLE



Collateral Sensitivity to β-Lactam Drugs in Drug-Resistant Tuberculosis Is Driven by the Transcriptional Wiring of BlaI Operon Genes

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ABSTRACT The evolution of resistance to one antimicrobial can result in enhanced sensitivity to another, known as "collateral sensitivity." This underexplored phenomenon opens new therapeutic possibilities for patients infected with pathogens unresponsive to classical treatments. Intrinsic resistance to β-lactams in *Mycobacterium tuberculosis* (the causative agent of tuberculosis) has traditionally curtailed the use of these low-cost and easy-to-administer drugs for tuberculosis treatment. Recently, β-lactam sensitivity has been reported in strains resistant to classical tuberculosis therapy, resurging the interest in β-lactams for tuberculosis. However, a lack of understanding of the molecular underpinnings of this sensitivity has delayed exploration in the clinic. We performed gene expression and network analyses and *in silico* knockout simulations of genes associated with β-lactam sensitivity and genes associated with resistance to classical tuberculosis drugs to investigate regulatory interactions and identify key gene mediators. We found activation of the key inhibitor of β-lactam resistance, *blaI*, following classical drug treatment as well as transcriptional links between genes associated with β-lactam sensitivity and those associated with resistance to classical treatment, suggesting that regulatory links might explain collateral sensitivity to β-lactams. Our results support *M. tuberculosis* β-lactam sensitivity as a collateral consequence of the evolution of resistance to classical tuberculosis drugs, mediated through changes to transcriptional regulation. These findings support continued exploration of β-lactams for the treatment of patients infected with tuberculosis strains resistant to classical therapies.

IMPORTANCE Tuberculosis remains a significant cause of global mortality, with strains resistant to classical drug treatment considered a major health concern by the World Health Organization. Challenging treatment regimens and difficulty accessing drugs in low-income communities have led to a high prevalence of strains resistant to multiple drugs, making the development of alternative therapies a priority. Although *Mycobacterium tuberculosis* is naturally resistant to β-lactam drugs, previous studies have shown sensitivity in strains resistant to classical drug treatment, but we currently lack understanding of the molecular underpinnings behind this phenomenon. We found that genes involved in β-lactam susceptibility are activated after classical drug treatment resulting from tight regulatory links with genes involved in drug resistance. Our study supports the hypothesis that β-lactam susceptibility observed in drug-resistant strains results from the underlying regulatory network of *M. tuberculosis*, supporting further exploration of the use of β-lactams for tuberculosis treatment.

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Understanding collateral sensitivity to β-lactams in tuberculosis using network analysis and in silico growth studies @anna_t_g

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Collateral antimicrobial sensitivity occurs when the evolution of resistance to one or more antimicrobials directly or indirectly causes increased sensitivity to unrelated antimicrobials (1). There are now numerous examples of this phenomenon in the literature (2, 3), and while direct mechanisms are sometimes evident based on our understanding of individual genes or pathways (4), there is a lack of knowledge to explain collateral sensitivity between drugs of unrelated function. An improved understanding of such mechanisms can inform novel treatment strategies that limit or delay the development of resistance (1).

Tuberculosis (TB) remains a significant cause of global mortality, causing an estimated 1.4 million deaths annually (5). It can be successfully treated through combination antimicrobial therapy targeting the causal pathogen, *Mycobacterium tuberculosis*. However, successful treatment is hampered by the continuing rise of antimicrobial-resistant *M. tuberculosis*, particularly strains resistant to multiple drugs (5). According to the World Health Organization, multidrug resistance among *M. tuberculosis* strains is defined as resistance to isoniazid and rifampicin, with or without resistance to other first-line drugs, and extensive drug resistance is defined as resistance to isoniazid and rifampicin plus any fluoroquinolone and any of the three second-line injectable drugs (amikacin, capreomycin, and kanamycin).

The potential application of clinical regimens including β-lactams for the treatment of TB is of particular interest due to the comparative low-cost, ease of treatment, and accessibility of these drugs (6). However, *M. tuberculosis* has generally been considered intrinsically resistant to β-lactams due to (i) the inclusion of nonclassical peptidoglycan linkages in its cell wall, by a combination of distinct penicillin binding proteins (7, 8), and (ii) the presence of BlaC β-lactamases that break down β-lactams with drug-specific efficiencies (9–11). However, sensitivity to certain subclasses of β-lactams in *M. tuberculosis*, namely, faropenem (12) and carbapenems (7, 13), was recently reported, although coadministration with a β-lactamase inhibitor, such as clavulanate in a combination known as Augmentin, is likely needed to be effective *in vivo* (6, 10, 14). Unfortunately, patient treatment trials of this combination have been far from promising (15, 16), and recent reports of novel β-lactamases (17) have added another layer of complexity to the use of β-lactams in the clinic.

Multidrug- or extensively drug-resistant clinical *M. tuberculosis* strains and isolates experimentally evolved to be resistant to aminoglycosides have been shown to exhibit enhanced sensitivity to the β-lactams with and without the addition of β-lactamase inhibitors (18–20), suggesting that β-lactam sensitivity may be associated with the evolution of resistance to classical TB drugs as a result of a process of collateral sensitivity. Therefore, exploration of the potential mechanism(s) linking β-lactam sensitivity with resistance to the classical drugs may help elucidate the biological underpinnings of β-lactam sensitivity.

Technological and algorithmic advances have facilitated the high-throughput measurement of gene expression as well as the inference and analysis of large-scale protein-protein interaction and DNA-protein interaction networks for *M. tuberculosis*, which can facilitate systems-level investigations into the transcriptional and regulatory mechanisms. These methods have been successfully adopted in the study of *M. tuberculosis* infection (21), latency (22), and response to the β-lactam plus β-lactamase inhibitor treatment (23) and for the identification of novel drug resistance mechanisms (24, 25). However, a systems-level understanding of collateral sensitivity in drug-resistant *M. tuberculosis* is currently lacking.

Here, we leverage network and transcriptomic analyses for the exploration of collateral β-lactam sensitivity in *M. tuberculosis*. We combine gene expression analyses with protein-protein interaction, gene regulatory network data, and functional *in silico* growth simulations. Our analyses suggest that collateral β-lactam sensitivity is the

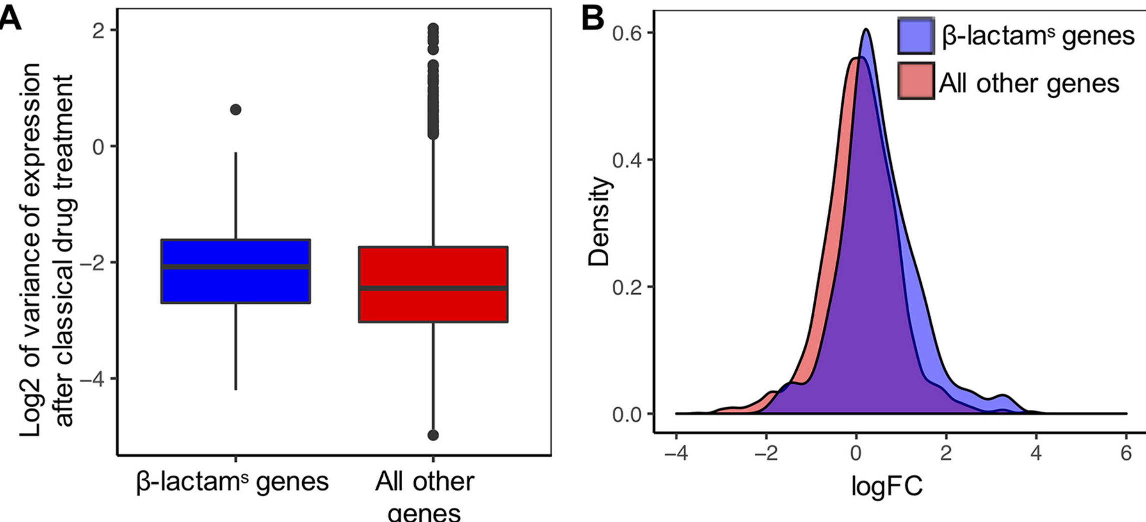

FIG 1 The expression of β-lactam sensitivity (β-lactam^s) genes is affected by treatment with classical TB drugs. (A) β-lactam^s genes tend to be more variable than non-β-lactam^s genes, suggesting these genes are affected by drug treatment (KS test *P* value, 0.0027). (B) Log fold change (logFC) of β-lactam^s genes and all other non-β-lactam^s genes after classical drug treatment. β-lactam^s genes tend to have a more positive logFC than other genes, suggesting preferential activation.

result of direct transcriptional regulation between genes associated with β-lactam sensitivity and those mediating resistance to classical TB drugs. This wiring promotes the inhibition of β-lactamases as a response to drug treatment, with genes of the BlaI operon that inhibit the blaC β-lactamases (*blaI*, *sigC*, and *atpH*), playing key roles.

RESULTS

Treatment with classical TB drugs induces the expression of β-lactamase inhibitors. If β-lactam sensitivity in *M. tuberculosis* is truly a consequence of classical drug resistance (i.e., truly collateral), we expect that genes/proteins implicated in β-lactam sensitivity (β-lactam^s genes) (see Table S1 in the supplemental material) should have close biochemical and/or regulatory connections to those that are implicated in classical drug resistance (DR genes) (see Table S2). We hypothesized that such connectivity may be detected as differential expression of β-lactam^s genes in response to classical drug treatment. Therefore, we investigated the differential expression of 199 genes with reported involvement in β-lactam sensitivity in *M. tuberculosis* (or the closely related species *Mycobacterium smegmatis* or *Mycobacterium bovis*) in response to incubation with classical TB drugs (ethambutol [EMB], ethionamide [ETH], two fluoroquinolones [FLQs]; levofloxacin and ofloxacin), (aminoglycosides [AMI], streptomycin [SM], isoniazid [INH], pyrazinamide [PZA], and rifampicin [RIF]) (26). Since we aimed to find commonalities between drug treatments, gene expression data across single-agent drug treatments were pooled. Given that many of the reported β-lactam^s genes have not yet been subjected to experimental validation, we primarily focused on a subset of 63 high-confidence genes (Table S1), which included canonical genes such as *blaI*, *bla*, and *atpA-G*, which were shown to have β-lactamase activity, are downstream of *blaI*, or have been identified through functional assays as being associated with sensitivity to β-lactams. We excluded genes solely identified through synthetic lethality or transposon mutant screens or with circumstantial evidence of involvement (see Materials and Methods). However, all analyses were repeated using the full set of 199 genes, reported in Table S6.

We found that β-lactam^s genes showed a greater variability of expression than non-β-lactam^s genes (Kolmogorov-Smirnov [KS] test *P* value = 0.027; Wilcoxon test *P* value = 0.014) (Fig. 1A), indicating that classical drug treatment disproportionately

affects the activity of these genes. We further validated this result by assessing the variability of randomly selected subsets of non-β-lactam^s genes matching the number of β-lactam^s genes (10,000 permutations, $P = 0.023$).

We next performed differential expression analysis using limma (27), revealing 483/3,947 differentially expressed genes after drug treatment (q value < 0.05, |fold change [FC]| > 2) (Fig. 1B; see also Table S3), with 268 of these being upregulated (6.79%). Inspection of the β-lactam^s genes revealed that 18.52% (10 of 54 in the data set) of β-lactam^s genes were significantly upregulated. This indicated an enrichment of β-lactam^s genes among the differentially expressed genes (Fisher exact test $P = 0.0075$).

To assess whether the activation of β-lactam^s genes may be a reflection of a stress response, we also investigated the expression levels of β-lactam^s genes in acidic environments (pH 5.5 and 6.5) (using data from reference 28, GEO accession GSE8827) and during a time course hypoxia experiment from 4 h to 8 days (using data from reference 29, GEO accession GSE9331). β-Lactam^s genes did not show patterns of increased expression under acidic conditions compared to that in the control (see Fig. S1). Only two β-lactam^s genes (Rv3290c and Rv0849) showed increased expression as an acute response to hypoxia within the first 8 h of treatment, while the rest either maintained similar levels or decreased their expression (see Fig. S2). This supports the notion that drug treatment is a more specific inducer of the expression of β-lactam^s genes than acidic stress conditions.

Interestingly, *blaI* (Rv1846c), the major repressor of the *blaC* β-lactamase, and *atpH* (Rv1307) and *sigC* (Rv2069), members of the BlaI regulon (30), were all upregulated after classical drug treatment (pooled results: *blaI* fold change = 2.85, q value = 0.0095; *atpH* fold change = 2.25, q value = 0.030; *sigC* fold change = 2.70, q value = 0.0004) (Table S3; Fig. S3). Given that an increased activity of the *blaI* repressor would lead to a loss of activity of *blaC* and therefore a reduction in β-lactamase production (30), these data indicate that classical TB drug treatment may inhibit the main β-lactamase responsible for *M. tuberculosis*'s intrinsic β-lactam resistance. Among the upregulated genes we also found Rv1884c (*rpfC*) (fold change = 3.33, q value = 0.0324), which has also been associated with β-lactam sensitivity (31). Overall, our data suggest that treatment of *M. tuberculosis* with classical anti-TB drugs used in the clinic promoted the upregulation of key inhibitors of β-lactam resistance.

Strong coexpression between β-lactam^s and DR genes. The findings from our gene expression analyses were consistent with our hypothesis that there is a transcriptional association between β-lactam^s genes and those encoding the classical drug targets. To further explore this hypothesis, we searched for coexpression associations between the β-lactam^s and DR genes, the latter of which include those that encode the classical drug target proteins.

First, we investigated module comembership of β-lactam^s genes with DR genes among previously defined coexpression modules derived from 437 perturbation experiments with different drugs and growth-inhibitory conditions (26). We found that 50% of coexpression clusters with at least 2 β-lactam^s genes also contained DR genes, with permutation analysis indicating that this was unlikely to occur by chance (P = 0.12, based on 10,000 randomizations of cluster membership), suggesting that these genes are controlled by similar regulatory networks.

Next, we compared the strength of correlation of expression of DR genes with β-lactam^s genes in these perturbation experiments (see Materials and Methods). We found that of the 37 DR genes, 30 (81.1%) had a stronger correlation with the high-confidence genes of the β-lactam^s cluster than with all other genes (Fig. 2, genes located above the diagonal line). These DR genes were disproportionately associated with INH resistance (13 genes) or with resistance to multiple drugs (7 genes), suggesting that these DR genes likely exert a strong influence on genes associated with β-lactam sensitivity.

Overall, DR and β-lactam^s genes were found to be highly coexpressed in the transcriptional network of *M. tuberculosis*, supporting our hypothesis of a transcriptional association of these genes.

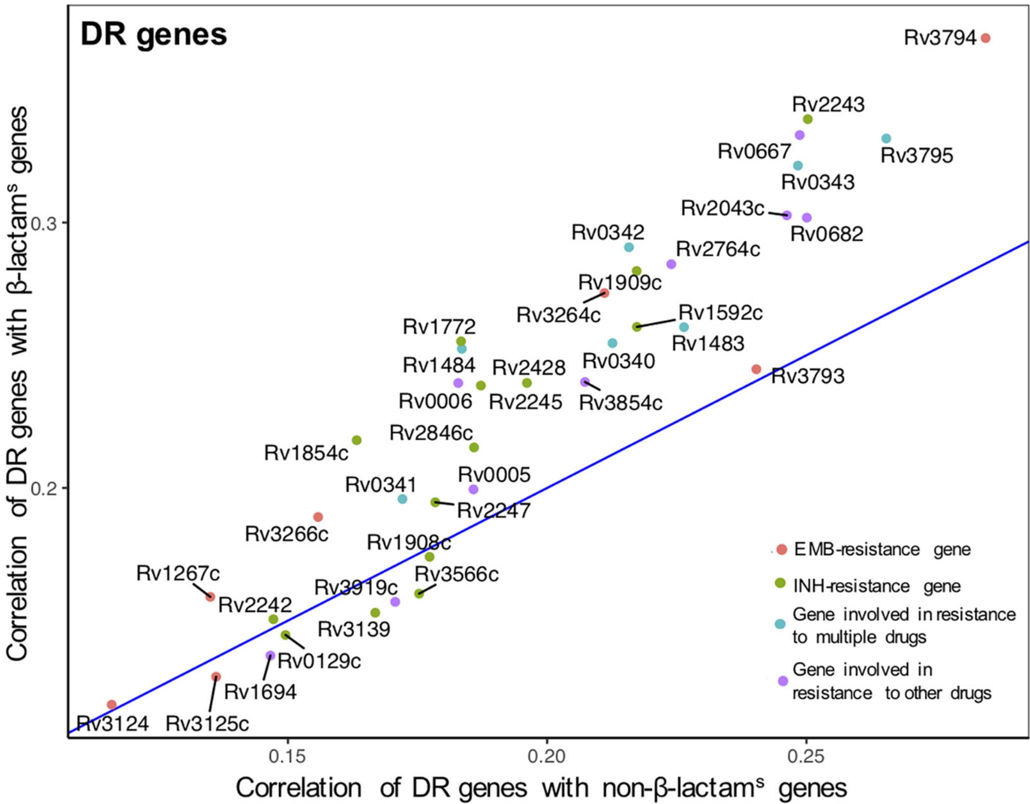

FIG 2 Upperquantile of expression correlation of drug resistance (DR) genes with β-lactam sensitivity (β-lactam^s) genes (y axis) and non-β-lactam^s genes (x axis). Genes above the diagonal line are more strongly coexpressed with β-lactam^s genes than with other genes, and vice versa. The genes with the strongest positive correlation of expression (well above the diagonal line) are implicated in isoniazid (INH) and ethambutol (EMB) resistance.

***β*-lactam^s and DR nodes (genes/proteins) are highly linked in the molecular network of *M. tuberculosis*.** To determine whether the transcriptional associations between β-lactam^s and DR genes were the result of direct regulation between these genes as opposed to indirect associations, we investigated their localization and interaction in the *M. tuberculosis* protein-protein and gene regulatory networks, where genes/proteins are represented as nodes and interactions are represented as edges. We integrated the STRING database (32) (here referred to as the protein-protein interaction [PPI] network) and transcription factor-target gene data published in reference 33 (here referred as to the gene regulatory [GR] network), excluding duplicated edges and self-loops. The resulting network contained 4,181 nodes (genes/proteins) and 37,313 edges, including experimentally validated physical and transcription factor-target associations between genes or proteins. There was no evidence to suggest that the combined PPI and GR network was not drawn from a power-law distribution (P = 0.065, i.e., indicating that we cannot reject the null hypothesis that the degree of distribution follows a power-law distribution), supporting the view that the network structure is consistent with a true biological network (34).

We found that 26 of the 63 (41%) β-lactam^s nodes were localized in a highly specific network region, interacting with each other (Fig. 3). We assessed the significance of the interactions between gene sets modeling the distribution of cross talk expected under a random model of a given network as a hypergeometric distribution (35) (see Materials and Methods). We found that β-lactam^s nodes were more likely to interact with each other than expected by chance in the largest interconnected component of the network region (q value $= 1 \times 10^{-35}$). Interestingly, within this subnetwork, β-lactam^s nodes were clustered based on the gene/protein functional role (Fig. 3), with

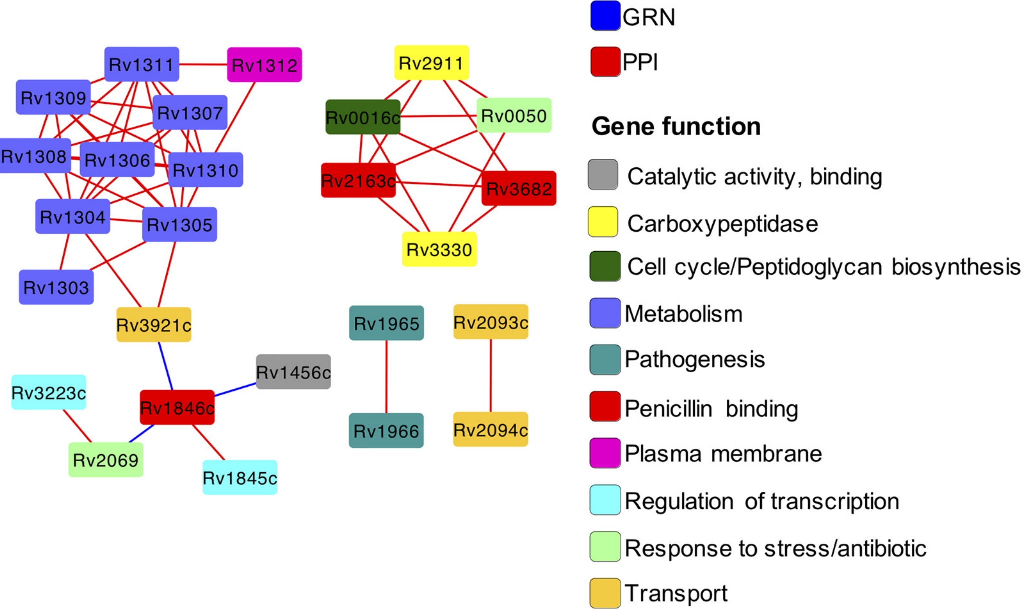

FIG 3 Network of interactions between genes/proteins associated with β-lactam sensitivity. β-Lactam^S (β-lactam sensitivity) genes form a single interconnected network, with a few exceptions, indicating a high degree of localization in the global *M. tuberculosis* network. Nodes are colored by predicted functional categories. The network shown is a combination of the protein-protein interaction (PPI; red edges) and the gene regulatory (GR; blue edges) networks.

clusters of nodes representing genes/proteins involved in similar functions, such as metabolism, consistent with previous findings that gene function is related to network localization (36, 37). However, the broader clustering of β-lactam^s nodes suggests a high degree of association between these genes even when these are functionally highly varied (Table S1), suggesting involvement in similar protein complexes or enzymatic reactions.

Next, we investigated the interactions between β-lactam^s nodes and DR nodes (Fig. 4; see also Table S4). We noted that the β-lactam^s nodes were located near the core or center of the subnetwork, with DR nodes organized in clusters at the periphery grouped by drug type. The degree of cross talk in the most highly connected component of the network was significant (q value = 0.0002). We found significant cross talk in the GR network between RIF and SM resistance and β-lactam^s genes (q value = 0.0003 and 0.003, respectively). These data support direct links between β-lactam^s and DR genes, which together with their strong transcriptional associations, support our hypothesis of direct regulatory interactions between β-lactam^s genes/proteins and the genes/proteins implicated in resistance to at least three of the first-line treatments used to treat TB in the clinic.

Mediators of the interactions between DR and β-lactam^s genes. To identify the key genes linking β-lactam^s and DR genes/proteins, which likely mediate collateral β-lactam sensitivity, we performed random walks between the β-lactam^s and DR nodes in the PPI and GR networks to determine the influence of one node over another (access times). Random walks correspond to the possible paths taken by a random walker on a network between a pair of nodes, and access times represent the ease with which information (e.g., signal transduction and gene regulation) flows from one node to another, as it is proportional to the number of connections and available paths between nodes.

Since the importance of a node highly depends on the underlying network structure, we first determined how the structural differences between the PPI and GR networks would affect the random walks by comparing access times between pairs of DR nodes and the full set of β-lactam^s nodes. We considered both directions of

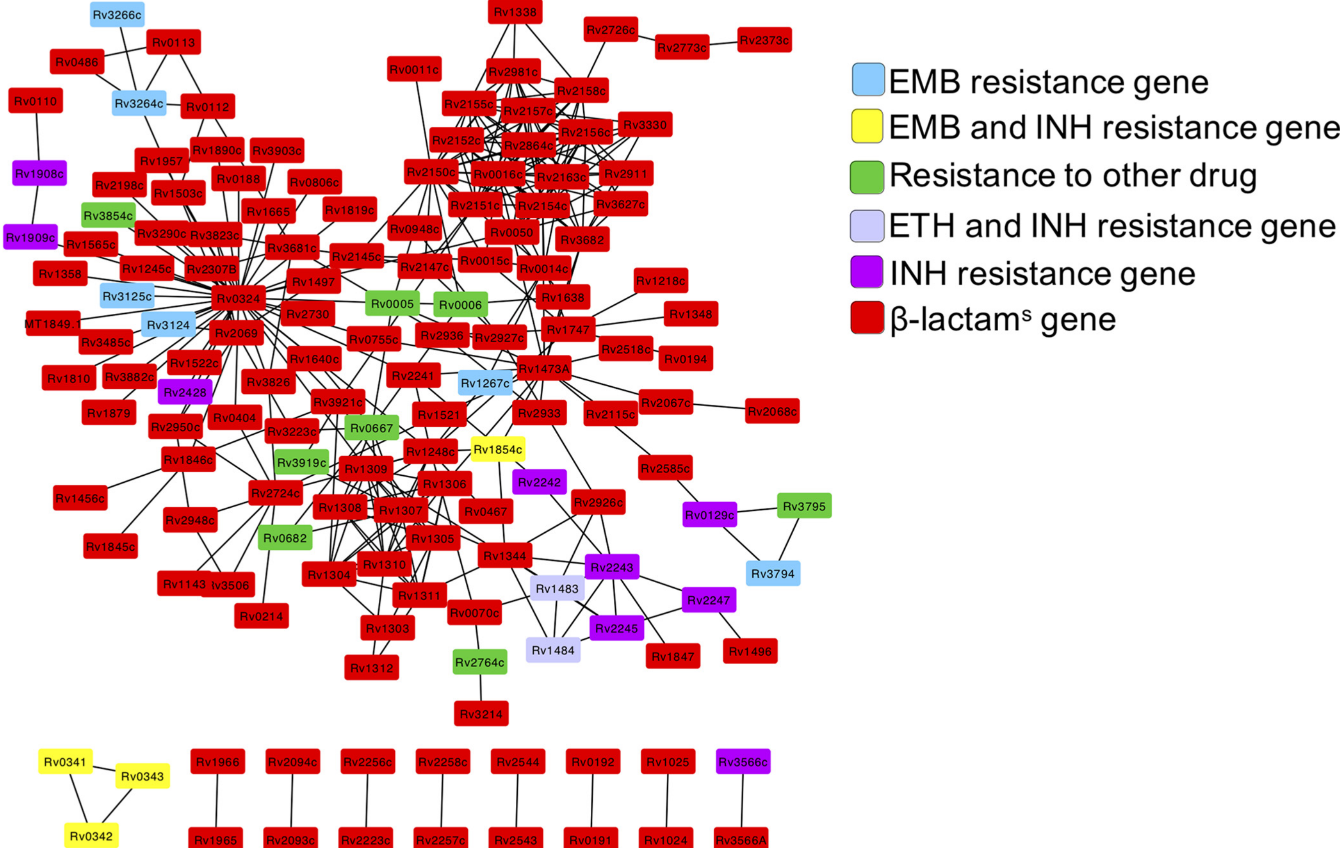

FIG 4 Network of interactions between β-lactam sensitivity (β-lactam^S) and drug resistance (DR) genes/proteins. β-Lactam^S genes/proteins tend to be located toward the core of the network, connecting distinct subgroups of DR genes. The network shown is a combination of the protein-protein interaction and the gene regulatory networks. EMB, ethambutol; INH, isoniazid; ETH, ethionamide.

information flow, from DR nodes to β-lactam^s nodes and vice versa. We ranked the access times between all pairs of nodes in each database separately and used bivariate Spearman's ρ to calculate the concordance of edges with similar access times (38, 39) (see Fig. S4). We found that consistency between the PPI and GR networks occurred in the top 29.65% of edges, with the smallest access times in the β-lactam^s-to-DR node direction and just 9.46% of those in the DR-to-β-lactam^s node direction. Similar results were obtained when we repeated the analysis with only the high-confidence β-lactam^s nodes, with the consistency in the β-lactam^s-to-DR node direction being 31.09% and no consistency identified in the DR-to-β-lactam^s node direction. This result is consistent with the notion that PPI networks and GR networks represent different types of associations between genes and proteins. Therefore, we used PPI and GR networks separately for subsequent random walk analyses.

Ranking pairs of β-lactam^s and DR nodes by their access times revealed discrete sets of node pairs with similar influence (Fig. 5A to D), consistent with the modular organization of the PPI and GR networks (40). We selected the set of node pairs with the smallest access times based on their distribution (red lines define threshold for each case): 30 and 35 pairs were identified from the PPI and GR networks, respectively, in the DR-to-β-lactam^s direction and 98 and 55 pairs, respectively, in the opposite direction (see Table S5). These node sets represent pairs of β-lactam^s and DR genes/proteins that are likely to modulate or influence each other's activity.

To ensure that the set of gene pairs with smallest access times represented biologically meaningful associations, we compared the coexpression of DR-β-lactam^s gene pairs with the smallest access times to DR-β-lactam^s gene pairs with higher access times (Fig. 5E). The distribution of coexpression of DR-β-lactam^s gene pairs with the

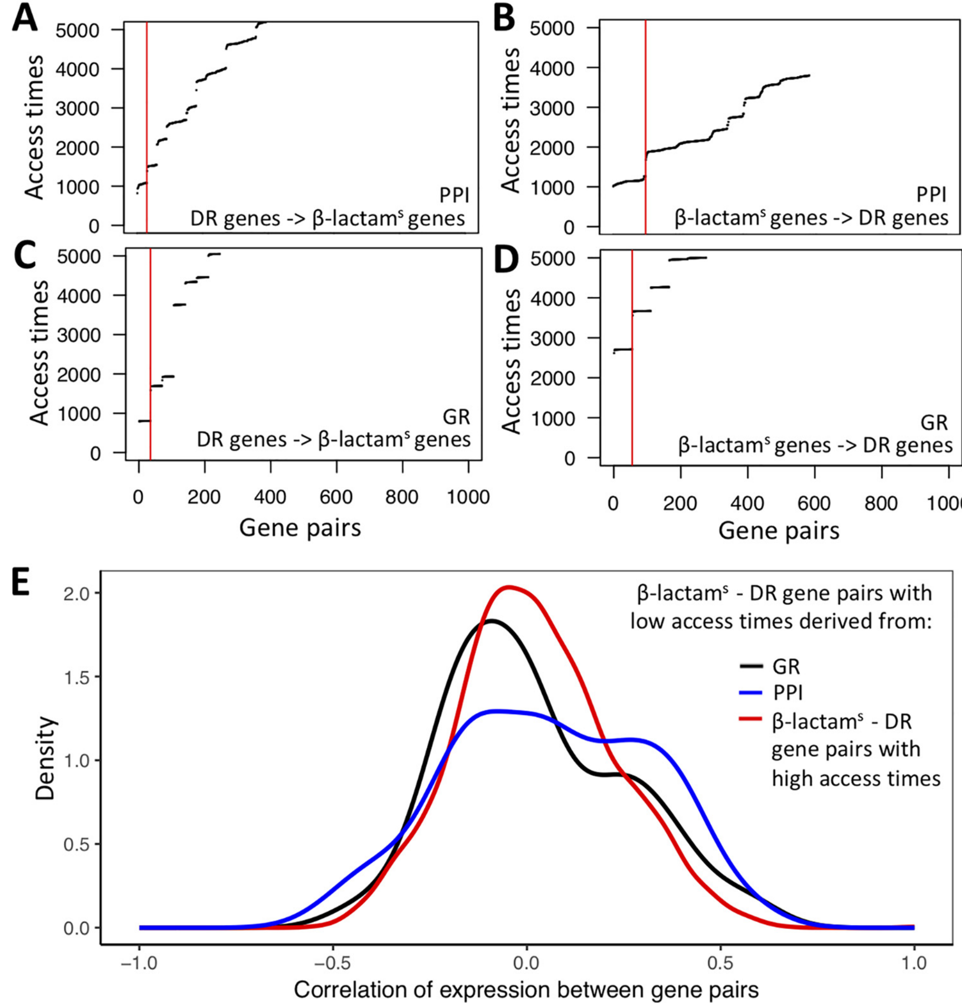

FIG 5 Highly influential pairs of β-lactam sensitivity (β-lactam^s) and drug resistance (DR) nodes identified by random walks in the networks. Access times for gene pairs in the protein-protein interaction (PPI) (A and B) and gene regulatory (GR) (C and D) networks in the DR→β-lacam^s direction (A and C) and β-lactam^s→DR direction (B and D). Due to a modular network structure, discrete clusters of highly influential pairs are identified. The set of genes with smallest access times (high influence between each other) to the left of the vertical red lines were used in the subsequent analysis. (E) Strength of coexpression between DR-β-lactam^s node pairs. DR-β-lactam^s node pairs with lowest access times are more strongly correlated than pairs of genes with higher access times (wider distribution).

smallest access times was wider than the reference distribution (Kolmogorov-Smirnov test $P = 0.016$ for the pairs derived from the PPI network and $P = 0.021$ for the pairs identified in the GR network), with a similar trend for β-lactam^s-DR gene pairs ($P = 0.063$ in the PPI network and $P = 0.08$ in the GR network). This indicates stronger magnitudes of coexpression between gene pairs with smaller access times.

Examination of DR-β-lactam^s gene pairs with the smallest access times revealed two key nodes in the paths of information flow. All small access time pairs derived from the PPI network were centered around AtpH (encoded by *atpH*, *Rv1307*), and those derived from the GR network were centered around WhiB4 (encoded by *Rv3681c*). *atpH* is transcriptionally regulated by BlaI (30); hence, this result once more implicates *blaI* and its transcriptional network in *M. tuberculosis* β-lactam collateral sensitivity. WhiB4 is a transcription factor involved in the regulation of a large number of PE/PPE genes (41) and has been implicated in the response to β-lactam/β-lactam inhibitor combination efficacy in *M. tuberculosis* through its role in modulating internal redox potential (23).

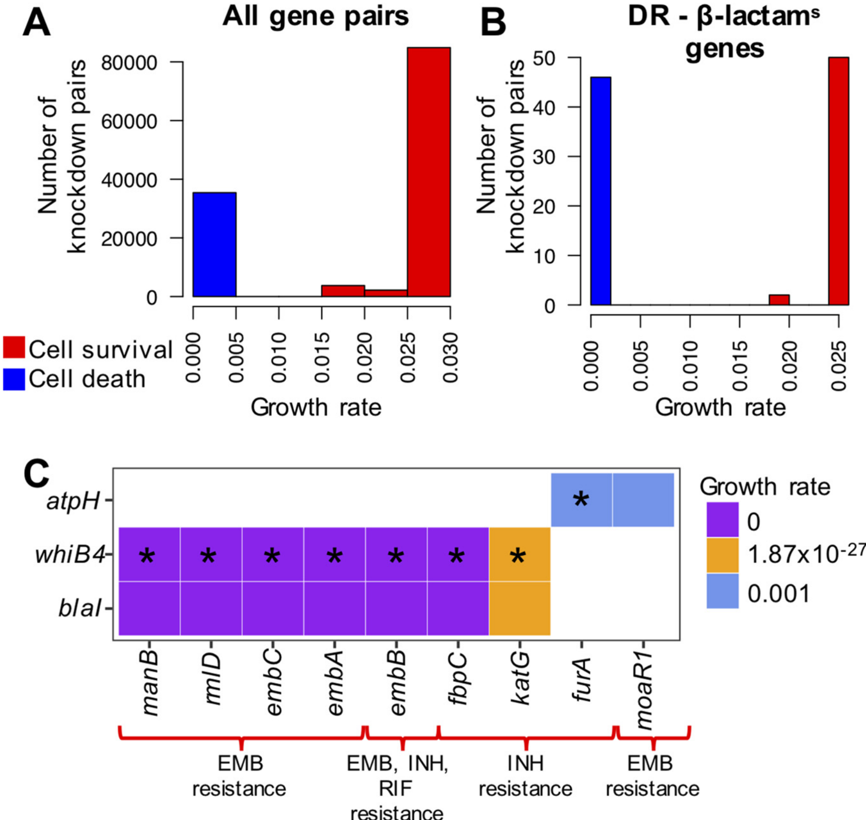

FIG 6 *In silico* double knockout of drug resistance (DR) and β-lactam sensitivity (β-lactam^s) gene pairs reduces *M. tuberculosis* growth rate. Effect on the growth of *M. tuberculosis* after *in silico* knockout of all gene pairs (A) or DR-β-lactam^s gene pairs (B). DR-β-lactam^s gene pairs are enriched in those that lead to lethality (growth rate < 0.010) after knockout (Fisher test $P = 7.00 \times 10^{-5}$). Knockouts resulting in cells with a growth rate of <0.010 were considered lethal (blue), and above this cutoff, nonlethal (red). (C). Growth rate of pairs of β-lactam^s and DR genes after double knockouts. The knockouts of *atpH*, *whiB4*, and *blaI* together with DR genes implicated in the resistance to commonly used anti-TB drugs (e.g., EMB and INH) led to cell death. Gene pairs identified by random walk network analyses as being highly influential pairs are indicated with an asterisk. EMB, ethambutol; INH, isoniazid; RIF, rifampicin.

***In silico* functional validation of a dependence mechanism between β-lactam^s and DR gene pairs.** We sought to validate the functional association between β-lactam^s and DR genes/proteins by exploring their role in cell growth. We simulated the growth effects of β-lactam^s and DR gene pair knockouts using an *in silico* regulatory model that incorporates both transcriptional data and metabolic modeling (42). We found that simultaneous knockout of DR and β-lactam^s gene pairs caused a marked reduction in growth rate (growth rate < 0.010) or resulted in cell death more often than expected by chance (Fig. 6A and B) (88.46% β-lactam^s and DR gene pairs compared to only 39.0% in other pairs, Fisher exact test $P = 7.00 \times 10^{-5}$), suggesting synthetic lethality and functional dependency between these genes. Of note, we found synthetic lethality between each of the *whiB4*, *blaI*, and *atpH* genes with the key DR genes *embB*, *katG*, and *furA* (Fig. 6C).

DISCUSSION

In this work, we applied a novel combination of systems biology approaches to investigate *M. tuberculosis* β-lactam collateral sensitivity. We combined gene expression and network analyses and show that the inhibitor of intrinsic β-lactam resistance, *blaI*, is activated after treatment with classical anti-TB drugs (e.g., isoniazid, rifampicin, amikacin, streptomycin, levofloxacin, ofloxacin, ethambutol, ethionamide, and pyrazinamide). Two genes transcriptionally regulated by *blaI*, *atpH* and *sigC* (30), as well as

Rv1884c (*rpfC*), whose knockout mutants suffer increased sensitivity to β-lactams (31), were also upregulated. These findings are concordant with a model whereby classical anti-TB treatment drives cells toward a loss of β-lactam resistance, consistent with previous reports that drug-resistant *M. tuberculosis* strains were more likely to be susceptible to β-lactams (18, 19). Our gene expression analyses showed that DR and β-lactam^s genes were coexpressed, and our network analyses (transcription factor-target associations and PPI network analysis) indicate that this coexpression may result from a tight coregulatory association between DR and β-lactam^s genes. However, further experimental evidence would be needed to confirm the latter result, such as investigating the activity of *blaC* in multidrug-resistant and extensive drug-resistant strains. While one previous study indicated that β-lactamase activity was qualitatively conserved among 10 *M. tuberculosis* strains with various amoxicillin/clavulanate MICs (18), it remains unclear if there are quantitative differences in expression that may impact susceptibility.

Previous studies have demonstrated the utility of random walks across networks to identify putative treatment cotargets for *M. tuberculosis* (24). Here, we applied random walks to identify key mediators of the communication between DR and β-lactam^s genes and identified *atpH* and *sigC* as key regulators. In addition, *in silico* growth models revealed synthetic lethality after simultaneous knockout of either *blaI*, *atpH*, or *sigC* in combination with the genes conferring resistance to isoniazid, ethambutol, or rifampicin, further supporting a functional association between these gene classes.

The analysis conducted in this work primarily focused on a set of 63 high-confidence β-lactam^s genes, i.e., those with experimental evidence demonstrating an association with β-lactam sensitivity in *M. tuberculosis*. To ensure that our results were robust to changes in this set of genes, we repeated all analyses using an extended list of 199 β-lactam^s-associated genes, including genes that were associated only through computational approaches (see Table S6 in the supplemental material). We find that all trends remain consistent, providing greater confidence that our analyses are robust to changes in the underlying list of genes.

Studies have shown that pathways regulating cell wall formation and β-lactam-associated genes are affected by stress in *M. tuberculosis* (43, 44), which would suggest that the induction of β-lactam^s gene expression and the subsequent acquisition of β-lactam susceptibility could be the result of a generic stress response. Our differential expression analysis did not reveal a preferential induction of β-lactam^s genes under acidic stress. In contrast, drug treatment was a more specific inducer of β-lactam^s genes, with 18.52% of induced genes being β-lactam^s genes. This result is consistent with the tight regulatory links between β-lactam^s and DR genes implicated by our network permutation and random walk analyses and *in silico* growth simulations. However, other types of stress, such as that induced by reactive oxygen species and reactive nitrogen species and macrophage stress, should also be investigated to rule out their role in the upregulation of β-lactam^s genes, and studies have reported regulation of β-lactam genes by the oxidant cumene hydroperoxide (23). Although we cannot exclude that some sources of stress might induce a response that modulates β-lactam susceptibility due to its effect on pathways regulating cell wall formation, our results as a whole suggest a potentially direct and specific rather than passive association between drug treatment and the activation of β-lactam^s genes.

Our *in silico* study of knockouts of gene pairs suggested synthetic lethality and functional dependency between β-lactam^s and DR gene pairs, specifically between each of the *whiB4*, *blaI*, and *atpH* genes with the key DR genes *embB*, *katG*, and *furA*. The effect of *whiB4* mutations and gene expression alterations has been studied in the context of single-gene knockouts or overexpression of *whiB4* (23, 45–47), suggesting a modulatory role in response to classical TB antibiotics. However, experimental studies are required to investigate the effect of double knockouts involving β-lactam^s and DR genes, which may be otherwise difficult to predict on the basis of single-gene knockout data.

Our findings are consistent with a model of collateral β-lactam sensitivity in classical drug-resistant *M. tuberculosis*, involving a concerted effect of multiple genes. Others have also recently found that collateral sensitivity to β-lactams, mainly penicillins, develops in *M. tuberculosis* strains evolved *in vitro* to be resistant to the classical drug class aminoglycosides (20). Our results suggest that *blaI*, together with its downstream targets, *atpH* and *sigC*, is a key regulator of collateral sensitivity resulting from classical drug resistance, although we were not able to detect a direct effect on transcription of the *blaC* β-lactamase gene in these data. Nevertheless, our evidence supporting a strong transcriptional wiring between β-lactam^s genes and DR genes suggests a tight coevolutionary relationship, likely due in part to functional similarities between the genes, many of which are implicated in resistance to drugs that target *M. tuberculosis* cell wall biosynthesis, e.g., ethambutol and isoniazid (48). Thus, collateral sensitivity to β-lactams may represent a functional evolutionary trade-off to classical drug resistance.

The development of bacterial drug resistance is often accompanied by a fitness cost (49), which in some cases can be overcome by compensatory mechanisms. We speculate that β-lactam sensitivity arises in *M. tuberculosis* as a compensatory mechanism to regain fitness after disruption of the molecular network of *M. tuberculosis* due to the evolution of classical drug resistance. Indeed, genes associated with sensitivity to β-lactams (e.g., *murE*, *ponA1*, *murD*, *Rv2752c*, and *Rv1218c*) have been identified as being under convergent evolution in drug-resistant *M. tuberculosis* or harboring compensatory mutations (50–52). Although most studies have associated compensatory mechanisms with mutations (50–52), our results suggest that transcriptional changes might also be playing a role, e.g., the upregulation of *blaI*. This assertion is consistent with a recent report showing that gene expression changes were associated with an increased fitness in *M. tuberculosis* that had developed resistance to rifampicin, isoniazid, streptomycin, fluoroquinolone, ethionamide, and amikacin during a single patient infection (53).

Taken together, our findings support a potential role for β-lactam therapy in patients with classical drug-resistant TB to delay and/or prevent the development of resistance. Previous *in vitro* studies have demonstrated anti-TB activity for certain β-lactam plus β-lactamase inhibitor combinations (10) and other drugs (54). However, mixed success in the clinic (15, 16, 55) suggests that treatment effectiveness might be dependent on other factors, such as the genetic background of the *M. tuberculosis* strain. Consequently, it will be essential to continue to develop our understanding of this phenomenon using a combination of bioinformatic and experimental approaches, such that we can readily identify *M. tuberculosis* strains and therefore patients for whom β-lactam therapy may be appropriate.

MATERIALS AND METHODS

Genes associated with β-lactam sensitivity. A list of 199 genes putatively associated with β-lactam sensitivity in *M. tuberculosis*, and two closely related species, *M. smegmatis* and *M. bovis*, was obtained from multiple sources (17, 18, 23, 30, 31, 56–76). An initial set of 110 genes as reported in reference 18 was extended to 199 by further literature searches (see Table S1 in the supplemental material). These included a diverse set of discovery approaches (e.g., functional studies focused on single genes, high-throughput phenotypic screens, and computational analyses) with variable likelihood of spurious or false-positive results. To limit the potential influence of false-positive genes, we report separate analyses for all 199 genes and a subset of 63 genes with the highest confidence evidence (i.e., which included canonical genes such as *blaI*, *bla*, and *atpA-G* that were shown to have β-lactamase activity, are downstream of *blaI*, or were identified through functional assays as being associated with sensitivity to β-lactams). A full list of genes used in this study can be found in Table S1 (genes in the high-confidence subset are marked).

Genes implicated in resistance to classical TB drugs. We compiled a list of 40 genes implicated in classical TB drug resistance (here termed DR genes) from The Tuberculosis Drug Resistance Mutation Database (81) (Table S2). These included genes associated with resistance to rifampicin (RIF; $n = 2$), isoniazid (INH; $n = 22$), aminoglycosides (AMI; kanamycin, capreomycin, amikacin, and viomycin, $n = 2$), streptomycin (SM; $n = 3$), fluoroquinolones (FLQs; $n = 2$), ethambutol (EMB; $n = 13$), ethionamide (ETH; $n = 3$), *para*-aminosalicylic acid (PAS; $n = 1$), and pyrazinamide (PZA; $n = 1$).

Expression data. *M. tuberculosis* microarray gene expression data were obtained from sample series GSE1642 (26) from the NCBI GEO database. Data were available for *M. tuberculosis* exposed to 437 treatments, including the following *in vitro* treatment conditions: classical TB drugs as single agents

(isoniazid, rifampin, amikacin, streptomycin, levofloxacin, ofloxacin, ethambutol, ethionamide, and pyrazinamide) and control conditions (7H9-based growth medium without drug treatment).

We assessed the impact of classical drug treatment by comparing the variance of expression of β-lactam^s genes to that across all genes. Significance testing was performed by comparison to the null distribution generated by random subsampling of *M. tuberculosis* genes ($n = 111$ genes with 10,000 replicates) and counting the number of times we obtained a variance equal to or greater than the observed value. Differential expression analysis was performed using limma (27), where differential expression was considered significant if the *q* value (i.e., a *P* value that has been adjusted for the false-discovery rate [FDR] considering multiple testing) was <0.05 and $|\text{fold change}|$ was >2.

To compare the strength of correlation of expression of DR genes with β-lactam^s genes, we exhaustively calculated Spearman's ρ between the expression of each of the individual genes, generating (i) a distribution of correlations of each individual DR gene with all β-lactam^s genes, and (ii) a distribution of DR genes with all other genes. We then used the upper quantile of the correlation magnitude (absolute value of the correlation of expression) of each of these distributions to summarize the differences in the distribution of the strength of correlation magnitude, therefore comparing the most strongly correlated DR genes and β-lactam^s genes with the most strongly correlated DR genes and non-β-lactam^s genes.

***M. tuberculosis* molecular interaction networks.** We integrated molecular interaction networks from two sources: protein-protein interactions (PPIs) (22,308 interactions) from the STRING database (32) and transcription factor-target interactions experimentally obtained using chromatin immunoprecipitations (33) as a gene regulatory (GR) network (15,054 interactions). Note that although the STRING database has traditionally been considered to be solely composed of PPIs, there are a number of regulatory interactions supported by gene coexpression analysis (32). Only high-confidence edges were analyzed: PPIs with a weight greater than 700 (the cutoff suggested by STRING as being of high confidence) and statistically significant transcription factor-target gene interactions (as defined by reference 33) were considered. The power-law distribution of the combined network PPI and GR was verified using igraph (77) to ensure its biological plausibility. Network visualizations were obtained using Cytoscape v3.4.0 (78).

Significance of interactions between β-lactam^s and DR nodes (genes/proteins) in the molecular interaction network. The degree of cross talk, N_{AB}, between two gene sets, *A* and *B* (e.g., β-lactam^s and DR genes), expected under a random model of a given network was modeled using the hypergeometric distribution $N_{AB} \approx \text{hypergeometric}(N, K, n)$, where *n*, *K*, and *N* are the numbers of edges in gene set *A*, gene set *B*, and the entire network (35). A one-sided *P* value can be calculated as the probability of observing at least the observed number of interactions under this random model. Significance was corrected using the Benjamini-Hochberg method (and hence is reported as *q* values).

Random network walks to identify β-lactam^s nodes influenced by DR nodes. We performed random walks between all pairs of nodes in the PPI and the GR networks separately to determine the access times as an indicator for the influence of one node over another. Simulating the random walk was unnecessary, as the access time on a finite graph has an analytical solution (79) computed via eigenvalue decomposition of the edge matrices of the networks.

To assess the similarity of the access times obtained with the PPI (32) and the GR networks (33), we investigated the stability using a multivariate extension of Spearman's ρ (38, 39). This allows us to assess the similarity of the top-*k* access times and determine if there is a set of stable edges with low access time.

We selected pairs of nodes comprising one β-lactam^s node and one DR node and narrowed down sets of pairs with small access times in either the PPI or GR network. Given the nonsymmetry of access times obtained with random walks (the access time from A to B is not equal to that from B to A), we considered the results obtained in both directions independently. Cutoffs were determined from the empirical distribution: 1,054.74 for the PPI in the DR gene$\rightarrow\beta$-lactam^s gene direction, 1,336.58 for the β-lactam^s gene$\rightarrow$DR gene direction, 1,713.37 for the GR network in the DR gene$\rightarrow\beta$-lactam^s gene direction, and 2,741.49 in the β-lactam^s gene$\rightarrow$DR gene direction.

Simulating the effect on bacterial growth of double knockout β-lactam^s plus DR gene mutants. To identify pairs of β-lactam^s and DR genes whose knockout would have the largest effect on the growth of *M. tuberculosis*, we performed simulations using the iSM810 model of *M. tuberculosis* with the PROM framework (42) and the COBRA toolbox (80), which incorporate both gene-regulatory and metabolic processes to predict growth rates after double knockdown simulations. As input, we used the GR network (33) and expression data described above (26).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, DOCX file, 0.2 MB.
FIG S2, DOCX file, 0.2 MB.
FIG S3, DOCX file, 0.3 MB.
FIG S4, DOCX file, 0.1 MB.
TABLE S1, DOCX file, 0.1 MB.
TABLE S2, DOCX file, 0.1 MB.
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TABLE S4, DOCX file, 0.1 MB.

TABLE S5, DOCX file, 0.1 MB.
TABLE S6, DOCX file, 0.1 MB.

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A.S.T. conceived of, designed, and performed research, analyzed data, and wrote the manuscript. B.W.G. provided the computational framework, analyzed data, supervised research, and edited the manuscript. J.B. provided the computational and statistical framework for network analyses and edited the manuscript. T.C.C. and N.G.F. supervised the research. K.L.W. conceived of and supervised the research and edited the manuscript.

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