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# Transcriptomic responses to antibiotic exposure in *Mycobacterium tuberculosis*

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**ABSTRACT** Transcriptional responses in bacteria following antibiotic exposure offer insights into antibiotic mechanism of action, bacterial responses, and characterization of antimicrobial resistance. We aimed to define the transcriptional antibiotic response (TAR) in *Mycobacterium tuberculosis* (Mtb) isolates for clinically relevant drugs by pooling and analyzing Mtb microarray and RNA-seq data sets. We generated 99 antibiotic transcription profiles across 17 antibiotics, with 76% of profiles generated using 3–24 hours of antibiotic exposure and 49% within one doubling of the WHO antibiotic critical concentration. TAR genes were time-dependent, and largely specific to the antibiotic mechanism of action. TAR signatures performed well at predicting antibiotic exposure, with the area under the receiver operating curve (AUC) ranging from 0.84-1.00 (TAR <6 hours of antibiotic exposure) and 0.76–1.00 (>6 hours of antibiotic exposure) for upregulated genes and 0.57–0.90 and 0.87–1.00, respectfully, for downregulated genes. This work desmonstrates that transcriptomics allows for the assessment of antibiotic activity in Mtb within 6 hours of exposure.

**KEYWORDS** *Mycobacterium tuberculosis*, antibiotic response, transcriptomics, novel diagnostics

The early diagnosis of tuberculosis (TB) and universal drug-susceptibility testing are essential components of the WHO's END-TB strategy (1). Molecular diagnostics that rely on detecting targeted antibiotic resistance mutations in the *Mycobacterium tuberculosis* (Mtb) genome have been rapidly integrated into national TB programs and are currently standard-of-care (2–5). However, commercially available molecular assays test a limited number of drugs and have yet to replace the traditional slow and costly process of growth-based phenotypic drug susceptibility testing (pDST). Pathogen whole-genome sequencing (WGS) is a promising replacement for pDST and is currently performed on culture-positive samples in several high-resource clinical laboratories (6). The diagnostic accuracy of WGS is excellent for drugs like isoniazid (INH) and rifampicin but still lags behind phenotypic methods for other key drugs like pyrazinamide and the novel/repurposed TB drugs (7–9).

Like all cells, bacteria, including Mtb, demonstrate transcriptional changes in response to environmental stressors such as hypoxia or pH changes and chemical pressure like antibiotic exposure (10–15). Compared with *Escherichia coli*, *Mycobacteria* are notable for an RNA-polymerase that forms unstable open promoter complexes that appear more easily prone to regulation by repressors or stimulating factors (16). At the same time, Mtb demonstrates a longer mRNA half-life on average (9.5 min) compared with *E. coli* (3–8 min) (17). Whereas this may delay the observation of transcriptional changes after they occur, especially for abundant transcripts, the delay will be in the order of minutes rather than hours. Hence, despite Mtb's substantially slower growth *in vitro* (doubling

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The authors declare no conflict of interest.

See the funding table on p. 14.

**Received** 14 September 2023
**Accepted** 6 March 2024
**Published** 8 April 2024

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time ~16–22 hours) than *E. coli* (doubling time ~20 min), its transcriptional response offers a much more rapid window than growth inhibition to predict antibiotic response.

A major use of transcriptomics in bacteria has been to characterize the mechanism of action of drugs, study the impact of resistance mutations, and predict the response of individual and antibiotic combinations (14, 18–25). The transcriptomic antibiotic response (TAR) may result from the direct antibiotic effect on a specific set of genes or pathways or reflect complex or global stress response (13, 15). The TAR of antibiotic-sensitive bacteria differs from the TAR seen in antibiotic-resistant bacteria and can distinguish between clinical strains of antibiotic-sensitive and antibiotic-resistant *E. coli*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and Mtb and is independent of the specific DNA mutation encoding resistance to a given drug (15, 26, 27). Barczak et al. demonstrated that the TAR observed in susceptible Mtb isolates differs significantly from that in isolates resistant to ciprofloxacin, streptomycin, and isoniazid (26). For Mtb, the isoniazid TAR was able to differentiate between low-level isoniazid resistance due to mutations in the *inhA* promoter vs high-level isoniazid resistance mutations in the *katG* mutant (26).

Developing transcription-based antimicrobial susceptibility testing for patient care will require the identification of TAR profiles for a wider range of antibiotics, and the evaluation of the effect of antibiotic concentration, and duration of antibiotic exposure (27). In this work, we aimed to define antibiotic response signatures for 17 clinically important tuberculosis drugs by pooling publicly available *M. tuberculosis* transcriptomic data. We also aimed to study the extent of shared transcriptional responses following antibiotic exposure between and across drugs and determine the performance of the TAR signatures for differentiating antibiotic exposed from unexposed *M. tuberculosis*.

## MATERIALS AND METHODS

### Search strategy

We searched NCBI's Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) and the European Nucleotide Archive (ENA) using the term "Mtb" and identified microarray and RNA sequencing (RNA-seq) studies' data sets available until 30 June 2022 for further review. We included studies that used strains grown in liquid mycobacterial culture media (Middlebrook 7H9 media) and used both antibiotic-exposed and antibiotic-unexposed conditions. We included all antibiotic concentrations and durations of culture. We excluded studies done using strains grown on solid culture media (Lowenstein-Jensen or Middlebrook agar), *in vivo* studies with cell lines or mouse studies, experiments that used knockout strains or stress conditions (such as hypoxia, low pH, or UV light), and those that used experimental drugs or drugs not in current clinical use for TB treatment. We were made aware of unpublished data meeting our inclusion criteria shared before publication by collaborators that we included in this analysis. These data have now been uploaded to NCBI under PRJNA932181.

### Metadata curation

We identified the Gene Expression omnibus Series (GSE) accession ID of studies of interest and downloaded the metadata for each study (Table S1). We extracted sample details including accession ID, Mtb strain, antibiotic, the concentration of antibiotic, duration of culture, and two-channel vs one-channel microarray or single-end or pair-end RNA-seq data. Where necessary, we converted the antibiotic concentration from moles to micrograms per milliliter (µg/mL). For each sample, we calculated the ratio of the antibiotic concentration used for that isolate to the WHO critical concentration. We used this metadata to create antibiotic or control conditions, with each condition a unique permutation of strain, antibiotic (or control), antibiotic concentration, and culture duration. These terms have been defined in the glossary (please see supplementary text).



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## Annotation

We used the H37Rv reference sequence NC_000962.3 for annotation. For CDC1551 strains, we used the H37Rv CDC1551 correspondence table in the metadata of GSE1642 to convert CDC1551 locus tags to H37Rv locus tags. For those CDC1551 locus tags that mapped to multiple H37Rv locus tags or *vice versa*, we retained the CDC1551 locus tags. Transcriptomic data from clinical strains were aligned against H37Rv. We excluded 384 genes coding for PE/PPE genes, repetitive regions, insertion sequences, and phage-related sequences (28).

## Bioinformatics pipelines and generation of transcription profiles

All analyses were performed using R (v4.1.0) (29) and RStudio (v1.4.1717) (30) with tidyverse (v1.3.1) (31). Transcriptomic data are generated from biological replicates of a specific experimental condition or state (32). Downstream analysis requires aggregation of data from biological replicates, and differentially expressed genes are identified by comparing transcriptomic data from all biological replicates of two different conditions (32). We analyzed each study independently, using separate bioinformatics pipelines for microarray and RNA-seq data to generate antibiotic and control transcription profiles (TPs) as detailed in the supplement. As data from individual samples cannot be used for downstream analysis or model building, the TP was used for all further analyses. We defined the antibiotic TP as all differentially expressed genes (at a false-discovery rate cutoff of 0.1) obtained from the comparison of all biological replicates of a specific strain of Mtb exposed to a particular antibiotic concentration for a defined duration with transcriptomic data obtained from multiple biological replicates of same strain incubated in culture without antibiotic exposure for identical duration. We generated two types of control TPs to contrast with antibiotic TPs. The first was differential expression from baseline (time zero) comparison of a strain of Mtb exposed to antibiotic vs those not exposed to antibiotic that we expect reflects noise in inoculum size. The second control TP was generated by identifying differential expression in antibiotic unexposed strains from two different time points that we expect reflect the temporal effect of growth in culture.

## Microarray analysis

For microarray analysis, we used R packages GEOquery (v2.6.0) (33) and Limma (v3.48.3) (34). Processed microarray data from one-channel and two-channel array experiments were downloaded with GEOQuery. Probe intensities were converted to log2fold, and the mean intensity for genes with multiple probes was used to create a single value for each gene. After removing the genes outlined above, Limma was used to perform quantile normalization across all arrays, followed by differential expression analysis to generate antibiotic and control transcription profiles.

## RNA-seq analysis

GSE and SRA accession IDs of identified studies were used to download metadata from SRA explorer (https://sra-explorer.info/); FASTQ files were downloaded from the ENA, and md5sums were checked. FASTQC (v0.11.5) (35) and multiQC (v1.5) (36) were used for quality control. Reads were aligned to H37Rv with BWA (v0.7.15) (37); Samtools (v1.9) (38) was used to create sorted BAM files and calculate the percent of the genome covered. Sorted BAM files were analyzed using featureCounts from Subread (v2.0.3) (39) to count reads with separate flags for single-end and paired-end reads. Experiments with <80% of genome coverage were excluded. Antibiotic and control transcription profiles were generated using DESeq2 (v1.32.0) (40), with quality control and analysis workflow adapted from the bulk-RNA-seq analysis by the Harvard Chan Bioinformatic Core (41).



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### Transcription profile analysis and generation of transcriptional antibiotic response

TPs generated using single samples without replicates or those where the ratio of the antibiotic concentration to the WHO critical concentration (antibiotic concentration ratio) was under 0.5 were excluded. As the duration of antibiotic exposure was significantly associated with the number of differentially expressed genes in response to antibiotic exposure and because of the heterogeneity in the duration of culture across different studies, we categorized the duration of culture into <3 hours, 3–6 hours, 6–24 hours, and >24 hours. This would allow us to explore the impact of the duration of antibiotic exposure on gene expression. However, there were not enough TPs within each of these time categories (Table 1) to assess the ability to discriminate between antibiotic from control TPs using a logistic regression model. The <6 and >6 hours categories allowed us to develop such models. The antibiotic concentration ratio was categorized into 0.5–1, 1–2, 2–4, and >4 times the WHO critical concentration.

To control for the heterogeneity in log2fold values across transcriptomic technology and studies from different laboratories, we ranked genes within each transcription profile by the magnitude of log2fold expression, assigning the lowest rank to the gene with the highest log2fold value. The transcription profiles for an antibiotic within each time category were used to calculate the median rank for each gene across those profiles, and the genes with the top 10 median ranks were selected to create the transcriptional antibiotic response (TAR). This was done separately for upregulated and downregulated genes. We chose 10 genes to develop the TAR based on previous studies that have used transcriptomics-based susceptibility testing methods (26, 27).

On TARs across antibiotics and time categories, we noted certain genes to be common to antibiotics with similar mechanisms of action across multiple time categories or common to multiple antibiotics with different mechanisms of action within the same time category. To explore this further, we calculated the median rank for specific combinations of antibiotics and genes across multiple time categories to determine temporal changes related to the mechanism of action for these antibiotic combinations. Heatmaps were created using R package pheatmap (v1.012) for all antibiotics within each time category. After assigning the value 0 to genes that were not differentially expressed, the median log2fold value for each gene for each antibiotic within a time category was calculated. The median log2fold value was obtained for upregulated and downregulated TARs for each antibiotic within a time category; genes that were significantly expressed in other antibiotics (but not within the TAR or not differentially expressed for that antibiotic) were also included.

### Statistical analysis

We compared the number of genes differentially expressed between microarray and RNA-seq data for antibiotic and control TPs using *t* tests. We used linear regression to determine the relationship between the number of genes differentially expressed as a function of the duration of antibiotic exposure, adjusting for the transcriptomic technology used (microarray or RNA-seq), study (batch effects), antibiotic, and antibiotic concentration. We repeated the analysis for control TPs.

For antibiotics with four or more TPs within a time category, we determined the classification accuracy of the TAR signature for that time category to distinguish between antibiotic TPs within that category and all control TPs. We filtered the antibiotic and control TPs to include only log2fold values of the 10 selected TAR genes for simplicity and due to the differences in the number of TPs, the proportion of genes differentially expressed, as well as the magnitude of log2fold values between studies. We performed a random 75%–25% split, used 75% of the data to train a logistic regression model, and tested the model classification accuracy on the remaining hold-out 25% of the data. The area under the receiver operating curve (AUC) as well as the sensitivity and specificity at the optimum decision threshold (Youden's J-statistic) was calculated using the R package pROC (42). We repeated this cross-validation procedure 10 times and calculated the



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**TABLE 1** TAR signature < 6 hours antibiotic exposure

| Antibiotic | Number of studies | | | Number of transcription profiles | | | Genes in TAR signature | |
|---|---|---|---|---|---|---|---|---|
| | Microarray | RNA-seq | Total | Microarray | RNA-seq | Total | Upregulated | Downregulated |
| Isoniazid | 3 | 3 | 6 | 5 | 4 | 9 | *iniB*, *iniA*, *accD6*, *acpM*, *fabD*, *kasA*, *kasB*, Rv1592c, *iniC*, and *efpA* | Rv0950c, *fbpB*, Rv0516c, Rv1885c, Rv2190c, *desA2*, Rv2189c, *desA1*, *rpfE*, and Rv0263c |
| Bedaquiline | 2 | 1 | 3 | 6 | 2 | 8 | *hsp*, *sigB*, *cydB*, Rv1461, Rv1462, Rv1463, *cydD*, *bkdB*, *csd*, and *cysD* | Rv3922c, Rv3921c, *hadC*, *trpA*, Rv2949c, *rnpA*, *rpsH*, *rplE*, Rv3920c, and *hadA* |
| Pyrazinamide | 1 | 1 | 2 | 5 | 1 | 6 | Rv3173c, Rv3531c, Rv1460, Rv0004, *cyp138*, Rv0791c, *clpB*, *hsp*, Rv0792c, and Rv0790c | *esxM*, *pks11*, Rv0531, *ahpD*, Rv2971, Rv1928c, *cysA3*, Rv0794c, *trxC*, and *kshB* |
| Clofazimine | 1 | 1 | 2 | 2 | 2 | 4 | Rv3161c, *furA*, *mbtC*, *mbtD*, *mbtB*, *mmpL5*, Rv0678, *mmpS5*, *mbtI*, and Rv1057 | *bfrB*, Rv2949c, *pks15*, *fadD29*, *narK1*, Rv2959c, Rv0057, Rv0892, Rv2275, and *def* |
| | | | | 18 | 9 | 27 | | |
| TAR signature > 6 hours antibiotic exposure | | | | | | | | |
| Isoniazid | 2 | 2 | 4 | 2 | 5 | 7 | *iniB*, Rv1057, *iniA*, *whiB6*, Rv3861, Rv1592c, *iniC*, *fadD14*, *cmtR*, and Rv2640c | Rv0950c, *fbpB*, *desA2*, *nuoL*, *desA1*, *nuoF*, *nuoA*, *nuoC*, *rpfE*, and *nuoD* |
| Bedaquiline | 1 | 1 | 2 | 3 | 4 | 7 | *mbtB*, *mbtC*, *hsp*, *mbtD*, *csd*, Rv1463, Rv1460, Rv1462, *gpdA1*, and *dnaE2* | *rpfC*, Rv1697, Rv0430, *hspX*, *mctB*, *rplR*, *rpmD*, *rpsE*, *rpsQ*, and *rpsN1* |
| Pyrazinamide | 2 | 1 | 3 | 3 | 2 | 5 | Rv1460, Rv0188, Rv0792c, *ahpC*, Rv0678, *ahpD*, Rv1894c, *ald*, Rv0791c, and *panB* | Rv0227c, Rv3352c, *fbpB*, *sdaA*, *rplJ*, *nuoA*, *atpB*, Rv2949c, *nuoD*, and *rpsN1* |
| Moxifloxacin | 1 | 1 | 2 | 1 | 4 | 5 | Rv3395c, Rv3074, Rv2719c, Rv0607, Rv1378c, Rv3394c, Rv3776, Rv3517, Rv1833c, and Rv2734 | *tgs1*, *ripA*, Rv2816c, Rv2030c, Rv1697, Rv1738, *fdxA*, Rv1478, *hrp1*, and Rv0312 |
| Pretomanid | 1 | 1 | 2 | 1 | 4 | 5 | *dnaE2*, *iniB*, Rv1378c, Rv3074, Rv3776, *iniA*, Rv3741c, Rv1460, Rv0068, and Rv0196 | *groES*, *tgs1*, *groEL2*, Rv2190c, Rv1815, *rplB*, Rv3922c, Rv0079, *cspA*, and *rpsS* |
| Linezolid | 0 | 2 | 2 | 0 | 5 | 5 | *rpfE*, *whiB7*, *whiB6*, Rv2451, *alkA*, Rv2415c, Rv0654, Rv0841, Rv0196, and Rv1258c | *tgs1*, Rv1738, *hspX*, *groEL2*, Rv3131, *fdxA*, *nuoL*, *nuoM*, *nuoH*, and Rv1752 |
| Clofazimine | 0 | 1 | 1 | 0 | 4 | 4 | *mbtI*, *mbtC*, *mbtB*, *mbtD*, *mbtJ*, *mbtE*, *mbtA*, Rv3402c, *hisE*, and *icl1* | *hspX*, Rv3131, *tgs1*, *hrp1*, Rv1738, Rv2030c, *fdxA*, *acg*, *nuoA*, and Rv0315 |
| | | | | 10 | 28 | 38 | | |
| TAR signature for antibiotics with four transcription profiles across all time categories | | | | | | | | |
| Streptomycin | 2 | 1 | 3 | 3 | 2 | 5 | Rv2190c, *lppU*, *sigB*, Rv0516c, Rv0813c, *pckA*, Rv0692, Rv2817c, Rv3127, and Rv1779c | *acpM*, *esxR*, *kasB*, Rv0324, *kasA*, *ethA*, *eccD3*, *pks16*, Rv0826, and *lprJ* |
| Delaminid | 0 | 2 | 2 | 0 | 5 | 5 | Rv2488c, Rv2490a, *ethA*, *ethR*, *lexA*, Rv3776, Rv2491, Rv3074, Rv1378c, and Rv3161c | *hspX*, *hrp1*, Rv2030c, Rv3129, TB31.7, *fdxA*, *tgs1*, *eis*, Rv1733c, and *rplW* |
| Rifampicin | 3 | 0 | 3 | 4 | 0 | 4 | Rv2669, *epiA*, Rv3204, Rv2372c, *citA*, Rv0089, *usfY*, *trcR*, Rv1691, and *papA5* | Rv1505c, Rv3403c, Rv2959c, Rv3651, *rpsK*, *acpM*, *espC*, Rv1987, Rv2633c, and Rv2147c |
| | | | | 7 | 7 | 14 | | |
| TAR signature for antibiotics less than four transcription profiles across all time categories; performance of signature not evaluated | | | | | | | | |
| Ethambutol[a] | 2 | 0 | 2 | 3 | 0 | 3 | *iniB*, *iniA*, *hsp*, *sigB*, *fadE24*, *cysK2*, Rv3354, *lpqS*, 35kd_ag, and *sigE* | Rv3269, *groES*, *groEL2*, Rv1021, *erg3*, *mce1B*, Rv1752, Rv1815, *nuoF*, and *fadE33* |

(*Continued on next page*)



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**TABLE 1** TAR signature < 6 hours antibiotic exposure (*Continued*)

| Antibiotic | Number of studies | | | Number of transcription profiles | | | Genes in TAR signature | |
|---|---|---|---|---|---|---|---|---|
| | Microarray | RNA-seq | Total | Microarray | RNA-seq | Total | Upregulated | Downregulated |
| Capreomycin[a] | 2 | 0 | 2 | 3 | 0 | 3 | *furA*, *higB*, *eis*, Rv2707, Rv2466c, *canA*, *rplU*, Rv1986, *lppU*, and *erm*(37) | *esxH*, *esxR*, *esxG*, *esxQ*, *groEL2*, *nuoM*, *atpC*, *esxS*, *nuoK*, and *atpD* |
| Ethionamide[a] | 2 | 0 | 2 | 3 | 0 | 3 | *iniB*, *acpM*, *fadE24*, *efpA*, *ahpC*, *kasB*, Rv1592c, *iniA*, Rv0885, and *kasA* | *fbpB*, Rv0947c, Rv0950c, Rv0516c, *rpfC*, *desA1*, *mce1B*, Rv2190c, *rpfE*, and *nuoH* |
| Kanamycin[b] | 1 | 0 | 1 | 1 | 0 | 1 | Rv1048c, Rv0990c, *hsp*, Rv0077c, Rv2516c, *whiB7*, *higB*, Rv0991c, MT1802, and *icl1* | Rv0600c, *narK1*, *glbN*, *lipF*, *papA1*, *nuoH*, MT1025.3, *lgt*, *oppD*, and *engA* |
| Amikacin[b] | 1 | 0 | 1 | 1 | 0 | 1 | *hsp*, *furA*, Rv2707, Rv2517c, *alkB*, *sthA*, *bfrB*, *rplY*, Rv2699c, and *higB* | *kasB*, *esxH*, *atpC*, *atpD*, Rv0247c, *esxS*, *esxR*, *qcrA*, *esxG*, and *nuoM* |
| Levofloxacin[b] | 1 | 0 | 1 | 1 | 0 | 1 | Rv3074, Rv3395c, Rv0184, *alkA*, Rv3642c, Rv3776, Rv1226c, Rv2024c, Rv1277, and Rv3645 | *esxR*, *esxH*, *esxQ*, Rv3094c, *esxG*, *esxS*, *atpC*, *hupB*, Rv2454c, and *whiB2* |
| Rifapentine[b] | 1 | 0 | 1 | 1 | 0 | 1 | *cdd*, *serC*, *moaB2*, *Rv1084*, *moaA1*, *relG*, *mbtM*, *ileS*, Rv2293c, and *mbtI* | *groES*, *vapB47*, *groEL2*, *esxM*, *fbpA*, *hupB*, *atpF*, *esxH*, Rv1871c, and Rv2840c |
| | | | | 13 | 0 | 13 | | |

[a]<4 transcription profiles across all time categories; TAR signature generated using profiles from all categories.
[b]Only one transcription profile.



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mean and the 95% CI (using the *t*-distribution) for the AUC, sensitivity, and specificity. We determined classification accuracy separately for upregulated and downregulated TARs, as well as for a model that combined upregulated and downregulated TARs.

## RESULTS

### Data curation and quality control

We identified 30 studies that met our inclusion criteria (Fig. 1; Table S1). We further excluded individual experiments that did not meet our criteria for experimental replicates or quality control criteria for microarray and RNA-seq data (Fig. 1, Supplementary text, Table S1). This left data from 562 experiments across 216 conditions (Fig. 1), of which 94% of experiments used laboratory reference strain H37Rv. Data from these experiments were used to generate 192 TPs. We excluded 18 TPs where the antibiotic concentration used was <0.5 times the WHO critical concentration for that antibiotic, 5 TPs for antibiotics not recommended for treatment of TB, and 13 TPs that were supported by only one experiment per condition (Fig. 1 and Supplementary text) (43, 44). After all exclusions, 99 antibiotic TPs and 57 control TPs from 24 studies were retained.

### Transcription profiles

Of the 99 antibiotic TPs across 17 antibiotics, 33 profiles were from first-line antibiotics, while 46 were from WHO Grades A or B drugs for treatment of multidrug-resistant tuberculosis (MDR-TB) (including eight pretomanid TPs) (Table 1) (45). The duration of exposure ranged from 30 min to 96 hours. Most TPs were generated from antibiotic exposure ranging from 3 to 6 hours (41%) or 6 to 24 hours (35%) (Table 1; Table S2 and S6; Fig. 2A and B). The experimental drug concentration (expressed as a ratio to the WHO critical concentration) ranged from 0.5 to 50 (Fig. 2A and C). Forty-nine profiles (49%) reflected antibiotic exposure within one doubling of the WHO critical concentration (drug concentration ratio 0.5–2, Table 1; Fig. 2C and D). We generated 57 control TPs: seven from time zero for either antibiotic exposure or unexposed inoculum and 50 from longitudinal experiments of antibiotic unexposed strains at two different time points ranging from 1 to 96 hours (Table S4). Tables S3 and S5 contain the antibiotic and control TPs, respectively.

The mean number of genes differentially expressed was lower for microarray than for RNA-seq for both antibiotic (1,227 vs 2,646, respectively; $P < 0.0001$) and control TPs (589 vs 1,888, respectively; $P < 0.0001$). Regression demonstrated that after controlling for the transcriptomic technology used, study/batch, antibiotic, and antibiotic concentration, a longer duration of exposure was associated with a larger number of differentially expressed genes ($P < 0.001$). Conditioning on the duration of exposure and the aforementioned experimental variables, we measured no significant effect of antibiotic

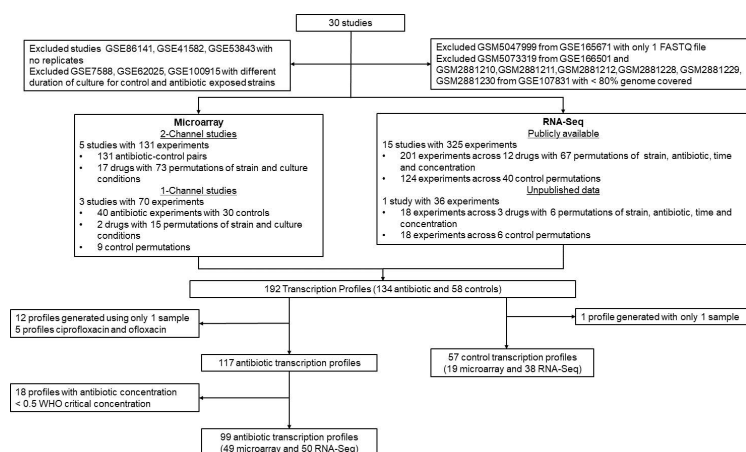


**FIG 1** Study flow diagram.



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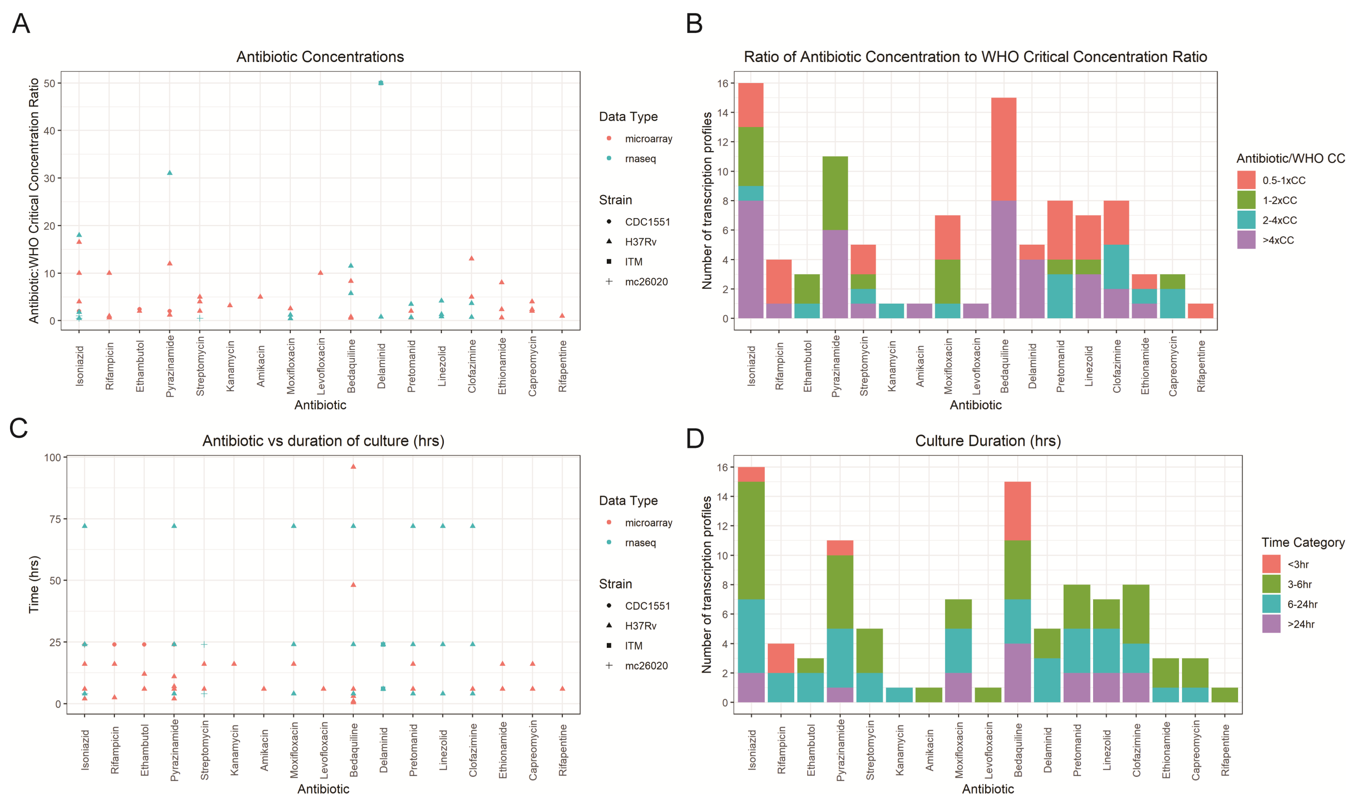


**FIG 2** Antibiotic TPs panel 2A: scatterplot of the duration of antibiotic exposure for TPs of each antibiotic, with different colors for microarray (orange) and RNA-seq (blue), and shape of the point representing different strains. Panel 2B: stacked bar chart of the count of TPs in each time category by antibiotic. Panel 2C: scatterplot of the ratio of antibiotic concentration to the WHO critical concentration for TPs of each antibiotic, with different colors for microarray (orange) and RNA-seq (blue), and shape of the point representing different strains. Panel 2D: stacked bar chart of the count of TPs within each category of the antibiotic concentration to the WHO critical concentration.

concentration on the number of differentially expressed genes within the range of evaluated concentrations ($P = 0.52$). After controlling for the study or batch and type of transcription technology used, the number of genes differentially expressed in control TPs was significantly associated with the time interval between the control conditions used to generate the TP ($P < 0.001$). The range of median log2fold values differed for microarray and RNA-seq in antibiotic (3.88 to −3.08 vs 6.06 to −4.43, respectively) but not control TPs (3.40 to −4.4 to vs 3.63 to −4.68).

## Transcriptional antibiotic response

We examined the top 10 most upregulated and the top 10 most downregulated genes based on the pooled median rank of the log2fold values within a time category for each antibiotic and considered this subset the TAR genes (Table S7). In Fig. 3, we report the timing of expression observed for some of the top transcripts and their known links to drug or drug resistance mechanisms. For brevity, we focus on genes differentially expressed in response to two or more anti-TB drug classes. A detailed description of genes shared across time categories for individual drugs is provided in the supplementary text, Table S7; Fig. S1 and S2. Table S6; Fig. S2 explore temporal changes in median ranks for various combinations of antibiotics and genes

### *Upregulated genes shared across drug classes or mechanisms*

The TAR gene upregulated in response to the largest number of drugs was the heat shock protein *hsp*, upregulated after 3–6 hours of exposure to pyrazinamide, bedaquiline, streptomycin, ethambutol, and amikacin and after 6–24 hours for



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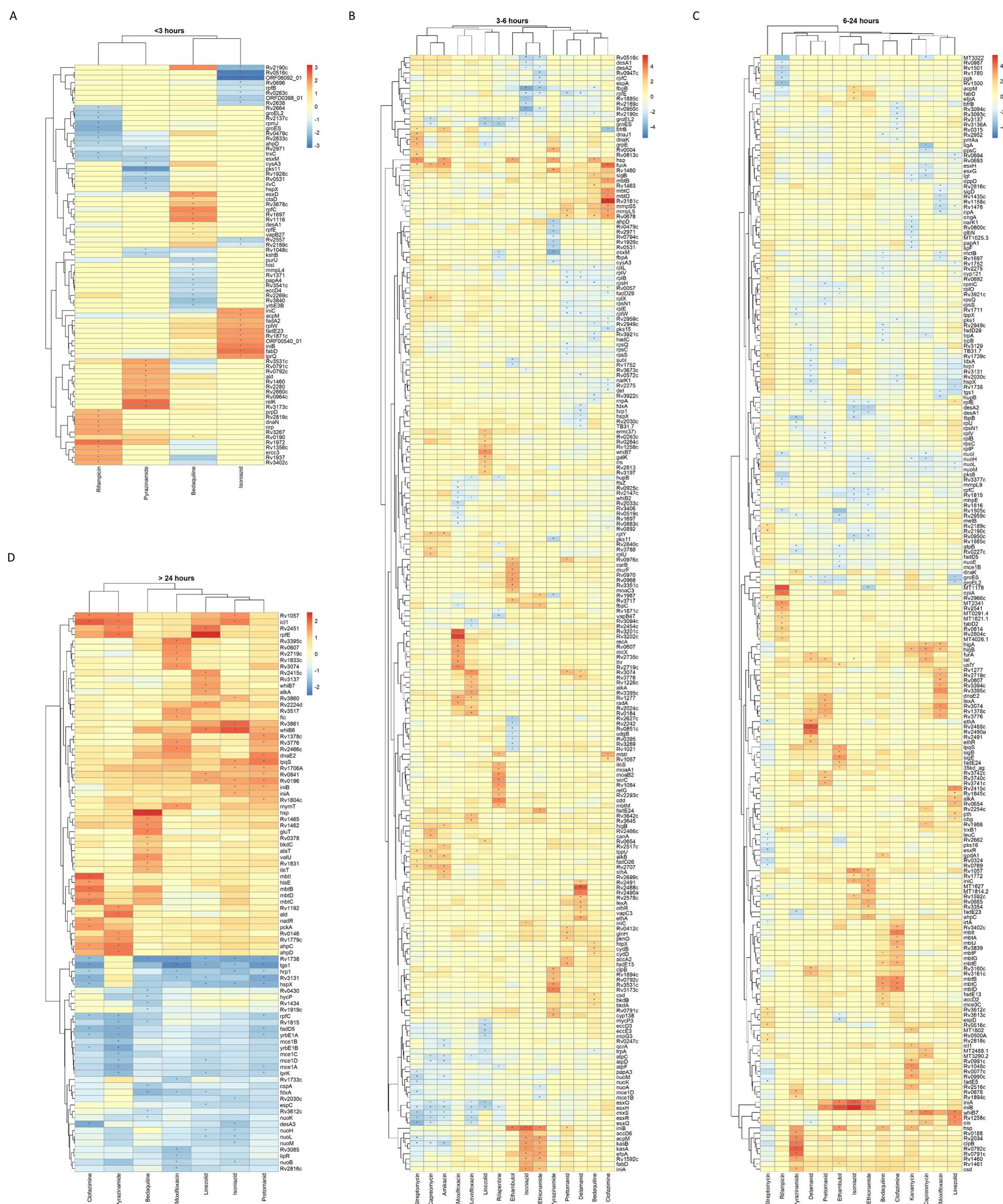


**FIG 3** Time category-specific heatmaps constructed using median log2fold values of the upregulated and downregulated TARs for each antibiotic within a time category; median log2fold values for genes that were significantly expressed in other antibiotics (but not within the TAR or not differentially expressed for that antibiotic) were also used. The median log2fold values were calculated for each gene across all TPs for each antibiotic after assigning a value of 0 for genes that were not differentially expressed and scaled for each antibiotic within a time category. The heatmaps were constructed for TPs in the (A) less than 3 hours, (B) 3–6 hours, (C) 6–24 hours, and (D) >24 hours time categories.

bedaquiline, ethambutol, and kanamycin, consistent with its response to multiple types of stress (46). We identified transcripts associated with the *lexA* DNA damage response to fluoroquinolones and nitroimidazoles (moxifloxacin and levofloxacin, delamanid and

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pretomanid, respectively) (47, 48). *whiB7*, a known transcriptional regulator of *eis* that is known to inactivate kanamycin, was noted in the TARs for the aminoglycosides, capreomycin, and linezolid, along with Rv1258c, *erm* (37), *eis*, and Rv0263c, all genes that are known to be regulated by *whiB7* (49, 50). Transcripts coding for efflux pumps were noted for isoniazid and ethionamide (*efpA*), pretomanid (*mmpL5*), bedaquiline and clofazimine (*mmpL5* and *mmpS5*), and linezolid (Rv1258c), suggesting the role of antibiotic efflux as a bacterial response to antibiotic exposure.

One or more transcripts from the *iniBAC* operon (*iniA*, *iniB*, and *iniC*; of which *iniA* is linked to isoniazid and ethambutol resistance putatively by facilitating the activity of an efflux pump as well as by maintaining plasma membrane structure) were among the top 10 most upregulated genes in one or more time category for the cell wall inhibitors isoniazid, ethambutol, ethionamide, and pretomanid (51, 52). In line with their role in inhibiting mycolic acid biosynthesis, isoniazid and ethionamide both upregulated FAS-II complex genes (*kasA*, *kasB*, and *acpM*) and *iniBAC* (*iniB* and *iniA*) operons at 3–6 hours of exposure.

One or more TARs for bedaquiline and clofazimine included members of the *mbt-1* locus consisting of genes *mbtA-mbtJ* responsible for the synthesis of the siderophore mycobactin, a molecule necessary for the intracellular transport of extracellular iron and for intracellular survival of Mtb in host cells (53, 54). The 6–24 hours TAR for bedaquiline and 3–6 TAR for clofazimine also included Rv0678, and the transcriptional repressor of the Mmps5-Mmpl5 efflux pump involved in the export of mycobactins; mutations in Rv0678 confer resistance to azoles, bedaquiline, and clofazimine through drug export by the MmpS5-MmpL5 efflux pump (55–58). The primary activity of MmpS5-MmpL5 system is the export of the siderophores synthesized by the *mbt-1* and *mbt-2 loci*; interruption of its activity leads to toxicity from the intracellular accumulation of iron (59).

## *Downregulated genes shared across drug classes or mechanisms*

The genes identified in downregulated TARs were consistent with a global response to antibiotic effect rather than an association with a specific pathway or mechanism of resistance for that antibiotic. Genes from the ESX gene family, the *mce1* operon, and he chaperone genes *groES* and *groEL2* were seen in antibiotic TARs across multiple time categories. The ESX family is involved in virulence, and six genes from this family (*esxR*, *esxH*, *esxQ*, *esxG*, *esxS*, *esxM*) were downregulated in response to levofloxacin, amikacin, streptomycin, capreomycin, rifampicin, rifapentine, pyrazinamide, and linezolid (60). *mce1* operon genes also code for proteins involved in virulence and were noted in TARs for pyrazinamide, linezolid, pretomanid, clofazimine, streptomycin, ethionamide, and

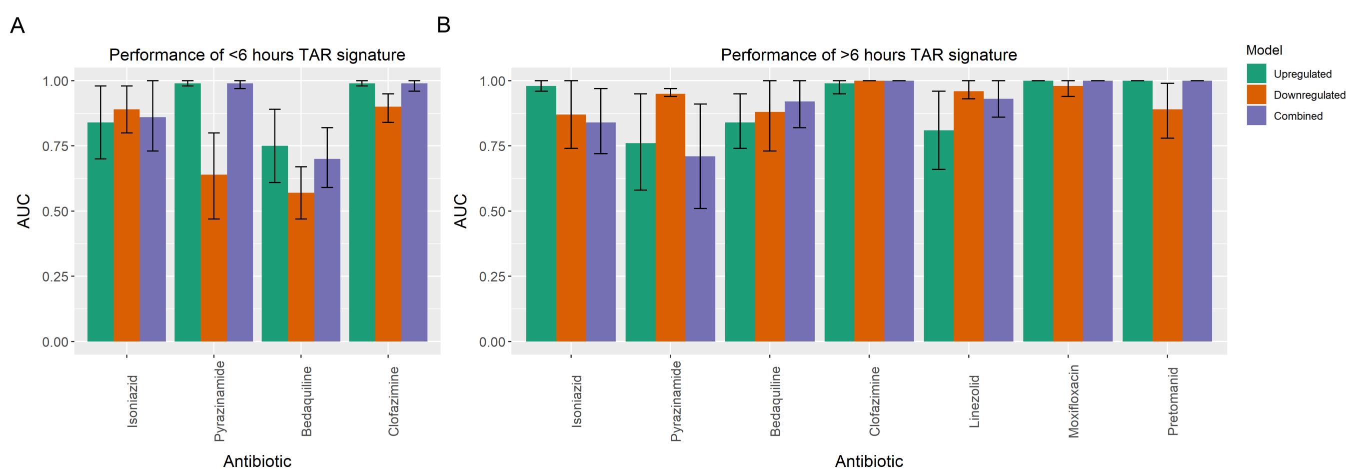


**FIG 4** The AUC used to determine the classification accuracy of a logistic regression model in distinguishing antibiotic from control TPs for antibiotics with at least four TPs in the (A) less than 6 hours and (B) greater than 6 hours time category, with the color of the bars corresponding to model constructed using genes from the upregulated (green), downregulated (red), or combined (purple) TAR.



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ethambutol (61). Stress response chaperone protein encoding genes *groES* and *groEL2* were present in TARs for pretomanid, delamanid, rifapentine, linezolid, ethambutol, and capreomycin. We identified the presence of genes coding for the 30S and 50S ribosomal subunit proteins and ribonuclease *P* protein (*rplL*, *rplW*, *rplB*, *rplV*, *rplP*, *rplE*, *rplR*, *rpsN1*, *rpsH*, *rpsC*, *rpsS*, *rpsE*, *rpsQ*, *rpmD*, *rpmC*, and *rnpA*) in the signatures for pretomanid, delamanid, bedaquiline, and linezolid (62).

We also noted the downregulation of genes involved in respiration in all-time categories. Three NADH dehydrogenases (Ndh, NdhA, and Nuo) responsible for NADH oxidation as part of the mycobacterial respiratory chain have been identified as potential drug targets. We identified the presence of genes from the *nuo* family (*nuoM*, *nuoL*, *nuoI*, *nuoH*, *nuoB*, *nuoC*, *nuoD*, *nuoF*, and *nuoE*) for isoniazid, ethionamide, linezolid, bedaquiline, moxifloxacin, capreomycin, kanamycin, streptomycin, amikacin, rifampicin, and genes involved in ATP synthase (*atpB*, *atpC*, *atpD*, and *atpF*) for pyrazinamide, capreomycin, amikacin, and levofloxacin. The >24 hours signature was striking for the presence of one or more genes involved in hypoxic stress response and dormancy genes (*hspX*, *tgs1*, Rv3131, Rv1738, Rv2030c, *hrp1*, and *fdxA*) for six of the seven antibiotics (bedaquiline, isoniazid, moxifloxacin, pretomanid, linezolid, and clofazimine) (63–67). The delamanid TARs from earlier time periods (3–6 hours and 6–24 hours) also had multiple genes associated with the hypoxic response.

Transcripts from the *desA1* and *desA2* genes involved in mycolic acid desaturation were noted for isoniazid and ethionamide (68–70). Resuscitation-promoting factors (*rpfA-E*) are virulence factors required for Mtb to recover from a dormant state (71). *rpfB*, *rfpC*, or *rpfE* are downregulated in response to the cell wall inhibitors, isoniazid, ethionamide, pretomanid, and delamanid, and also noted in a single bedaquiline TAR.

### Antibiotic TAR performance

Given the strong temporality of the transcriptional signal and the lesser effect of drug concentration, we generated two antibiotic TAR signatures by pooling across drug concentrations for the ≤6 hours and >6 hours categories, respectively (Table 1; Fig. 4). We focused on the drugs with at least four TPs for either time interval: isoniazid, bedaquiline, pyrazinamide, clofazimine, moxifloxacin, pretomanid, and linezolid. There was a median of seven TPs per drug for these agents for the ≤6 hours interval and a median of five TPs per drug for the >6 hours time interval. We built a logistic regression model that used the log2fold expression values of the top 10 upregulated and downregulated genes for a particular time interval and drug to distinguish drug exposure from all pooled controls (i.e., both longitudinal and time zero controls), as a more extreme test of classification specificity. We used 10-fold 75%–25% splits of the data for training and cross-validation (Materials and methods). The upregulated TAR signatures performed well with AUC for discriminating exposure *vs.* no exposure ranging from a minimum of 0.84–1.00 across antibiotics for the ≤6 hours time category and 0.76–1.00 for the >6 hours time category (Fig. 4A). The downregulated TAR had comparable AUC for most drugs in both time points (Fig. 4B). Combining upregulated and downregulated TAR signatures did not improve AUC over the upregulated or downregulated TAR signatures alone across drugs and time categories. Using TARs of either 5 or 15 genes did not significantly change performance (Table S9; Fig. S3).

For the antibiotics delamanid, streptomycin, and rifampicin, there were fewer than five TPs in total per drug, so we built a model for resistance classification by pooling TPs across the <6 hours or >6 hours time categories. The AUC for predicting antibiotic exposure was 0.99–1, 0.82–0.85, and 0.68–0.71 for streptomycin, delamanid, and rifampicin, respectively (Table S9).

## DISCUSSION

We analyzed microarray and RNA-seq data from 562 experiments across 17 antibiotics to identify Mtb gene expression signatures useful for determining *in vitro*



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antibiotic susceptibility. We demonstrated that transcriptionally responsive genes vary by antibiotic and over time. Time appears to affect the number of differentially expressed genes to a larger extent than drug concentration in the available data. Though we found genes common to TAR signatures across and between antibiotic classes, most TAR signatures were drug-specific. TAR signatures performed well at discriminating between gene expression due to antibiotic effect and gene expression seen in antibiotic unexposed Mtb. Our results contribute significantly to the literature on this topic as we pool all publicly available data to-date. We focused on drugs currently approved for the treatment of TB, including several newer and repurposed drugs for which the molecular mechanisms of resistance are not yet well-delineated. The culture media, time, and antibiotic concentration categories studied reflect the expected workflow of a clinical microbiology laboratory.

The upregulated TARs largely included genes related to the mechanism of action rather than genes associated with resistance mutations for antibiotics isoniazid (*katG* and *inhA* promoter), ethionamide (*inhA* promoter), and fluoroquionolones (*gyrA* and *gyrB*) (72). Genes known to be associated with resistance were seen in the TARs for bedaquiline and clofazimine (Rv0678), kanamycin (*whiB7*), and capreomycin (*eis* and *whiB7*); however, these genes were also seen in pretomanid and pyrazinamide (Rv0678), delamanid (*eis*), and linezolid (*eis* and *whiB7*), which are not known to be associated with Mtb resistance to these antibiotics (72). Similarly, *whiB6*, associated with Mtb resistance to amikacin, streptomycin, and capreomycin, was not present in the TAR for these drugs but was noted in the TARs for isoniazid, pretomanid, and linezolid (72). Mutations in the efflux pump coded by Rv1258c are associated with resistance to isoniazid, streptomycin, and pyrazinamide (72); however, we only identified it in the TARs for linezolid along with other genes (*eis* and Rv0263c) induced by *whiB7*. Thus, the TAR likely reflects drug-specific mechanisms of action that result in characteristic perturbations in cellular processes. These data confirm that transcriptomic responses enable a better understanding of the drug's mechanism of action. Although isolates with drug resistance mutations are not studied here, the overlap of TAR genes with known drug resistance encoding genes suggests that the transcriptomic response to antibiotics will enable the characterization of phenotypic and genotypic resistance in *M. tuberculosis.*

The transcriptional response to antibiotic exposure had notable overlap across drug classes. We observed both upregulation and downregulation of certain stress response genes, including heat shock (*hsp*), DNA repair (*lexA*-regulated), stress response chaperone proteins (*groES* and *groEL2*), and hypoxic response and dormancy genes across multiple antibiotics. We noted downregulation of virulence genes aerobic respiration (*nuo* and *atp*) and ribosomal protein subunits across drug classes. Efflux pumps were noted in the upregulated TARs, but the specific efflux genes differed by drug class. These may reflect global responses in bacterial physiology due to antibiotic effect (12, 15). While the data available for our analysis did not allow us to explore this hypothesis in detail, it may be possible to use a single TAR to demonstrate antibiotic activity among agents of the same class or multiple classes.

Our study was not without limitations. As this is a meta-analysis, transcriptional changes were measured across a heterogeneous set of antibiotic concentrations and duration of time, and some antibiotics were not well represented in the available data. There was not enough data to identify class-specific TARs for aminoglycosides and fluoroquinolones. Our selection of 10 genes is arbitrary albeit based on previous work with gram-negative pathogens; the optimal number of genes may defer (27). Most experiments were performed using the reference strain H37Rv, so we could not evaluate the impact of background genetic variation seen in clinical isolates or the role of lineage in antibiotic response. Assessment of the performance of transcriptional response for predicting antibiotic effect is preliminary as it used cross-validation without the ability to validate an independent hold-out test data set due to the data set size available. Transcriptional data on antibiotic-exposed resistant isolates were unavailable on NCBI/ENA when we conducted this study. Assessment of performance also assumes



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that the transcriptional profile of resistant isolates exposed to a drug is like that of a susceptible isolate unexposed to the same drug. Although one prior study confirmed that this assumption holds for three drugs and mechanisms of resistance, it may not hold true for all drugs (26). Future studies will need to validate TAR signatures using antibiotic-resistant isolates to determine if transcriptomic responses can be used as a rapid drug-susceptibility method.

In conclusion, our study demonstrates the rapid and drug-specific responses of Mtb to antibiotic exposure. The overall patterns of differential expression are consistent with the published literature for the effect of specific antibiotics, including studies from which data were not available publicly for inclusion in this meta-analysis and in the sputum of patients treated for drug-susceptible tuberculosis (19, 66, 73–75). This approach allows for the characterization of mechanisms of action of new compounds that affect a range of cellular processes. Because transcriptomic methods can detect both coding mutations and transcript abundance, they have the potential to combine phenotypic and genotypic methods into a single test. Compared with DNA-based diagnostics this approach lessens the dependence on a comprehensive catalogue of resistance mutations (26, 27). Further work is needed to confirm the accuracy of this approach, including the study of more genetically diverse clinical strains, the standardization of growth conditions and antibiotic concentrations, and refining and validating the signature using resistant strains of Mtb.

## ACKNOWLEDGMENTS

H.P. would like to thank the members of the Harvard Chan Bioinformatics Core for offering bioinformatics training.

Research reported in this publication was supported by Tufts Medical Center Department of Medicine and National Center for Advancing Translational Sciences of the National Institute of Health through Tufts Clinical and Translational Sciences Institute under award 1K12TR004384 (H.P.), the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number 1R01AI176498-01 (M.R.F.) and F32AI161793 (A.G.G.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. For this publication, the research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement FP7-223681 to D.M.C. Portions of this research were conducted on the O2 High Performance Compute Cluster, supported by the Research Computing Group, at Harvard Medical School. See https://it.hms.harvard.edu/our-services/research-computing for more information.

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## FUNDING

| Funder | Grant(s) | Author(s) |
|---|---|---|
| HHS \| NIH \| National Institute of Allergy and Infectious Diseases (NIAID) | 1R01AI176498-01 | Maha R. Farhat |
| HHS \| NIH \| National Institute of Allergy and Infectious Diseases (NIAID) | F32AI161793 | Anna G. Green |
| EC \| Seventh Framework Programme (FP7) | FP7-223681 | Daniela Maria Cirillo |
| HHS \| NIH \| National Center for Advancing Translational Sciences (NCATS) | 1K12TR004384 | Husain Poonawala |

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Husain Poonawala, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review and editing | Yu Zhang, Formal analysis, Investigation, Software | Sravya Kuchibhotla, Formal analysis, Investigation, Software | Anna G. Green, Methodology, Validation, Writing – review and editing | Daniela Maria Cirillo, Resources | Federico Di Marco, Resources | Andrea Spitlaeri, Resources | Paolo Miotto, Resources, Writing – review and editing | Maha R. Farhat, Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing, Funding acquisition

## DATA AVAILABILITY

Links to microarray and RNA-Seq data are available in Table S1. Code and metadata used to analyze these data will be available on GitHub following a 6 months embargo from the date of publication to allow for commercialization of research findings.

## ADDITIONAL FILES

The following material is available online.

### Supplemental Material

**Figure S1 (AAC01185-23-s0001.tif).** Antibiotic heatmaps.
**Figure S2 (AAC01185-23-s0002.tif).** Temporal changes in select antibiotic-gene combinations.
**Figure S3 (AAC01185-23-s0003.tif).** Performance of 5-gene and 15-gene signatures.
**Supplemental material (AAC01185-23-s0004.docx).** Supplemental text and legends for supplemental figures and tables.
**Supplemental tables (AAC01185-23-s0005.xlsx).** Tables S1–S9.

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