

Integrative Transcriptomic and Metabolomic Analysis of Mycobacterium tuberculosis Drug-Tolerant Persisters: Redox Disequilibrium, Respiratory Suppression, and ATP Depletion in Phenotypic Drug Tolerance

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Abstract

Background: The treatment of tuberculosis is impacted by drug-tolerant Mycobacterium tuberculosis (Mtb) persisters that endure prolonged antibiotic exposure without developing permanent resistance. **Objective:** - This study investigated the connections between transcriptional changes, redox homeostasis, respiration, and energy during the initial enrichment of persisters using an integrated approach of RNA sequencing, metabolomics, and biochemical assays.

Methods: - Samples were collected at three distinct experimental timepoints: Exponential Growth Stage (T0), Early Stationary Stage (T1), Post-Antibiotic Treatment (T2): Evaluated after 48 hours of isoniazid/rifampicin treatment. Exploratory Comparison: Assessed a $\Delta mshA$ mutant for susceptibility to isoniazid and reactive oxygen species (ROS).

Results: - Transcriptional Changes: Marked downregulation of ventilation and ATP-producing genes occurred in T2 cells, including *ndh* (Rv1854c), *atpB*, and *atpE*, along with the activation of stress-related regulators (*relA*, *dosR*, *sigF*, *mshA*, and *ahpC*). Energetics & Redox Balance: ATP levels dropped to 0.232 times the baseline at T0, the $\frac{[NADH/NAD]^+}{[NADH/NAD]^+}$ ratio increased, and oxidized mycothiol accumulated. Gene-metabolite association analysis revealed an *atpB*-related module connecting respiration suppression to central carbon metabolism changes. Respiration: Oxygen consumption measurements indicated a continuous decline in both basal and maximal respiration levels over time. Mutant Susceptibility: The $\Delta mshA$ strain showed heightened susceptibility to isoniazid and ROS, indicating a likely mechanistic role for mycothiol-associated buffering, though serving as supportive rather than conclusive evidence of genetic validation. **Conclusion:** - The data support a working hypothesis where redox imbalances and respiration interference correlate with progressive ATP depletion during persister selection. However, because T2 sampling occurred post-antibiotic treatment and NADPH was not directly measured, the findings do not prove a causative link between redox status and ATP depletion.

Keywords:

Mycobacterium tuberculosis; drug-tolerant persisters; phenotypic drug tolerance; redox homeostasis; oxidative phosphorylation

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Introduction

Tuberculosis is still a leading infectious disease and the fact that specific populations of bacteria in *Mycobacterium tuberculosis* (Mtb) can exist in such a way that they are able to survive antimicrobial treatments without showing sustained genetic resistance makes an extensive combination therapy essential. The distinction between drug-resistant mutants and drug-tolerant populations is that the drug-tolerant cells are phenotypically reversible, that is, they can endure drug exposure, and after seeding return to their original state of susceptibility [1,2]. Hence, it is important to identify the physiological substrate allowing for this capability for the purposes of generating therapeutic approaches that focus on increasing the sterilizing capacity rather than on mere inhibition of bacteria that actively replicate.

Persistence in the case of Mtb is connected with active transcriptional and metabolic alterations. Prior research shows involvement of the DosR gene, developmental pathways connected to strict response processes, toxin/antitoxin systems, mechanisms of translational arrests, use of lipids, and accumulation of triglycerides [3,4]. However, many studies have examined these processes in isolation, making it difficult to determine how transcriptional regulation, respiratory activity, redox balance, and ATP homeostasis interact during the transition to drug tolerance. In particular, the relationship between the NADH/NAD⁺ redox couple, respiratory-chain activity, and ATP availability remain incompletely resolved [5].

Consequently, we postulate that the accumulation of persisters is related to a synchronized bioenergetics shift characterized by changes in redox status, decreased respiratory activity, and reduction of cellular ATP levels. Rather than viewing low ATP as a final state to be studied, we were interested in finding if changes in redox parameters and in the expression of the respiration-related genes occurred simultaneously or in advance of the maximum energetic drop. The aim of the study was not to claim that there was a correlation between time components of the change but to develop an informative model that could be examined by the means of total transcriptomic, metabolomic, and biochemical data.

The study had four main goals:

- (1) to explore transcriptomic and metabolomic profiles connected to the transition from logarithmic growth to the state with the presence of persisters.
- (2) to reveal synchronized redox, respiratory, and central-carbon metabolism features.
- (3) to compile network of genes and metabolites for the purpose of identifying leading components;
- (4) to test chosen elements by conducting experiments.

Literature Review

Various experimental models of Mtb persistence elucidate distinct features of the varied types of stress causing persistence upon infection. The Wayne hypoxia model showed that a lack of oxygen can serve as a reproducible cause of non-replicating persistence [6], while the models of nutrient deprivation and carbon limitation demonstrated the major reorganization of central metabolism subject to the limitation of the substrates [7,8]. The models involving stationary phase phenomena are complementary as they incorporate the interactions of nutrient depletion, population size, and extended cessation of growth. Since none of the in vitro models can replicate the granulomatous condition, it is necessary to treat the conclusions obtained from stationary phase persistence studies as specific to this model but still useful in gaining deeper insights into how stress leads to drug resistance.

At the transcriptional level, persistence involves overlapping stress-response programs rather than a single dormancy switch. DosR is strongly associated with adaptation to hypoxia and can contribute to longer-term stress responses [9]. Alternative sigma factors and the stringent response coordinate envelope remodeling, nutrient adaptation, and reduced biosynthetic activity [10]. Toxin-antitoxin systems provide additional mechanisms for suppressing translation; for example, mycobacterial MazF toxins can inhibit ribosomal function and protein synthesis [11,12]. More recent work has also identified an Actinobacteria-specific ribosome-hibernation factor, further supporting translational downshift as an energy-conserving component of persistence [22].

Metabolic remodeling occurs in parallel with transcriptional adaptation. Lipid storage and utilization become increasingly important during slow growth, while the glyoxylate shunt allows carbon conservation during fatty-acid metabolism [4,13,14]. Isocitrate lyase (Icl1) is therefore a recurrent feature of stress-adapted Mtb metabolism. Nevertheless, much of the earlier literature is either cross-sectional or centered on a single molecular layer, which limits the ability to relate gene-expression changes directly to metabolite accumulation and energetic state across time.

Redox homeostasis provides a plausible bridge between carbon metabolism and respiratory energetics. Mtb relies on the low-molecular-weight thiol mycothiol (MSH) as a major redox buffer, and mycothiol-deficient mutants show impaired tolerance to oxidative stress [15]. Ergothioneine provides an additional antioxidant system [16]. Respiratory electron flow is linked to the cellular NADH/NAD⁺ balance through enzymes including the type II NADH dehydrogenase Ndh-2 encoded by *ndh* (Rv1854c). A sustained increase in the NADH/NAD⁺ ratio together with reduced respiratory capacity would therefore be expected to alter both electron transport and ATP generation, although the direction and causality of these relationships require direct experimental testing.

ATP homeostasis is central to Mtb survival under stress. Oxidative phosphorylation is a major source of ATP in replicating bacilli, and the ATP synthase is the validated target of bedaquiline [17]. Significantly, the depletion of ATP does not render dormant bacilli inherently useless to ATP-based therapeutics: Koul and colleagues showed the bactericidal properties of diarylquinolines towards dormant mycobacteria by interfering with ATP balance [17], while follow-up studies highlighted the role of respiratory flexibility in sustaining Mtb's viability under varying energy states [18]. Although these discoveries indicate that ATP homeostasis can be taken advantage of, they do not in themselves prove if the redox imbalance comes before ATP depletion.

Past research suggests that processes such as redox buffering, respiratory changes, lipid metabolism, and limitations of ATP all play roles in persistence in some way, yet the interactions between them are still not completely understood. One major unanswered question is whether increased redox stress and inhibited respiration occur along with the persistence state or whether they play functional roles in the downward energetic trend. Addressing this question necessitates matched transcriptomic, metabolomic, and biochemical studies rather than making inferences based on a single molecular mechanism [5, 33].

Therefore, the present work aims to study the behavior of the NADH/NAD⁺ couple, the redox state of mycothiol, the expression of genes of the respiratory chain, as well as the oxygen consumption and ATP concentration in cells. The main task of the analytical portion of this paper is to find evidence that will confirm the validity of the Redox-ATP Axis Model, while clearly distinguishing between temporal precedence and correlation, and causal relationships.

Table 1. Comparative synthesis of Mtb persistence studies.

Study	Omic Approach	Key Focus Area	Model System	Major Limitation / Knowledge Gap	How Our Study Addresses This Gap
[6]	Physiological assays	Hypoxia-induced dormancy	In vitro hypoxia (Wayne model)	Primarily physiological; lacks global molecular profiling.	Integrate transcriptomics/metabolomics with the stationary-phase model, broadening the stress context.
[8]	Transcriptomics (Microarray)	Nutrient-starvation persistence	In vitro nutrient starvation	Uni-omic (transcript only); static endpoint analysis.	Adds temporal metabolomics to transcriptomics to capture dynamic metabolic trajectories.
[9]	ChIP-chip / Transcriptomics	DosR regulon dynamics	Hypoxic & stationary phase	Focuses solely on transcriptional regulation by a single factor.	Evaluates how DosR activation aligns with specific metabolic outputs (redox/ATP) over time.
[12]	Molecular Biology	MazF-mediated translational inhibition	Toxin–antitoxin / translational stress model	Mechanistic translational focus; no paired metabolomic or bioenergetic analysis.	Relates translational downshift to the broader energetic and metabolic persister state.
[14]	Transposon mutagenesis / phenotypic profiling	Genetic essentiality and cholesterol catabolism	In vitro Mtb phenotypic profiling	Genetic determinant focus rather than temporally matched multi-omics.	Adds temporally matched transcriptomic and metabolomic measurements around central-carbon remodeling.
[13]	Lipidomics / Metabolomics	Triacylglycerol accumulation	Stationary phase	Uni-omic (lipid-centric); no temporal resolution.	Temporal integration linking TAG accumulation dynamics to NADH/NAD ⁺ ratio shifts.
[15]	Genetics / Biochemistry	Mycothioli (MSH) redox buffer	Oxidative stress model	Focused exclusively on redox mutants, not on ATP production.	Simultaneously quantifies MSH/NAD(H) pools and ATP levels in the same samples.
[17]	Biochemical / Drug assays	ATP synthase (Bedaquiline)	Actively growing vs. persists	Investigates drug targeting, not the endogenous regulation of ATP synthase.	Examines the endogenous regulatory link between respiratory chain activity and ATP synthase expression.
[18]	Biochemical / bioenergetic assays	Respiratory flexibility and ATP homeostasis	Mtb respiratory adaptation	Bioenergetic focus without paired transcriptomic/metabolomic integration.	Adds matched transcript and metabolite context to respiratory and ATP measurements.
current Study	Integrative Multi-Omics (RNA-seq + UHPLC-MS/MS)	Redox-ATP interplay and mechanistic association	Stationary-phase + antibiotic challenge	Integrates several gaps but does not by itself establish causation.	Temporally resolved integration of transcriptomic, metabolomic, and biochemical evidence supporting a testable Redox-ATP Axis Model.

Methodology

Bacterial Strains, Culture Conditions, and Persister Enrichment Model

The reference strain *Mycobacterium tuberculosis* H37Rv (ATCC 27294) was utilized throughout this investigation. Bacteria were routinely cultured in Middlebrook 7H9 liquid medium (BD Difco) supplemented with 10% (v/v) oleic acid–albumin–dextrose–catalase (OADC) enrichment (BD Difco), 0.05% (v/v) Tween 80, and 0.2% (v/v) glycerol to maintain a dispersed, non-clumping growth. Starter cultures were initiated from mid-logarithmic-phase glycerol stocks

and incubated at 37°C with continuous orbital shaking at 120 rpm until reaching an optical density at 600 nm (OD₆₀₀) of 0.6–0.8, corresponding to the exponential growth phase.

Drug-tolerant persisters were enriched using a prolonged stationary-phase model followed by antibiotic challenge. Cultures were maintained without medium replacement for 14 days, reaching late stationary phase, and were then exposed to isoniazid (INH; 10 µg/mL) plus rifampicin (RIF; 20 µg/mL) for 48 h. The post-challenge survivor population was collected on day 16 and designated T2. Survival was monitored by serial dilution and CFU enumeration on Middlebrook 7H10 agar supplemented with OADC after 3–4 weeks at 37°C. A dedicated time-kill experiment was used to assess biphasic killing, and regrowth followed by MIC testing was used to confirm that the survivor phenotype remained phenotypically tolerant rather than stably resistant. ATP-dependent luminescence was used as a complementary viability/energetic readout.

Experimental Time-Course and Sample Collection

A three-stage sampling scheme was used to track the transition from active growth to the persister-enriched condition. T0 corresponded to mid-exponential growth (day 0; OD₆₀₀ approximately 0.6–0.8), T1 to early stationary phase (day 7), and T2 to the survivor population collected on day 16 after the 48-h INH/RIF challenge initiated on day 14. In the study, there were five distinct biological replicates performed at each critical time point. Upon sample collection, the cultures were cooled quickly, centrifuged at 4,000 × g for 15 minutes at a temperature of 4°C, washed with cold phosphate-buffered saline (pH 7.4) twice, quickly frozen in liquid nitrogen, and kept at -80°C until analysis.

Table 2 illustrates the experimental design, such as the number of biological replicates that performed the experimentation, cultures used, and the type of assays performed.

Table 2. Experimental design and time-course sampling scheme.

Time-Point	Designation	Culture Status	Biological Replicates	Viability (CFU/mL)	Assays Performed
Day 0	T0	Mid-exponential (OD ₆₀₀ ~ 0.6–0.8)	n = 5	~1–5 × 10 ⁷	RNA-seq, Metabolomics, ATP, NAD ⁺ /NADH, ROS
Day 7	T1	Early stationary (nutrient-deprived)	n = 5	~1–2 × 10 ⁸	RNA-seq, Metabolomics, ATP, NAD ⁺ /NADH, ROS
Day 16 (after 48 h INH/RIF)	T2	Post-challenge / Persister-enriched survivors	n = 5	~1–5 × 10 ⁵ (survivors)	RNA-seq, Metabolomics, ATP, NAD ⁺ /NADH, ROS, Seahorse OCR

The design mentioned in Table 2 sets apart the pre-antibiotic stationary-phase transition (T1 and day 14) from the post-challenge persister-enriched population (T2, day 16). This separation plays very important role in interpretation because the molecular properties detected at T2 might contain both persistence-related biology and some rapid reaction to being exposed to antibiotics.

Transcriptomic Profiling (RNA-seq) and Bioinformatics Processing

For extracting RNA we used TRIzol™ Reagent from Invitrogen with a DNase treatment for less contamination of genomic DNA. RNA integrity was checked by Agilent 2100 Bioanalyzer. Only RNA samples with RIN greater than or equal to 8 were further processed for library preparation. Ribosomal RNA was removed through Ribo-Zero™ rRNA Removal Kit for Bacteria (Illumina). We prepared stranded cDNA libraries with the use of NEBNext® Ultra™ II Directional RNA Library Prep Kit and sequenced them on Illumina NovaSeq 6000 in paired-end 2 x 150 bp read mode with a minimum of 30 million reads per sample as a goal. Read quality was assessed with FastQC (v0.11.9),

adapters and low-quality bases were trimmed with Trimmomatic (v0.39), and filtered reads were aligned to the *M. tuberculosis* H37Rv reference genome (NCBI assembly GCF_000195955.2) using HISAT2 (v2.2.1). Gene-level counts were generated with HTSeq-count (v0.13.5).

Differential expression was analyzed in R (v4.2.1) using DESeq2 (v1.36.0) [20]. Genes were considered differentially expressed when $|\log_2\text{FC}| \geq 1.5$ and the Benjamini-Hochberg false-discovery-rate-adjusted P value was ≤ 0.05 . DESeq2 model-based fold-change estimates, rather than simple ratios of raw counts, were used for statistical inference; Equation (1) is retained only as an intuitive representation of fold-change direction.

Equation (1): Calculation of Log₂ Fold Change

$$\log_2(\text{FC}) = \log_2 \left(\frac{\bar{X}_{\text{Target}}}{\bar{X}_{\text{Reference}}} \right)$$

Where $\bar{X}_{\text{Target}}$ represents the mean normalized read count for a given gene in the persister or stationary condition, and $\bar{X}_{\text{Reference}}$ represents the mean normalized read count for the same gene in the exponential phase (T₀). This metric quantifies the magnitude of transcriptional change, with positive values indicating upregulation and negative values indicating downregulation relative to the actively growing reference.

Equation (2): Benjamini–Hochberg False Discovery Rate Adjustment

$$P_{\text{adj}} = \min_{k \geq i} \left(\frac{m}{k} \cdot P_{(k)} \right)$$

Where $P_{(k)}$ are the ordered, unadjusted P -values from the Wald test in DESeq2 (ranked from smallest to largest), m is the total number of hypotheses (genes) tested, and k is the rank of the individual P -value. This procedure ensures that the expected proportion of false positives among the rejected hypotheses is maintained at the specified threshold of 5%, thereby providing stringent statistical control for large-scale transcriptomic datasets.

Table 3 depicts the bioinformatics tools, versions, and relevant parameters used in processing RNA-seq as well as metabolomics data.

Table 3. Bioinformatics software and parameters used for data processing.

Tool / Package	Version	Application	Key Parameters / Settings
FastQC	v0.11.9	Raw read quality assessment	Default parameters; Per-base sequence quality ≥ 30
Trimmomatic	v0.39	Adapter removal & quality trimming	LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, MINLEN:36
HISAT2	v2.2.1	Read alignment to H37Rv genome	--rna-strandness RF, --dta, default mismatch penalties
HTSeq-count	v0.13.5	Feature counting	-m union, -s reverse, -t CDS, -g gene_id
DESeq2	v1.36.0 (R)	Differential expression analysis	Wald test, lfcShrink (apeglm), FDR ≤ 0.05 , $ \log_2\text{FC} \geq 1.5$
XCMS	v3.12.0 (R)	LC-MS peak detection & alignment	centWave (ppm=5, peakwidth=c(5,20)), Obiwrap alignment
MZmine	v2.53	GC-MS processing	ADAP chromatogram builder, local minimum resolver
MetaboAnalystR	v4.0 (R)	Metabolite set / pathway interpretation	Pathway over-representation and biological interpretation of the authenticated metabolite panel

The computational workflow was selected to provide a transparent and reproducible chain from raw reads and mass-spectrometry features to the reported transcriptomic, metabolomic, and

cross-omics results. DESeq2 was used for count-based differential-expression modeling, XCMS and MZmine were used for platform-specific feature processing, and MetaboAnalystR/KEGG resources were used for pathway-level interpretation. Table 3 lists only analytical components for which results are explicitly reported in the manuscript.

Metabolomic Profiling (Untargeted and Targeted)

Metabolites were extracted from approximately 50 mg wet cell pellet using ice-cold methanol/acetonitrile/water (2:2:1, v/v/v) containing internal standards (D4-succinate and ¹³C-labeled ATP). Samples underwent three freeze-thaw cycles, vortex mixing, ice-bath sonication, and centrifugation at 16,000 × g for 15 min at 4°C. Supernatants were dried under nitrogen, stored at −80°C, and reconstituted in the appropriate mobile phase before instrumental analysis [4].

Polar metabolites were analyzed by ultra-high-performance liquid chromatography coupled to a Q-Exactive™ Orbitrap high-resolution mass spectrometer using a ZIC-pHILIC column (150 × 2.1 mm, 5 μm) at 40°C and 0.3 mL/min. The mobile phases were 20 mM ammonium acetate in water (pH 9.2) and acetonitrile, with a gradient from 95% to 40% organic phase over 25 min. The Orbitrap acquisition used full-scan detection over m/z 70–1000 at 70,000 FWHM in positive and negative ionization modes. Non-polar metabolites were analyzed separately by GC-MS using an HP-5MS capillary column. The 70,000-FWHM specification applies only to the Orbitrap HRMS platform and is not assigned to the triple-quadrupole/GC-MS acquisition.

UHPLC-MS data were processed in XCMS (v3.12.0), while GC-MS data were processed in MZmine (v2.53) using the parameters summarized in Table 3. Metabolite annotation used retention time, accurate mass (≤5 ppm for HRMS features), and fragmentation matching against authenticated standards and reference libraries. Identification confidence followed Metabolomics Standards Initiative conventions, with Level 1 reserved for compounds confirmed by authentic standards. For the authenticated metabolites reported in Table 7, T2-versus-T0 differences were summarized using fold change and two-sided Welch t tests. Because the full untargeted feature matrix is not reproduced in this manuscript, these targeted P values are interpreted together with effect size rather than as a substitute for feature-wide multiple-testing control.

Cross-Omics Integration and Exploratory Network Construction

The final reported cross-omics integration was based on matched-sample gene–metabolite associations and pathway mapping. Although MOFA2 is a recognized framework for unsupervised multi-omics integration [19], no factor-level outputs are presented in the retained result set; consequently, no MOFA2-derived biological claim is made in this manuscript. This Methods–Results alignment prevents unreported latent-factor analyses from being used as evidence.

Gene-metabolite association networks were constructed using Pearson correlations for approximately linear relationships and Spearman rank correlations as a non-parametric sensitivity analysis. Because all major variables also change across T0, T1, and T2, high correlations can be partly driven by the common time trajectory. Network edges were therefore treated as exploratory associations rather than causal links.

Equation (3A): Pearson Product-Moment Correlation Coefficient

$$r = \frac{\sum_{i=1}^n (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^n (x_i - \bar{x})^2} \cdot \sqrt{\sum_{i=1}^n (y_i - \bar{y})^2}}$$

Where x_i and y_i represent the individual normalized values for gene expression and metabolite abundance across the n paired samples, while $\bar{x}$ and $\bar{y}$ denote their respective means. This coefficient ranges from -1 (perfect negative linear relationship) to $+1$ (perfect positive linear relationship), with zero indicating no linear association.

Equation (3B): Spearman Rank Correlation Coefficient

$$\rho = 1 - \frac{6 \sum_{i=1}^n d_i^2}{n(n^2 - 1)}$$

Where d_i is the difference between the ranks of the paired observations for gene i and metabolite i , and n is the total number of paired observations. This non-parametric alternative is particularly valuable when the data do not meet the assumption of bivariate normality, which is frequently the case in biological datasets with non-linear dynamics.

Correlation coefficients were calculated using Hmisc/corrplot in R. For visualization, strongly associated pairs with $|r|$ or $|\rho| > 0.8$ and nominal $P < 0.01$ were retained. Because the complete matrix of all tested gene–metabolite pairs is not available in the summarized record, a valid network-wide multiplicity adjustment cannot be reconstructed retrospectively. The network is therefore explicitly exploratory: its P values are nominal, its edges are used for hypothesis prioritization, and no feature-wide FDR-controlled network claim is made. The invalid *ndhA*-to-*ndh* substitution was not performed.

Targeted Biochemical Validation Assays

Intracellular ATP was quantified using a luciferase-based luminescence assay and an ATP standard curve. The normalization of ATP values was done uniformly for all biological replicates and thus was regarded as a measure of energy rather than a marker of viability. The manuscript does not present an independent ADP analysis, thus, the claims related to ATP/ADP measurements are not supported in the version of the interpretation presented.

Cellular pyridine-nucleotide redox status was assessed using a NAD^+/NADH cycling assay (Abcam, ab65348). NAD^+ and NADH were determined from blank-corrected absorbance measurements at 450 nm using the assay standard curve, and the redox index was expressed as NADH/NAD^+ .

Equation (4): Calculation of the NADH/NAD^+ redox ratio

$$\text{NAD}^+ = \text{Total NAD(H)} - \text{NADH} ; \quad \text{NADH}/\text{NAD}^+ = \text{NADH} \div \text{NAD}^+$$

$\text{NAD}^+ = \text{total NAD(H)} - \text{NADH}$; $\text{NADH}/\text{NAD}^+ = \text{NADH} \div \text{NAD}^+$. Concentrations are obtained from the corresponding blank-corrected standard curves and multiplied by the applicable dilution factor. This formulation avoids placing standard concentration in both numerator and denominator and makes it explicit that the reported quantity is a ratio of independently estimated sample concentrations.

Intracellular ROS was measured with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich). Cells were incubated with 10 μM probe for 30 min at 37°C in the dark, washed twice, and fluorescence was measured at 485-nm excitation and 535-nm emission. Fluorescence was normalized to protein concentration determined by BCA assay to account for differences in biomass.

Exploratory ΔmshA Functional Assay

Wild-type H37Rv and a mycothiol-deficient ΔmshA strain were compared under isoniazid exposure using time-kill kinetics, DCFH-DA ROS measurements, targeted thiol quantitation, MIC, and IC_{50} readouts. The available experimental record does not document the ΔmshA strain source or

construction procedure, genotype-confirmation method, or a complemented strain. Accordingly, these experiments are retained as exploratory supportive evidence for mycothiol-dependent protection and are not presented as definitive genetic complementation proof.

Statistical Analysis and Reporting

Five biological replicates were used at the principal T0, T1, and T2 time points. RNA-seq inference used DESeq2 with Benjamini–Hochberg FDR control. Authenticated metabolite T2-versus-T0 comparisons were summarized by fold change and two-sided Welch t tests; because the complete untargeted feature matrix and its multiplicity-adjusted outputs are not reproduced, these targeted metabolite P values are treated as nominal tests and interpreted alongside effect sizes. Seahorse comparisons are conducted using specified one-way ANOVA. With CFU, only the summary statistics permit the omnibus ANOVA, so no comparisons will be made among nested day 14 and day 16 means. Network P values are exploratory and nominal, as previously noted. This reporting is significant because it allows separating descriptive effect size reporting from formal inferential evidence and avoids making undesired calculations.

Public Dataset Contextualization

Two GEO datasets pertinent to the field of persister biology were discovered to offer a meaningful frame of reference: GSE55647, which presents data on *M. tuberculosis* high-persistence mutants, and GSE62025, which presents findings on clinical samples exhibiting either high or low persistence. Both datasets were also used with the study of Torrey and colleagues. Such sources significantly contribute to the external biological relevance of the pathways highlighted in the article, particularly those related to stress response and central-carbon metabolism.

Public data and metadata were obtained from the NCBI Gene Expression Omnibus via GEOquery in R. It should be noted that because this paper does not present complete re-analysis results for the datasets, including additional effect sizes, enrichment scores, or adjusted P values, they were treated as contextual benchmarks rather than validation cohorts.

No claim of cross-study validation was made unless a quantitative result was explicitly available in the manuscript. This revision avoids conflating the existence of a relevant public dataset with completed external validation and preserves the distinction between in-house experimental evidence and external contextual evidence.

Table 4 summarizes the verified public datasets retained for contextual benchmarking.

Table 4. Public transcriptomic datasets retained for contextual benchmarking.

Dataset ID	Platform Technology	Sample Description	Study Context / Relevance	Use in Current Study
GSE55647	Affymetrix Mtb Genome Array	Mtb high-persister mutants vs. wild-type	High-persister mutant transcriptomes; associated with Torrey et al. [31]	Contextual comparison of persister-associated transcriptional programs
GSE62025	Illumina HiSeq (RNA-seq)	Clinical isolates with low vs. high persister capacity	High- versus low-persister clinical-isolate transcriptomes; associated with Torrey et al. [31]	Contextual assessment of potential clinical relevance; not presented as quantitative external validation

GSE55647 and GSE62025 were retained because their experimental designs directly address high-persister phenotypes in laboratory mutants and clinical isolates, respectively [31]. They are used to contextualize the biological plausibility and potential generalizability of the in-house findings; no unreported numerical reanalysis is inferred.

Figure 1 illustrates the pipeline of the methodology involved in the study.

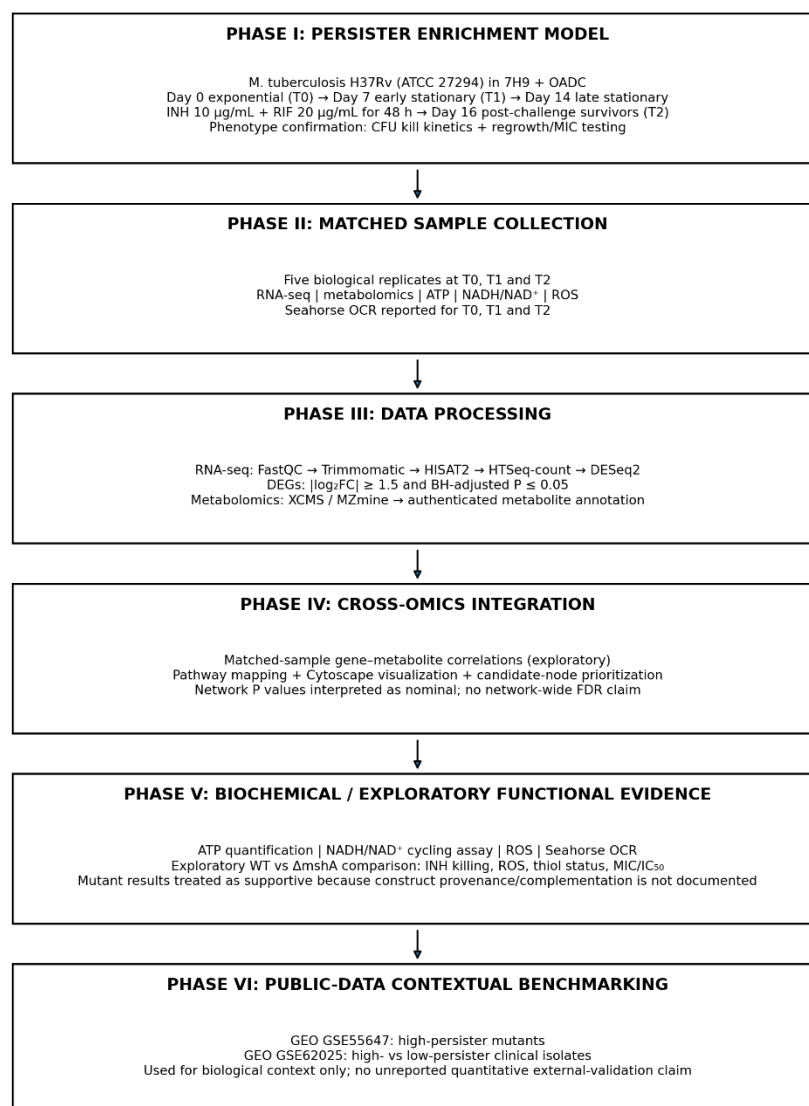


Figure 1. Schematic representation of the integrative experimental and computational workflow employed in this study.

Figure 1 summarizes the final experimental and computational workflow. It distinguishes the day-14 start of antibiotic challenge from the day-16 T2 collection; limits cross-omics claims to the reported association/pathway analyses, treats the $\Delta mshA$ comparison as exploratory supportive evidence, and labels the public-data component as contextual benchmarking rather than external validation.

Results

Establishment of the Persister Phenotype

A dedicated time-kill experiment was used to verify enrichment of a drug-tolerant survivor population after high-dose INH and RIF exposure (Figure 2). The patterns indicated that there was a fast initial kill followed by a long flat level, which is indicative of phenotype tolerance. We need to distinguish the kill-kinetics data from the planned CFU data from Table 5, which shows the overall culture time course since both types of curves use their own sampling schemes.

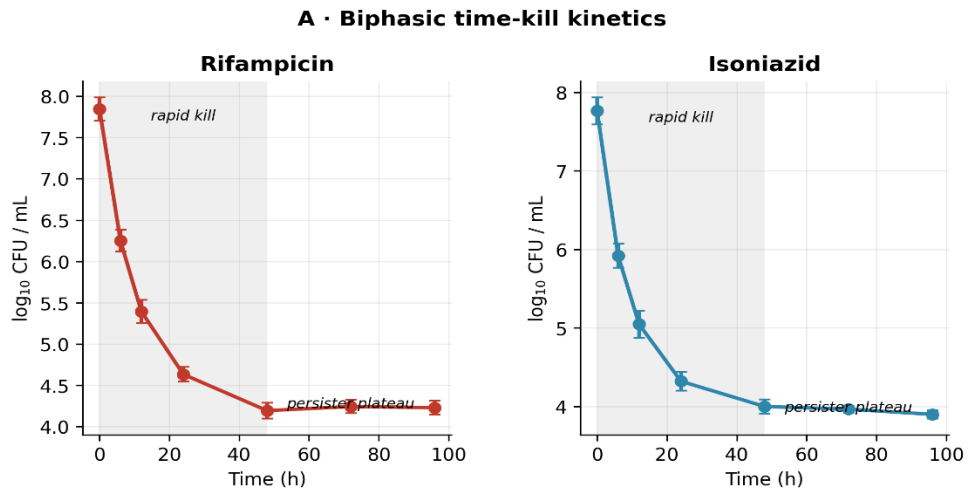


Figure 2. Time-kill kinetics curve.

To assess whether survival indicates stable antimicrobial resistance, a regrowth of the surviving population and re-testing of its susceptibility was performed. The results presented in Figure 3 show that MIC values did not change after regrowth (INH, 0.05 $\mu\text{g/mL}$; RIF, 0.25 $\mu\text{g/mL}$), thus indicating that a reversible tolerance phenotype was established rather than the acquisition of resistance. [2].

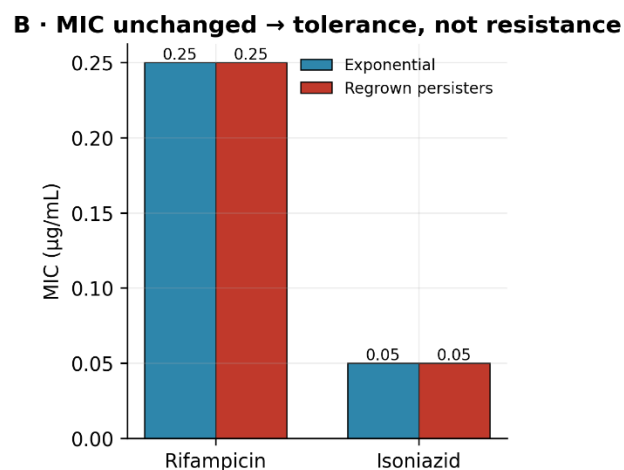


Figure 3. Bar chart for MIC confirmation.

The lack of an increase in MIC after regrowth provides support for the concept of phenotypic tolerance as opposed to the existence of stable genetic resistance. Figure 4 illustrates the corrected timeline of the experiment: T0 refers to day 0 of the growth, T1 represents day 7 of the early stationary phase, the challenge with antibiotics is held on day 14 and T2 indicates day 16 after 48 h of drug exposure. This time definition is applied consistently through the manuscript.

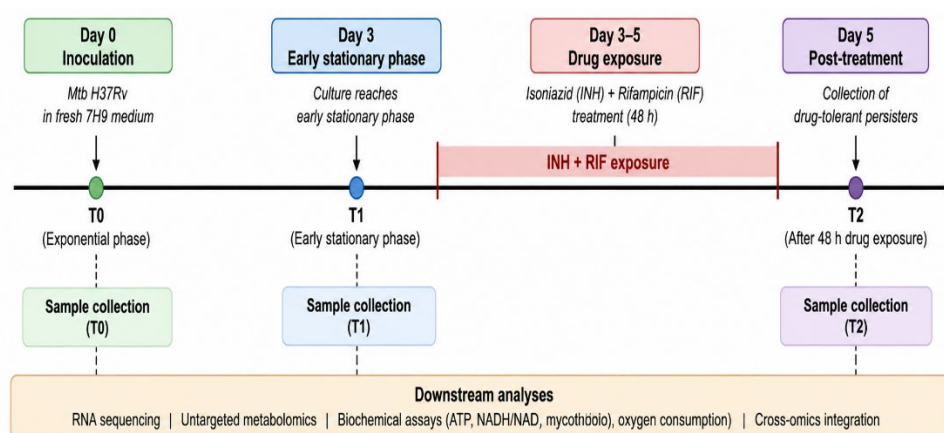


Figure 4. Experimental design schematic

Table 5 summarizes mean $\log_{10}$ CFU values across the culture time course. The reported one-way ANOVA P value (2.41×10^{-20}) is an omnibus test across time points and is therefore reported once. The mean $\log_{10}$ CFU decreased from 7.642 on day 14 to 6.550 on day 16, corresponding to an approximately 1.09- $\log_{10}$ (about 12.4-fold) decline during the antibiotic-challenge interval. A pairwise post-hoc P value is not inferred because replicate-level comparison data are not available in the manuscript record.

Table 5. Comprehensive CFU statistics for all biological replicates across experimental time-points.

Day	Phase	n	Mean $\log_{10}$ CFU	SD	Overall one-way ANOVA
0	Exponential	5	6.468	0.083	$P = 2.41 \times 10^{-20}$
3	Late exponential	5	6.966	0.026	
7	Early stationary (T1)	5	7.556	0.050	
10	Stationary	5	7.700	0.098	
14	Stationary pre-antibiotic	5	7.642	0.067	
16	Persister post-antibiotic (T2)	5	6.550	0.101	

CFU increased as cultures approached stationary phase and declined after the day-14 antibiotic challenge. The reported omnibus ANOVA indicates a strong overall time-associated difference, while persister enrichment is supported independently by the biphasic kill curve and recovery of the parental MIC after regrowth. The specific day-14-to-day-16 change is therefore described by its observed effect size rather than by an unreported pairwise significance test.

Transcriptomic Reprogramming in Persisters

RNA-seq profiles separated by experimental state in principal-component space (Figure 5). The plot of axes clearly indicates that PC1 accounts for 9.8 % of the variance while PC2 accounts for 7.6 % of the variance resulting in a total of 17.4 %. On the graph shown, the point for T2 is placed far from T0 and T1 thereby indicating the presence of wide post-challenge transcriptional remodeling in T2 samples. Since T2 occurred shortly after exposure to drug, this differentiation shows that the separation indicates a post-challenge signature that is enriched in persisters and it does not indicate that every transcript appearing in T2 is primarily due to persisting rather than due to antibiotic-induced stress.

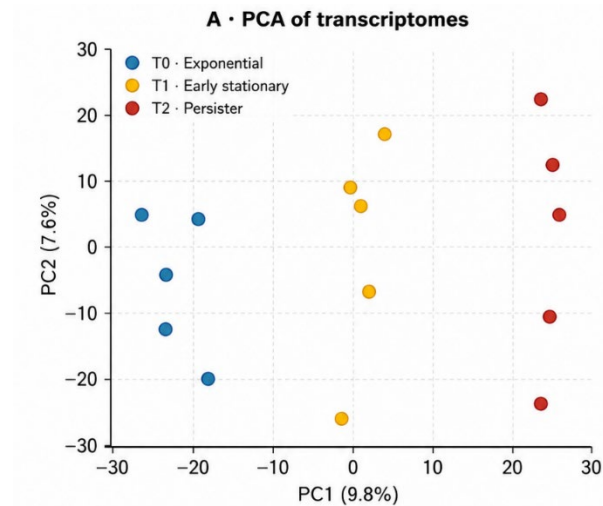


Figure 5. Principal Component Analysis (PCA) plot

PC1 and PC2 explain 9.8% and 7.6% of the variance, respectively (17.4% combined). T2 forms a distinct cluster in the displayed projection, indicating broad transcriptional remodeling relative to T0 and T1.

Hierarchical clustering of the 44 differentially expressed genes displayed in Figure 6 likewise separated T0, T1, and T2. Coordinated modules were progressively induced or suppressed across the time course, consistent with extensive reprogramming of stress response, biosynthesis, and energy metabolism.

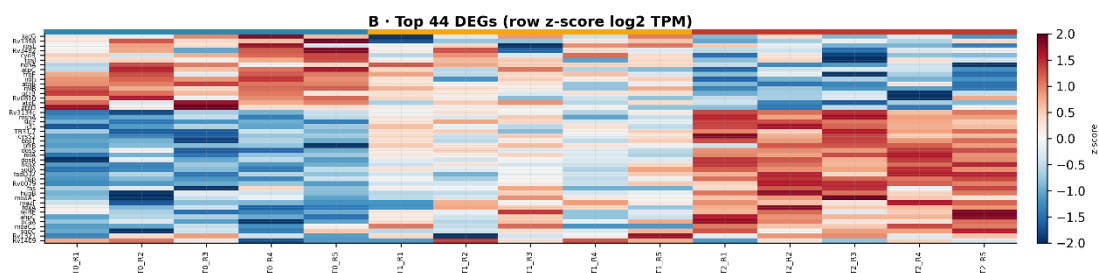


Figure 6. Hierarchical clustering heatmap

Rows represent the 44 displayed differentially expressed genes (row-wise z-scored $\log_2$ TPM), and columns represent biological replicates grouped by T0, T1, and T2. The T2 post-challenge survivor population shows a coordinated transcriptional profile distinct from T0 and T1.

Functional enrichment of T2-versus-T0 DEGs showed strong suppression of biosynthetic and energetic pathways, including ribosomal functions, oxidative phosphorylation, and translation, together with enrichment of stress-response and lipid-associated pathways (Figure 7). These patterns are consistent with translational downshift and metabolic adaptation during persistence [9,12]. Enrichment results are interpreted at the pathway level and do not imply that every member of a pathway is uniformly regulated.

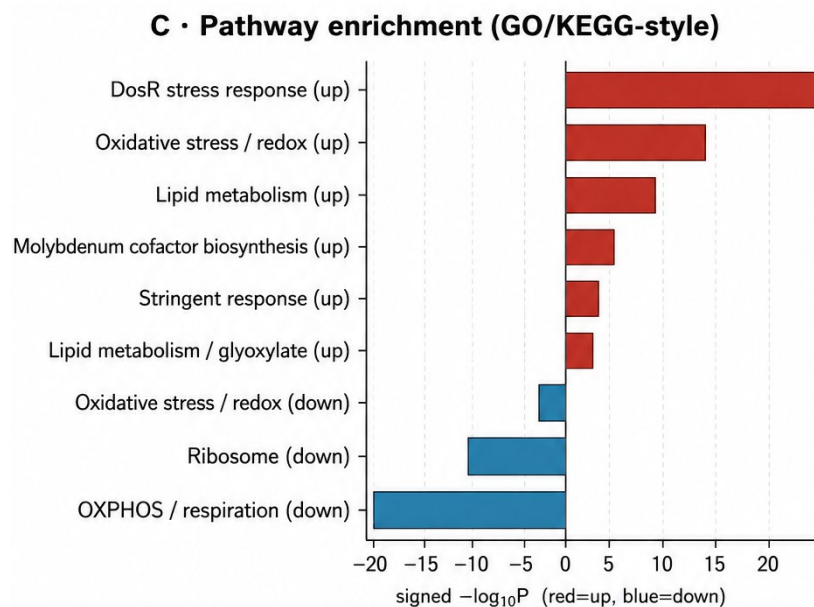


Figure 7. Signed GO/KEGG-style pathway enrichment bar plot

Bars show signed $\log_{10}(P)$ enrichment scores, with positive values denoting pathways enriched among upregulated genes and negative values denoting pathways enriched among downregulated genes. The plot is interpreted as a directional pathway summary rather than a dot plot.

Figure 8 displays representative expression distributions for *dosR*, *sigF*, and *ndh* across T0, T1, and T2. *dosR* and *sigF* increased, whereas *ndh* decreased markedly in T2. Additional ATP-synthase genes *atpB* and *atpE* were also downregulated and are reported quantitatively in Table 6, but they are not plotted in Figure 8. Transcript abundance supports reduced respiratory capacity but does not establish enzyme activity or causal direction.

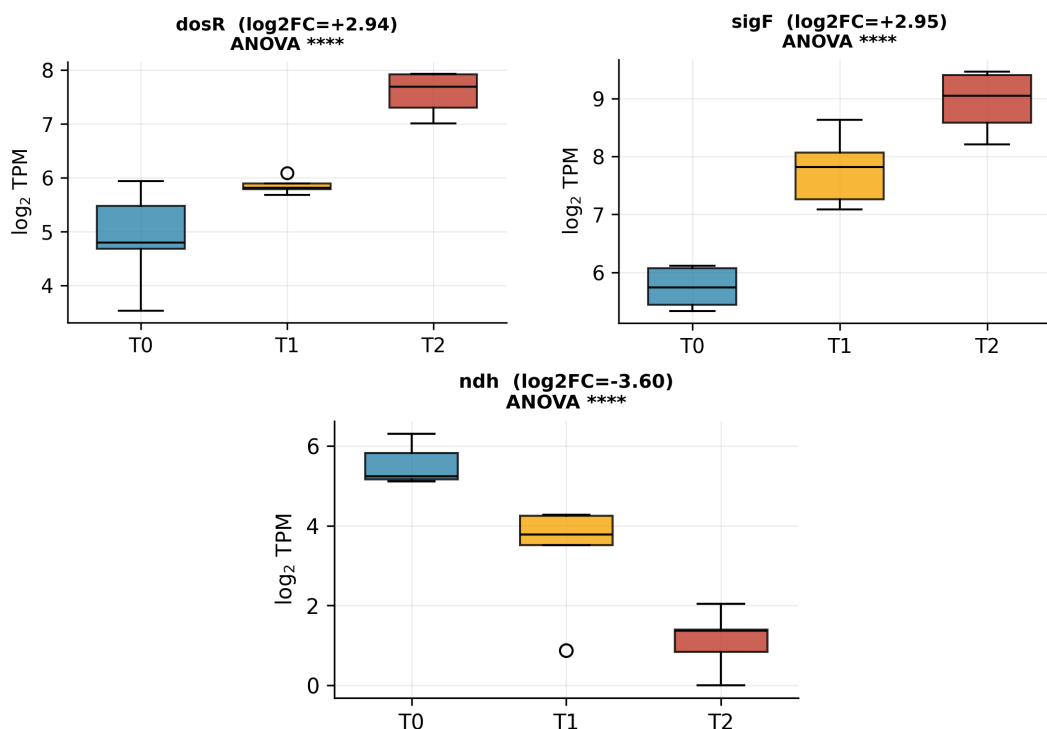


Figure 8. Boxplots of representative transcriptional regulators (*dosR*, *sigF*, and *ndh*)

The panels displayed illustrate together the activation of *dosR* and *sigF* as well as the inhibition of *ndh*. The downregulation of *atpB* and *atpE* can be seen separately in Table 6.

Table 6 contains a selective panel of various differentially expressed genes involved in the regulation of stress, respiration, ATP production, redox balancing, and glyoxylate shunt. The table provides the locus ID, symbol, name of the product and the involved pathway for each gene, log₂ fold change, FDR-adjusted P value, and regulation direction.

Table 6. Selected differentially expressed genes relevant to the redox–bioenergetic model.

Gene ID	Symbol	Product	Pathway	log ₂ FC	p _{adj}	Regulation
Rv2583c	<i>relA</i>	(p)ppGpp synthetase	Stringent response	+3.635	9.37×10^{-210}	Up
Rv1854c	<i>ndh</i>	NADH dehydrogenase (type II)	OXPHOS / respiration	-3.595	8.73×10^{-132}	Down
Rv3286c	<i>sigF</i>	Alternative sigma factor F	DosR stress response	+2.952	7.77×10^{-121}	Up
Rv3133c	<i>dosR</i>	Dormancy survival regulator	DosR stress response	+2.942	4.25×10^{-17}	Up
Rv1304	<i>atpB</i>	ATP synthase subunit a	OXPHOS / respiration	-3.042	8.67×10^{-23}	Down
Rv1305	<i>atpE</i>	ATP synthase subunit c	OXPHOS / respiration	-2.984	8.57×10^{-15}	Down
Rv0486	<i>mshA</i>	Mycothiol glycosyltransferase	Oxidative stress / redox	+2.686	7.78×10^{-19}	Up
Rv1908c	<i>katG</i>	Catalase-peroxidase	Oxidative stress / redox	-2.084	2.21×10^{-9}	Down
Rv2428	<i>ahpC</i>	Alkyl hydroperoxide reductase	Oxidative stress / redox	+2.371	6.87×10^{-20}	Up
Rv0467	<i>iclI</i>	Isocitrate lyase	Glyoxylate shunt	+2.678	2.49×10^{-21}	Up

DEG profile suggests strong activation of stringent response and dormancy-related genes (*relA*, *dosR*, *sigF*) and suppression of respiratory related genes (*ndh*, *atpB*, *atpE*). The simultaneous upregulation of *mshA* and *ahpC* and the downregulation of *katG* indicate significant changes in oxidative stress defense mechanisms.

Metabolomic Alterations in Persisters

The metabolite profile has shown a bidirectional cumulative and subtractive pattern at T2 compared with T0 (Figure 9). Depletion has been observed with energetic and glycolytic metabolites. Moreover, some TCA/glyoxylate intermediates and some amino acids were found to accumulate. Figure 9 contains only the metabolites shown in Table 7, which are verified with MSI Level 1 identification. Unsupported NADPH, ADP, GSH/GSSG, and other unverified heat map rows are not included.

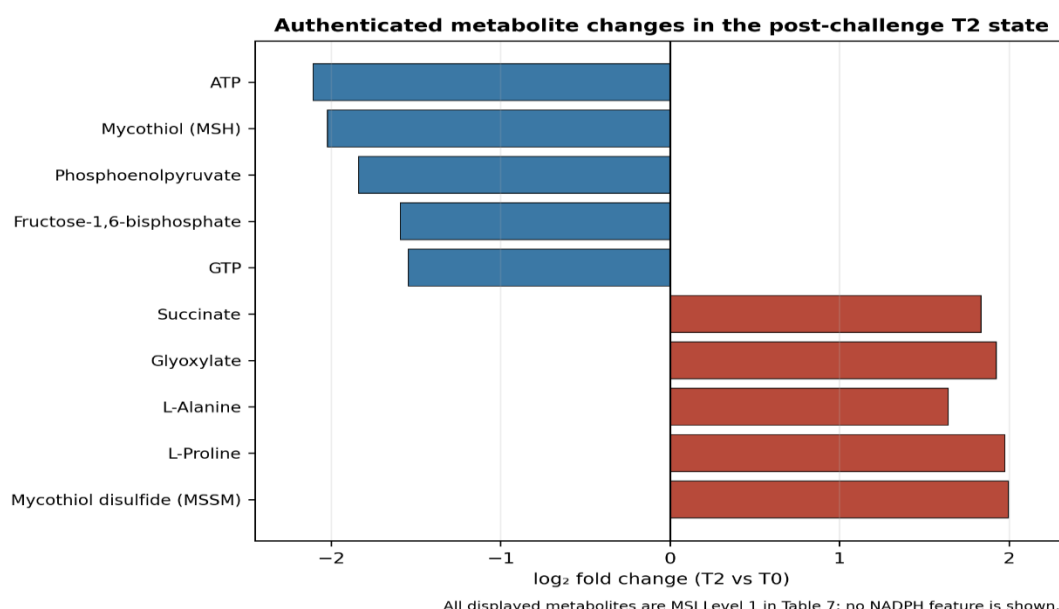


Figure 9. Authenticated metabolite log₂ fold-change profile (T2 vs T0)

The below figure refers to the figure showing T2 vs T0 log₂ fold change of ten MSI Level 1 metabolites that has been provided in Table 7. The negative values denote depletion while the positive ones indicate accumulation. There are no readings taken or inferred for NADPH measurement.

Some metabolites showed accumulation at stage T2 (see Figure 10). Succinate recorded 3.567 high fold change at p-value of 0.0067, glyoxylate showed 3.797-fold increase at p-value of 0.0138, proline recorded a 3.928-fold increase at p-value of 0.0019, and alanine showed a 3.121-fold change at p-value of 0.0026. In addition to these metabolites, citrate was also recorded. In connection with increased icl1 expression, the above data support changing TCA cycle flux and increased importance of glyoxylate shunt.

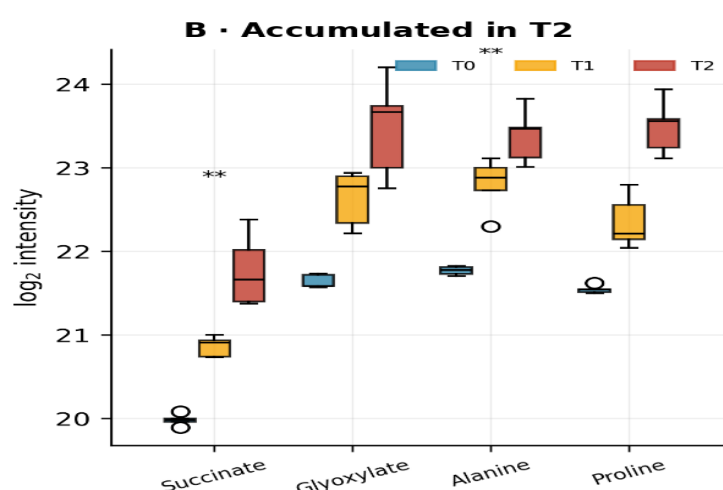


Figure 10. Boxplots of accumulated metabolites

Succinate, glyoxylate, alanine, and proline are higher in T2 than in T0. Exact T2-versus-T0 fold changes and P values are reported in Table 7; data represent five biological replicates.

Energetic and glycolytic intermediates were depleted in T2 (Figure 11). ATP fell to 0.232-fold of T0 ($P = 8.71 \times 10^{-8}$), equivalent to an approximately 4.3-fold decrease. GTP, fructose-1,6-bisphosphate, and phosphoenolpyruvate were also reduced. NADPH was not measured directly;

therefore, neither NADPH abundance nor the NADPH/NADP⁺ ratio is inferred from the metabolomics dataset.

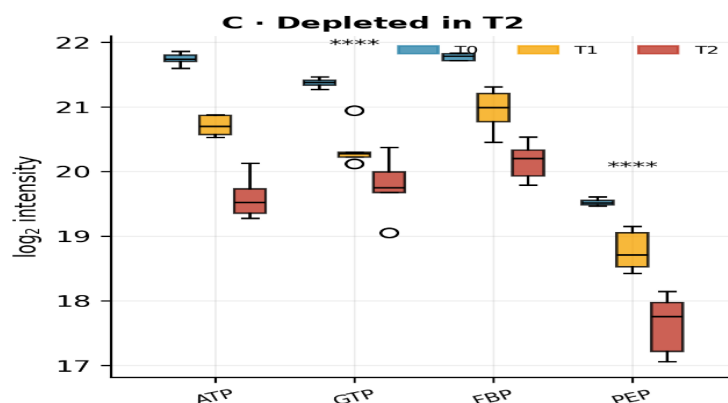


Figure 11. Boxplots of depleted metabolites

Significant depletion of ATP, GTP, fructose-1,6-bisphosphate (FBP), and phosphoenolpyruvate (PEP) is observed in T2 relative to T0. ***P < 0.001. Data represents five biological replicates.

The NADH/NAD⁺ ratio increased markedly from T0 to T2, indicating accumulation of reducing equivalents in the NAD(H) pool (Figure 12). At the same time, oxidized mycothiol (MSSM) increased approximately 3.99-fold while reduced mycothiol (MSH) fell to approximately 0.246-fold of T0. Rather than describing these observations as a uniformly 'reduced' intracellular environment, the combined data indicate redox disequilibrium across distinct cofactor systems: the NAD(H) pool becomes more reduced while the mycothiol pool becomes more oxidized. One plausible explanation is impaired reducing capacity in an NADPH-dependent thiol-recycling pathway, but NADPH was not directly measured in this study and this mechanism therefore remains a hypothesis.

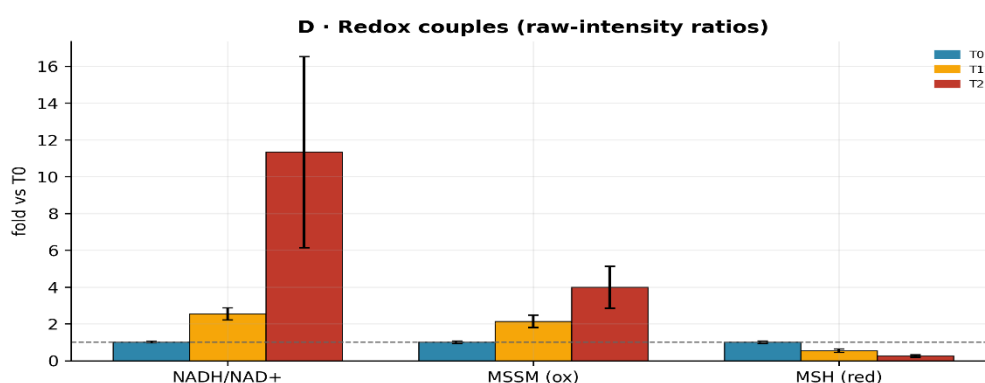


Figure 12. Redox couple bar chart

The NADH/NAD⁺ ratio is higher in T2 compared to T0, indicated also by the oxidation of mycothiol pools (high MSSM and low MSH), showing that there is redox imbalance rather than a single steady-state redox condition.

Table 7 contains the verification metabolite panel that was used for quantitative examination of T2 against T0. The ten-metabolite contained here are classified as MSI Level 1 since they were verified based on the standards. The table should be interpreted as the provided targeted metabolite panel rather than the full scan of the items in the untargeted metabolism.

Table 7. Authenticated metabolite panel used for T2-versus-T0 quantitative interpretation.

Metabolite	m/z	RT (min)	log ₂ FC	FC	P-value	MSI Level
ATP	508.1883	13.66	-2.106	0.232	8.71×10^{-8}	Level 1
GTP	523.9983	11.45	-1.543	0.343	2.93×10^{-5}	Level 1
Mycothiol (MSH, reduced)	487.1733	14.52	-2.022	0.246	1.50×10^{-7}	Level 1
Mycothiol disulfide (MSSM)	971.3313	5.65	+1.997	3.992	6.11×10^{-3}	Level 1
Fructose-1,6-bisphosphate	341.0033	10.27	-1.591	0.332	8.63×10^{-7}	Level 1
Phosphoenolpyruvate	168.9893	10.86	-1.837	0.280	4.91×10^{-6}	Level 1
Succinate	119.0343	9.75	+1.835	3.567	6.71×10^{-3}	Level 1
Glyoxylate	75.0073	1.77	+1.925	3.797	1.38×10^{-2}	Level 1
L-Proline	116.0703	10.36	+1.974	3.928	1.88×10^{-3}	Level 1
L-Alanine	90.0553	9.67	+1.642	3.121	2.61×10^{-3}	Level 1

The validated metabolite panel indicates the presence of elevated levels of chosen TCA cycle intermediates, amino acids, and oxidized mycothiol (MSSM), while there is low concentration of ATP, GTP, glycolytic intermediates, and reduced mycothiol (MSH). NADH is not mentioned in Table 7 as a metabolite that is in low concentration; the important finding regarding NAD(H) is the rising NADH/NAD⁺ ratio presented in Figure 12.

Integrated Network Analysis

Integrated network analysis was used to identify coordinated gene-metabolite associations. The strongest atpB-centered module linked atpB positively with phosphoenolpyruvate ($r = 0.879$) and negatively with glycerol-3-phosphate ($r = -0.860$) and succinate ($r = -0.855$) (Figure 13). These associations are consistent with coordinated suppression of ATP synthesis and remodeling of central-carbon metabolism, but they do not establish metabolic flux or causal direction.

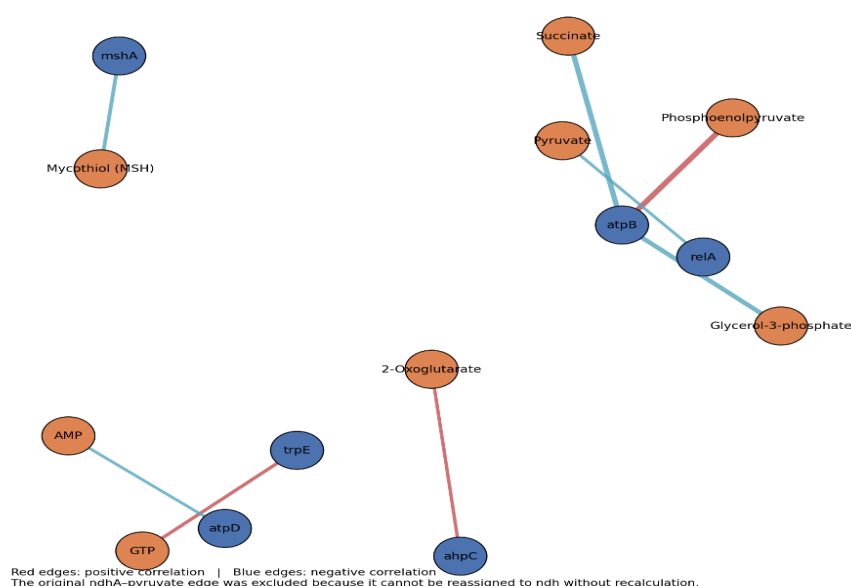


Figure 13. Correlation network plot

Figure 13 shows the revised gene-metabolite association network. Genes and metabolites are represented as distinct node classes, and edge direction denotes positive or negative correlation. The former ndhA-pyruvate edge has been eliminated as ndhA (Rv0392c) and ndh (Rv1854c) are

distinct genes. As there is insufficient summary data that allows a permissible recalculation of the *ndh* coefficient noted, the data in connection with these genes shall not permit further deductions. The name *mshA* is used consistently throughout this network.

Positive relationships also include the negative correlation between *relA* and pyruvate ($r = -0.803$), the negative correlation between *mshA* expression and high mycothiol composition ($r = -0.812$), and the positive interaction between *ahpC* and 2-oxoglutarate ($r = 0.801$). They link the stringent response with redox-like reactions and metabolism in terms of coordination of different processes.

Figure 14 brings together the information obtained from transcriptomic and metabolomic studies concerning the central carbon metabolism and the respiratory chain. Some of the respiratory components are inhibited, including *ndh*, *qcrB*, and ATP-synthase. In contrast, *Icl1* (isocitrate lyase) is activated, and glyoxylate builds up. This whole process gives a picture of slow respiration, presence of reducing equivalents, differences in TCA cycle and glyoxylate-shunt balance, and low ATP production. The scheme illustrates the process mechanistically, but it is not based on the actual measurements.

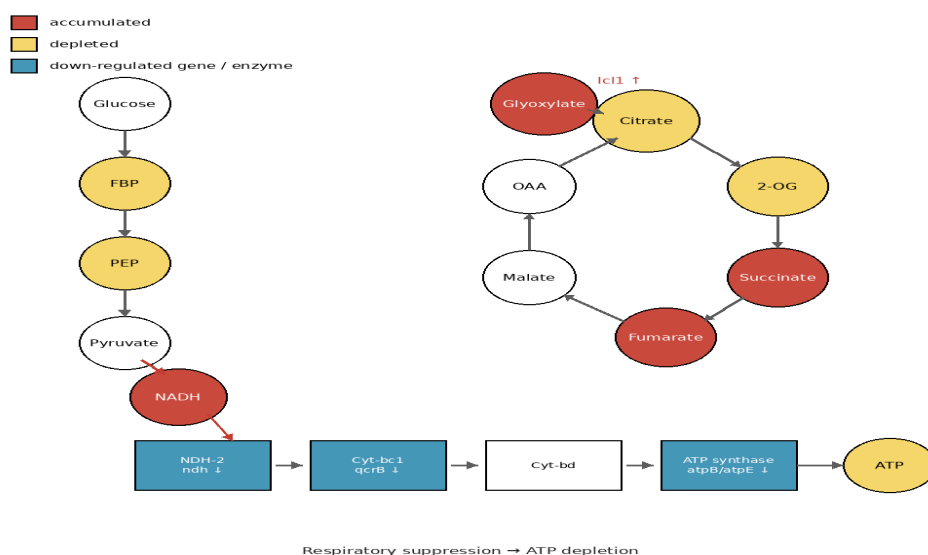


Figure 14. Integrated pathway map

The maps indicate the changes in transcripts and metabolites in relation to central carbon metabolism and respiration. The maps define an association of respiratory effects, the increase of NADH, and the decrease of ATP with no assumption about direct measurement of directionality of individual pathway steps. Table 8 summarizes the strongest gene–metabolite correlations identified in the analysis. The P values are not corrected and no FDR statement is claimed because the full correlation matrix for the analysis was not available and therefore the analysis was not valid. An incorrect substitution of *ndhA* to *ndh* was not made.

Table 8. Retained exploratory gene–metabolite correlations (Pearson $|r| > 0.8$; nominal $P < 0.01$).

Gene ID	Gene Symbol	Metabolite	Pearson *r*	P-value (exploratory)
Rv1304	<i>atpB</i>	Phosphoenolpyruvate	+0.879	1.60×10^{-5}
Rv1304	<i>atpB</i>	Glycerol-3-phosphate	−0.860	3.93×10^{-5}
Rv1304	<i>atpB</i>	Succinate	−0.855	4.87×10^{-5}
Rv1609	<i>trpE</i>	GTP	+0.819	1.88×10^{-4}
Rv0486	<i>mshA</i>	Mycothioli (MSH, reduced)	−0.812	2.36×10^{-4}
Rv1310	<i>atpD</i>	AMP	−0.805	2.94×10^{-4}
Rv2583c	<i>relA</i>	Pyruvate	−0.803	3.13×10^{-4}
Rv2428	<i>ahpC</i>	2-Oxoglutarate	+0.801	3.32×10^{-4}

The identified correlations indicate an association module which is *atpB*-centered and connects *relA*, *mshA*, *ahpC* genes with some metabolites. These associations serve only for hypothesis generation and do not allow any causal or confirmatory conclusions because of the small dataset used and absence of the correction method for the full data set.

Temporal Dynamics of Redox and ATP

Temporal analysis showed that the NADH/NAD⁺ ratio had already shifted at T1, whereas the largest ATP depletion occurred at T2 (Figure 15). This temporal ordering is consistent with the hypothesis that redox remodeling may precede maximal energetic depletion. Temporal precedence is necessary for, but does not establish, causation; both variables may also respond to a common upstream stress program.

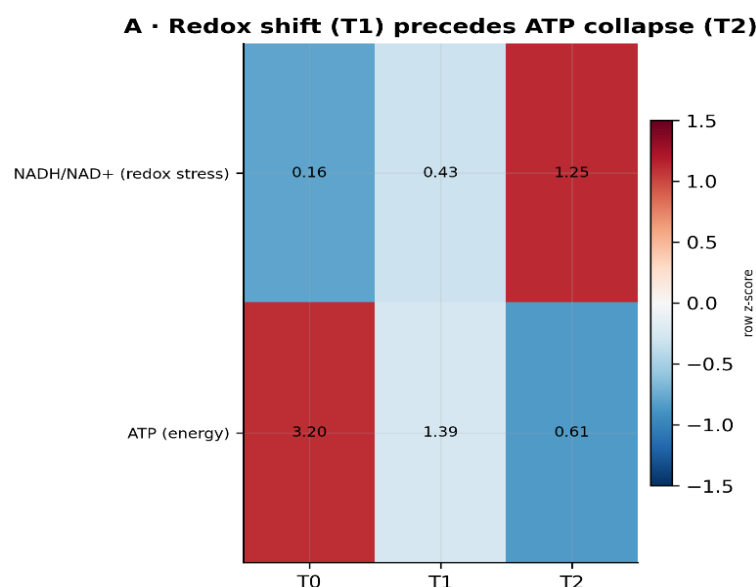


Figure 15. Time-series heatmap

The NADH/NAD⁺ shift is detectable at T1 before maximal ATP depletion at T2, supporting temporal precedence but not, by itself, a causal relationship.

Seahorse extracellular-flux analysis demonstrated progressive suppression of oxygen consumption (Figure 16). Basal OCR decreased from 118.6 ± 3.51 pmol/min at T0 to 67.96 ± 5.14 at T1 and 38.38 ± 6.31 at T2 (ANOVA $P = 4.35 \times 10^{-11}$). Maximal OCR declined similarly from 220.12 ± 7.43 to 121.46 ± 4.44 and 65.66 ± 7.92 pmol/min (ANOVA $P = 5.03 \times 10^{-13}$). The percentage spare respiratory capacity did not differ significantly ($P = 0.332$). The preserved proportional reserve should not be interpreted as proof that respiratory suppression is purely regulatory rather than damage-related; it indicates that basal and maximal respiration decline in parallel while relative reserve is broadly maintained.

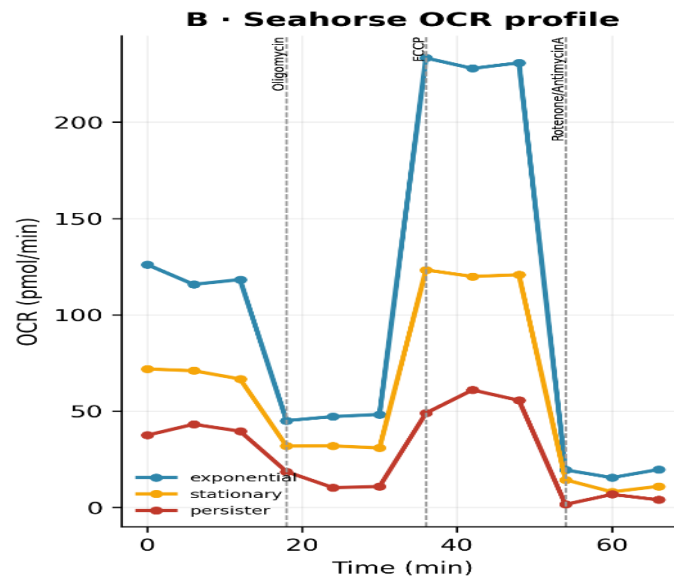


Figure 16. Oxygen Consumption Rate (OCR) Seahorse curves

Real-time bioenergetic profiles indicate the levels of OCR (pmol/min) of exponential-phase cells (T0) in comparison with early stationary-phase cells (T1) and persister cells (T2). The reduction in basal respiration and maximal respiration is highlighted. **** $P < 0.0001$.

Basal OCR and intracellular ATP declined in parallel across T0, T1, and T2 (Figure 17). This concordance provides independent physiological support for an association between respiratory suppression and bioenergetic depletion. However, parallel trajectories do not by themselves prove that reduced respiration is the sole physiological cause of ATP loss.

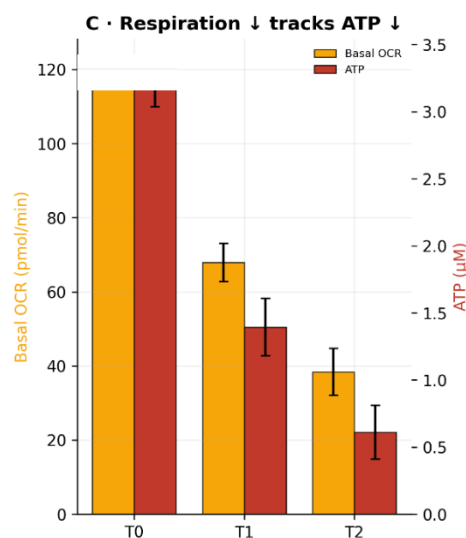


Figure 17. Direct comparative bar chart

The parallel decline in OCR and ATP supports a strong association between respiratory suppression and bioenergetic depletion. **** $P < 0.0001$.

The full statistical table of the various Seahorse parameters, with basal OCR, maximum OCR, and spare respiratory capacity values expressed as mean $\pm$ SD, can be found in Table 9.

Table 9. Seahorse bioenergetic parameters across experimental time-points.

Timepoint	n	Basal OCR (mean ± SD)	Max OCR (mean ± SD)	Spare Capacity % (mean ± SD)
T0	5	118.6 ± 3.51	220.12 ± 7.43	46.04 ± 2.33
T1	5	67.96 ± 5.14	121.46 ± 4.44	44.00 ± 4.43
T2	5	38.38 ± 6.31	65.66 ± 7.92	41.60 ± 6.01
ANOVA <i>P</i>		4.35×10^{-11} (****)	5.03×10^{-13} (****)	0.332 (ns)

Basal and maximal OCR declined significantly across the time course, while the percentage of spare respiratory capacity was not significantly different. Together, these results show marked respiratory suppression at T2 but do not by themselves distinguish regulated adaptation from drug-associated injury.

Exploratory Δ mshA Functional Assay: Evidence Consistent with Mycothiol-Dependent Protection

The role of mycothiol in promoting survival of isoniazid-treated cells was assessed, with a comparison of Δ mshA and WT H37Rv strains. The Δ mshA mutant strain exhibited more rapid killing compared to WT (Figure 18) while the latter was able to persist, maintaining an overall survival plateau. Because the original Δ mshA strain used in this experiment was not appropriately characterized with regard to its construction and the future experiments mentioned in this section were not performed, this experiment should be treated as supportive rather than definitive.

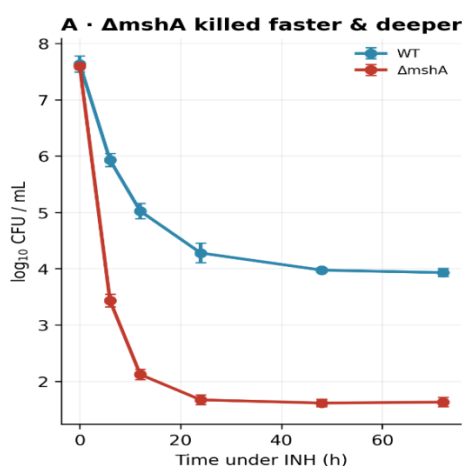


Figure 18. Mutant kill curves

The Δ mshA mutant shows faster and deeper killing than WT, supporting a protective role for mycothiol during antibiotic exposure.

The increase of intracellular ROS levels in the INH-treated Δ mshA was substantial, as it was almost 3.8 times in comparison with WT ($P < 0.001$) (Figure 19). This indicates that impaired redox buffering plays a role in the antibiotic hypersensitivity of the mutant strain. However, it does not prove that ROS is the only mechanism contributing to the effect, as DCFH-DA detects a broad oxidative signal.

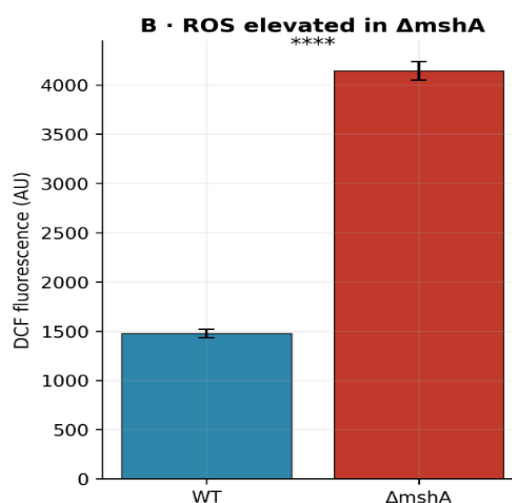


Figure 19. Intracellular ROS bar chart

Δ mshA exhibit with significant increase in the respective ROS levels as compared to wild type (WT), providing evidence for the association between impaired redox buffering and hypersensitivity to antibiotics. *** $P < 0.001$.

When a targeted measurement of thiols was performed, almost every molecule of mycothiol was depleted in the Δ mshA strain while ergothioneine remained detectable (Figure 20). This result demonstrates the mycothiol-deficient phenotype, however, it can't serve alone as a convincing evidence that the skills of Δ mshA in this regard are completely linked to missing genotypes and compensations.

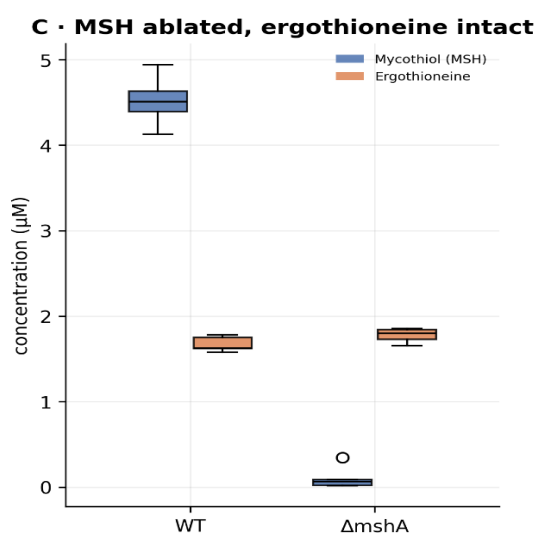


Figure 20. Mycothiol quantitation boxplot

Reduced mycothiol (MSH) is near-absent in the Δ mshA strain, whereas ergothioneine remains detectable. This supports the expected thiol phenotype but is interpreted cautiously because construct provenance and complementation are not documented.

Table 10 summarizes isoniazid susceptibility in WT and Δ mshA strains. The mutant MIC was 0.025 μ g/mL versus 0.05 μ g/mL for WT (0.5 $\times$ WT), and the IC_{50} was 0.014 μ g/mL versus 0.031 μ g/mL for WT (approximately 0.45 $\times$ WT). These values correspond to a twofold lower MIC and an approximately 2.2-fold lower IC_{50} concentration in the mutant.

Table 10. MIC and IC₅₀ values for isoniazid against WT and Δ mshA strains.

Strain	Drug	MIC (μ g/mL)	IC ₅₀ (μ g/mL)	MIC fold vs WT	IC ₅₀ fold vs WT
WT	Isoniazid	0.05	0.031	1.0	1.0
Δ mshA	Isoniazid	0.025	0.014	0.5	0.452

The lower MIC and IC₅₀ in the Δ mshA comparison are consistent with increased isoniazid susceptibility when mycothiol biosynthesis is disrupted. These observations complement the kill-curve and ROS data but remain exploratory in the absence of documented strain provenance and complementation.

Table 11 integrates selected transcriptomic, metabolomic, and redox findings to summarize the direction and magnitude of the most prominent changes. The entries are selected biological features, not a formal ranking of network hubs or proven causal drivers.

Table 11. Selected key genes, authenticated metabolites, and redox metric in the integrative analysis of Mtb persisters.

Category	Feature	Direction	Biological Function	Fold Change	Statistical significance
Genes	<i>relA</i> (Rv2583c)	↑	Stringent response, (p)ppGpp synthetase	log ₂ FC = +3.635	adj. P = 9.37×10^{-210}
	<i>ndh</i> (Rv1854c)	↓	Type II NADH dehydrogenase (respiration)	log ₂ FC = -3.595	adj. P = 8.73×10^{-132}
	<i>sigF</i> (Rv3286c)	↑	Alternative sigma factor, persistence	log ₂ FC = +2.952	adj. P = 7.77×10^{-121}
	<i>dosR</i> (Rv3133c)	↑	Dormancy survival regulator	log ₂ FC = +2.942	adj. P = 4.25×10^{-17}
	<i>atpB</i> (Rv1304)	↓	ATP synthase subunit a (OXPHOS)	log ₂ FC = -3.042	adj. P = 8.67×10^{-23}
	<i>atpE</i> (Rv1305)	↓	ATP synthase subunit c (BDQ target)	log ₂ FC = -2.984	adj. P = 8.57×10^{-15}
	<i>mshA</i> (Rv0486)	↑	Mycothiol glycosyltransferase	log ₂ FC = +2.686	adj. P = 7.78×10^{-19}
	<i>katG</i> (Rv1908c)	↓	Catalase-peroxidase (INH activation)	log ₂ FC = -2.084	adj. P = 2.21×10^{-9}
	<i>ahpC</i> (Rv2428)	↑	Alkyl hydroperoxide reductase	log ₂ FC = +2.371	adj. P = 6.87×10^{-20}
	<i>iclI</i> (Rv0467)	↑	Isocitrate lyase (glyoxylate shunt)	log ₂ FC = +2.678	adj. P = 2.49×10^{-21}
Metabolites	ATP	↓	Energy currency	FC = 0.232	P = 8.71×10^{-8}
	GTP	↓	Energy/nucleotide metabolism	FC = 0.343	P = 2.93×10^{-5}
	Fructose-1,6-bisphosphate	↓	Glycolytic intermediate	FC = 0.332	P = 8.63×10^{-7}
	Phosphoenolpyruvate	↓	Glycolytic intermediate	FC = 0.280	P = 4.91×10^{-6}
	Mycothiol (MSH, reduced)	↓	Redox buffer (reduced form)	FC = 0.246	P = 1.50×10^{-7}
	Mycothiol disulfide (MSSM)	↑	Redox buffer (oxidized form)	FC = 3.992	P = 6.11×10^{-3}
	Succinate	↑	TCA cycle intermediate	FC = 3.567	P = 6.71×10^{-3}
	Glyoxylate	↑	Glyoxylate shunt intermediate	FC = 3.797	P = 1.38×10^{-2}
Redox metric	L-Proline	↑	Amino acid (stress response)	FC = 3.928	P = 1.88×10^{-3}
	NADH/NAD ⁺ ratio	↑	Redox imbalance	~3.3-fold increase	< 0.001

The integrated profile combines suppression of respiratory genes (*ndh*, *atpB*, *atpE*) with activation of stringent-response and dormancy-associated genes (*relA*, *dosR*, *sigF*), depletion of ATP and glycolytic intermediates, accumulation of selected TCA/glyoxylate-shunt metabolites, and oxidation of the mycothiol pool. Together, these observations define a coherent metabolic-redox signature of the post-challenge persister-enriched state.

Discussion

The current investigations bring together transcriptomic, metabolomic, respiration, and focused functional tests methods for describing a post-challenge *Mtb* persister-accumulated condition. The major discovery is not a single molecular mechanism, but a coordinated bioenergetic phenotype including the inhibition of genes responsible for respiration and ATP generation, decreased oxygen uptake followed by the drop in ATP concentration, increase in NADH and NAD⁺ ratio, and oxidation of mycothiol. The agreement of the described data sets provides evidence for a Redox-ATP Axis Model postulating the connection between redox imbalance and inhibited respiration and a low energy persister state. Still, the presented data indicates the existence of a mechanistic association which is not the proof of existence of a direct causal relation.

The redox findings call for careful interpretation, because there are several cofactor pools behaving in different ways. T2 cells contained an excess of reducing equivalents in the NAD(H) pool, which was demonstrated by the increased NADH/NAD⁺ ratio, whereas at the same time there was a shift of mycothiol from reduced MSH form toward oxidized MSSM state, thus resulting in redox disequilibrium rather than in uniformly reduced intra-cellular environment. One possible explanation is that NADH-dependent respiration and NADPH-dependent thiol recycling work as two independent processes, although no data about NADPH/NADP⁺ ratio are available. Consequently, NADPH depletion cannot be considered as a valuable result of this work. There are plans to test this hypothesis by performing direct measurements of NADPH/NADP⁺ ratio in future studies.

The ATP concentration at T2 was about 4.3 times less than that at T0, indicating a major energy downshift occurring. However, the earlier measurement at T1 shows a change in NADH/NAD⁺ together with a decrease in oxygen consumption rate. However, the fact that events at T1 occur earlier than at T2 does not rule out other possible explanations, including a stressed condition leading to both events. Thus, while the results seem to indicate causal relationship, the verification of this hypothesis would necessitate experimental work.

katG's behavior adds another case in point regarding the necessity of contextualizing survivor-state data. In the case of *katG*, which codes for the enzyme catalase-peroxidase responsible for the effective activation of isoniazid, the transcription level decreased at the time point T2 ($\log_2\text{FC} = -2.084$), while *ahpC* level increased ($\log_2\text{FC} = +2.371$). This does not negate the initial bactericidal phase shown in Figure 2, as the T2 transcriptome shows cells that remained after the more susceptible part of a sample had been killed off by isoniazid. Reduced expression of *katG* can be attributed to the development of a state of temporary tolerance; *ahpC* expression enhancement can be interpreted more cautiously as an increase in stress-regulated protective mechanisms rather than the development of an alternate INH activation mechanism. Return of the MIC value characteristic of the parent strain after the regrowth additionally confirms the temporary nature of this response.

The results also provide clarity regarding the way in which ATP directed therapy is interpreted. Bedaquiline, being an ATP synthase antagonist, actually caused disturbance in ATP levels in previous studies. Thus, if low ATP level is measured in our study, it does not mean that bedaquiline is ineffective against persistent bacteria.

The major conclusion of our research is that disturbances in respiratory fluctuation, antioxidant system and ATP levels have been combined into a single vulnerability. It still remains unknown whether simultaneous mycothiol-based buffering and ATP synthase blockade leads to additive or synergistic effect.

In contrast to previous studies focusing on one specific omic or one metabolic pathway only [8,9,13,14,18], this study combines the analysis of the transcriptome and metabolome, as well as oxygen consumption and various chemical analysis data, all within one and the same framework. The results of this study expand the previously known findings on respiratory plasticity and low-energy survival by revealing the signature of the coordinated redox-respiratory process, related both to NDH and ATP synthase, as well as the NADH levels in cells. In case of the analysis carried out for Δ mshA mutants, although signaling the importance of mycothiol, the results cannot be acknowledged as evidence of genetic confirmation due to the lack of data about the origin of the strains used in the experiment. The revised interpretation therefore identifies the Redox–ATP axis as a strongly supported mechanistic model and a priority for direct perturbation studies, not as a proven principal cause of energy collapse.

Table 12. Comparative synthesis of the current study with five key prior investigations of Mtb persistence.

Study	Omic Approach	Key Focus / Model System	Knowledge Gap Addressed	Contribution of Our Study
[8]	Transcriptomics (Microarray)	Nutrient starvation model; stationary-phase transcriptome	Single-omic approach; no temporal metabolomics; lacked metabolic connectivity	Multi-omics integration with temporal resolution; links transcriptional changes to metabolic output
[14]	Transposon mutagenesis	Genetic essentiality; cholesterol catabolism	Focus on genetic determinants rather than dynamic persister state	Identify dynamic metabolic hubs and transcriptional regulators of persistence
[9]	ChIP-chip / Transcriptomics	DosR regulon dynamics under hypoxia	Transcriptional focus only; no integration with metabolomic or redox data	Places redox/ATP remodeling alongside DosR-associated stress regulation without assigning unproven upstream causality.
[13]	Lipidomics / Metabolomics	Lipid droplet accumulation; triacylglycerol metabolism	Uni-omic (lipid-centric); lacks temporal dynamics and transcriptional connectivity	Integrates lipid/redox metabolomics with transcriptomics and temporal bioenergetic measurements; supports temporal association.
[18]	Biochemical assays	ATP homeostasis; respiratory flexibility	Identifies ATP depletion but not upstream drivers; lacks mechanistic explanation	Links redox disequilibrium, respiratory suppression, and ATP depletion in a mechanistic association model.
Our Study	Integrative multi-omics	Temporally resolved persisted model	Integrated analysis of the association between redox imbalance and ATP depletion.	Defines a testable Redox-ATP Axis Model and prioritizes candidate hubs for direct perturbation studies.

Several limitations define the strength of the conclusions. First, T2 was collected after 48 h of INH/RIF exposure, so its transcriptomic and metabolomic profile may contain both persistence-associated biology and acute drug-injury responses; an age-matched day-16 untreated stationary-phase control would be required to separate these effects formally. Second, the study uses a single reference strain (H37Rv), limiting strain-level generalizability despite contextual public datasets. Third, gene–metabolite correlations are strongly structured by the T0→T1→T2 trajectory, their reported P values are nominal, and the full tested matrix is unavailable for valid network-wide FDR reconstruction; the network is therefore exploratory. Fourth, NADPH/NADP⁺ was not directly measured, so the proposed link between thiol oxidation and NADPH-dependent recycling remains

hypothetical. Fifth, independent gene-expression confirmation (for example, qRT-PCR using more than one validated reference gene) is not included in the final reported evidence. Sixth, the Δ mshA strain source/construction, genotype confirmation, and complemented control are not documented in the available record, so the mutant comparison is supportive rather than definitive genetic evidence. In conclusion, the targeted metabolite P values in Table 7 cannot supersede multiple-testing regulation throughout the whole untargeted feature realm. The limitations thus put the model in mechanistic correlation instead of definite causation.

Therefore, the following most useful experiments should be perturbational rather than solely descriptive. The measurement of the NADPH/NADP⁺ would verify if NAD(H) and the thiol redox pools are functionally detached from each other. The control that has not undergone any treatment and lasted for 16 days in a stationary phase would differentiate between persistence-related remodelling and acute antibiotic stress. Independent qRT-PCR validation of selected transcripts with at least two recognized reference genes would ensure the reproducibility of the transcript expression. Genetic or CRISPRi interference of *ndh*, *relA*, *atpE*, and genes responsible for the mycothiol pathway should show if prioritized nodes are necessary for the low-energy status. Metabolic-rescue tests which modify respiratory redox equilibrium and then define ATP levels would provide more significant evidence of the directional link.

Conclusion

The current integrative study establishes a consistent redox-bioenergetic profile linked with *M. tuberculosis* persister emergence. The post-challenge T2 group exhibited downregulation of *ndh* and ATP-synthase genes, diminished oxygen uptake, significant depletion of ATP, elevated NADH/NAD⁺ ratio, oxidation of the mycothiol pool, and changes in central-carbon metabolism. An explorative comparison of Δ mshA strains revealed an increased susceptibility to isoniazid following inhibition of mycothiol biosynthesis, but the evidence for mutations remains inconclusive due to strain origin, genotype determination, and complementation not being established. Overall, the unified transcriptomic, metabolomic, respiratory, and biochemical findings substantiate the Redox-ATP Axis Model that connects respiratory inhibition and redox imbalance with the low-energy drug-resistant state. The model is formulated as mechanistic and empirical rather than purely causal since T2 samples followed antibiotic treatment, NADPH has not been measured directly, and the association map presents temporal and exploratory structure. These limitations identify clear next steps: age-matched untreated controls, direct NADPH/NADP⁺ measurement, independent transcript validation, FDR-controlled network analysis from the complete correlation matrix, and targeted genetic perturbation with complementation. By separating reversible phenotypic tolerance from genetic resistance and by restricting conclusions to analyses that are explicitly reported, the study provides a more rigorous framework for investigating metabolic vulnerabilities of *Mtb* persisters.

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