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Examining the basis of isoniazid tolerance in nonreplicating *Mycobacterium tuberculosis* using transcriptional profiling

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Abstract

Background—Understanding how growth state influences *Mycobacterium tuberculosis* responses to antibiotic exposure provides a window into drug action during patient chemotherapy. In this article, we describe the transcriptional programs mediated by isoniazid (INH) during the transition from log-phase to nonreplicating bacilli, from INH-sensitive to INH-tolerant bacilli respectively, using the Wayne model.

Results—INH treatment did not elicit a transcriptional response from nonreplicating bacteria under microaerophilic conditions (NRP2), unlike the induction of a robust and well-characterized INH signature in log-phase bacilli.

Conclusion—The differential regulation (between drug-free NRP2 and log-phase bacilli) of genes directly implicated in INH resistance could not account for the abrogation of INH killing in nongrowing bacilli. Thus, factors affecting the requirement for mycolic acids and the redox status of bacilli are likely responsible for the reduction in INH efficacy. We speculate on additional mechanisms revealed by transcriptome analysis that might account for INH tolerance.

While effective at resolving active *Mycobacterium tuberculosis* (TB) disease, current tuberculosis chemotherapy requires multiple drugs to be taken over 6 months to clear infection of genotypically drug-sensitive bacilli. This long treatment period exerts huge demands on healthcare infrastructure and finances, and risks the emergence of drug resistance through poor patient compliance. The search for a more effective shortened drug regimen is complicated by the presence of persistent organisms, likely phenotypically drug-tolerant bacilli that require prolonged drug treatment to clear [1,2].

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Phenotypic drug tolerance, unlike genetically defined resistance, is a transient bacterial state in which many antibiotics are ineffective. Tolerance is hypothesized to be dependent upon pre-existing subpopulations of genetically susceptible bacteria in a slow/nonmultiplying state that are refractory to drug action [3]. This small fraction of phenotypically distinct bacteria enable bacterial populations to survive dramatic changes in the environment [4]. In addition to stochastic mechanisms that create subpopulations of nongrowing cells in a phenotypically heterogeneous bacterial community, recent evidence suggests that tolerant bacterial subpopulations are generated after antibiotic stress [5]. In TB clinical settings, phenotypically tolerant bacilli may be responsible for the persistence of *M. tuberculosis* bacilli through the drug-treatment period [6]. We speculate that phenotypically tolerant bacilli in patients may exist as a result of:

- Stochastic processes;
- Exposure of bacilli to multiple microenvironments imposing individual constraints on growth that may be temporally and spatially separated;
- Prolonged antibiotic stress.

There is sparse information defining subpopulations of *M. tuberculosis*, but both clinical data and the demonstration of subpopulations from *in vitro* culture and animal models [7,8] indicate that subpopulations exist. Further *in vivo* evidence is provided by quantitative variations in lipid inclusion body staining of human sputum-derived bacilli [9] and the observation that bacilli in different locations of human lung tissues display unique gene-expression patterns [10]. Furthermore, murine models of chronic infection show increased tolerance to isoniazid (INH), suggesting slow growth rates are associated with reduced drug efficacy [11].

Isoniazid is a front-line drug in TB treatment and prophylaxis. Bactericidal activity during the first 2 days of TB treatment is almost entirely due to INH eliminating approximately 90% of bacilli [12]. However, minimal killing is observed after exposure of nonreplicating *M. tuberculosis* bacilli to INH *in vitro* [13], so it is unlikely that INH contributes to the clearance of persistent bacilli in patients during the extended treatment phase. INH is a prodrug that enters the cell by passive diffusion where it is converted by a *katG*-encoding catalase–peroxidase to an active INH–NAD adduct (reviewed elsewhere [14]). The primary target of INH–NAD is InhA, an enoyl-ACP reductase, part of the FASII cycle, which is required for mycolic acid synthesis and maintenance of the mycobacterial cell wall structure [15,16]. The INH–NAD(P) adduct(s) are likely to inhibit multiple secondary targets, such as the NADPH-dependent dihydrofolate reductase, which may also contribute to INH-mediated killing [17]. Resistance to INH is most frequently determined by mutations in *katG*, inactivating the catalase–peroxidase required for INH prodrug conversion. Mutations in the promoter of *inhA*, resulting in the overexpression of the target (InhA) or affecting the InhA active site also confer resistance [18]. Mutations in a number of other genes have been identified in INH-resistant *M. tuberculosis* strains [19]. However, approximately 22–29% of INH-resistant strains do not possess mutations in genes known to affect INH-resistance [20,21], indicating that there may be unexplored mechanisms that influence INH efficacy in *M. tuberculosis*.

Transcriptional profiling by microarray analysis has been used extensively to define the *M. tuberculosis* genome-wide responses to antibiotic exposure (classifying drug action and identifying possible resistance mechanisms [22–24]) and to fluctuations in environmental factors such as limited oxygen [25–27] or nutrient starvation [28,29] are reviewed in [30]. In one such model of mycobacterial persistence, developed by Wayne and colleagues, oxygen levels are gradually reduced by stirring liquid cultures in sealed tubes [31]. Under these conditions, bacilli enter a physiological state known as nonreplicating persistence (NRP), which is characterized by two distinct stages. The first, NRP1, occurs in microaerophilic conditions when the concentration of oxygen in the medium descends to 1%. During this phase, bacilli shut down DNA synthesis and cell division, restrict biosynthetic activity and use alternative energy sources to stabilize and protect essential cell components. The second NRP2 stage, where general metabolism is reduced further and replication ceases, occurs in anaerobic conditions when the dissolved oxygen concentration drops below 0.06% [13]. Nonreplicating bacteria may become tolerant to antibiotic exposure [32,33]; bacilli in NRP2 exhibit some definite but reduced killing with rifampicin and are highly tolerant to INH and ciprofloxacin [31]. Furthermore, the absence of an INH-inducible transcriptional signature has been used to characterize *in vitro* and *in vivo* *M. tuberculosis* dormant states [34].

In an attempt to understand why nonreplicating bacilli are refractory to killing by INH and, thus, to identify putative INH-tolerance mechanisms mediated by changing transcript abundance, we defined the transcriptional responses to INH exposure in log-phase and Wayne model NRP2 bacilli. Having contrasted the changing pattern of gene expression through the transition from log-phase to NRP2 with the INH-induced transcriptome signature, we also discuss INH tolerance in nongrowing populations and consider mechanisms that may influence drug tolerance.

Experimental

■ Aerobic log-phase & Wayne model cultures

Seed cultures of *M. tuberculosis* H37Rv were incubated in 10 ml Dubos liquid medium (BD, Franklin Lakes, USA) supplemented with Dubos medium albumin, with shaking at 37°C for 6 days to an optical density 580 nm of 0.4. Both aerobic and NRP cultures were set up in glass tubes (125 × 20 mm) with screw-top lids containing 1.5 mm magnetic stir bars and stirred at 120 rpm as characterized by Wayne [35]. Log-phase bacilli were incubated for 3 days in 10 ml Dubos medium in tubes with loosened caps. The Wayne model cultures were incubated for 6 days (NRP1) or 21 days (NRP2) in 17 ml Dubos medium in tubes with sealed lids (with a resulting head-space ratio of 0.5). Growth was monitored by measuring optical density every 24 h. RNA was isolated from aerobic log-phase and Wayne model cultures on two independent occasions.

■ INH treatment

Cultures were treated with 0.2 µg/ml INH final concentration (from 100 µg/ml stock solution, Sigma, Saint Louis, USA) at days 6 and 21 in the Wayne model (for NRP1 and NRP2, respectively), and at day 3 in log-phase aerobic cultures (Supplementary Figure 1 [available at www.future-science.com/toc/fmc/2/8]). An equivalent volume of sterile water

was added to drug-free carrier control samples. Mycobacterial RNA was extracted after exposure to INH for 4 h. Prior to addition, syringes containing INH (or sterile water) were pre-incubated in an anaerobic cabinet overnight, to eliminate oxygen from the solutions and prevent the reintroduction of dissolved oxygen to the NRP cultures.

■ Mycobacterial RNA isolation

RNA was extracted using the GTC/TRIZOL method [36,37]; at each timepoint the culture volume (eight tubes per condition) was added to four volumes of 5 M guanidine thiocyanate solution and the bacteria harvested by centrifugation. The bacilli were lysed in TRIzol using a reciprocal shaker and the nucleic acid extracted with chloroform, before isopropanol precipitation. The RNA samples were purified and DNase I treated on RNeasy columns (Qiagen, Hilden, Germany). Total RNA concentration was determined by spectrophotometry (NanoDrop ND-1000 3.1, Thermo Fisher Scientific, Waltham, MA, USA) and size distribution assessed using an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, USA).

■ Microarray hybridization

Whole-genome microarrays generated by the Bacterial Microarray Group at St George's (ArrayExpress accession number A-BUGS-23 [101]) were hybridized as described previously [37, 38]. Briefly, 5 µg of cDNA derived from each RNA sample and 2 µg of *M. tuberculosis* H37Rv genomic DNA were labeled with Cy5-dCTP and Cy3-dCTP, respectively. RNA extracted from two biological replicates were hybridized in triplicate, resulting in six hybridizations per condition. Microarrays were scanned at 532 and 635 nm corresponding to Cy3 and Cy5 excitation maxima, respectively, using the Affymetrix 428™ Array Scanner (MWG Biotech, Ebersberg, Germany). Comparative spot intensities from the images were calculated using Imagen 5.5 (BioDiscovery, El Segundo, USA) and the data imported into GeneSpring GX 7.3.1 (Agilent Technologies, Santa Clara, USA) for analysis. The data were normalized to the 50th percentile of all genes detected to be present and filtered to include genes flagged to be present in 80% of the arrays.

■ Transcriptional profiling analysis

Genes that were significantly differentially expressed between growth states or after INH treatment were identified using a t-test with Benjamini and Hochberg multiple testing correction ($p < 0.05$) and a minimum fold-change of 1.5. Genes were hierarchically clustered using Cluster and the results displayed using Treeview [39]. The hypergeometric distribution was used to determine if previously identified gene-expression signatures or genomic functional categories were significantly enriched in each comparison [40]. Significantly differentially expressed genes are detailed further in Supplementary Tables 1 & 2 (Available at www.future-science.com/toc/fmc/2/8). Fully annotated microarray data from this study have been deposited in BµG @Sbase (accession number: E-BUGS-104 [102]) and ArrayExpress (accession number: E-BUGS-104).

■ Quantitative RT-PCR

Primers and probes for *cydA*, *efpA*, *icl*, *inhA*, *iniA*, *kasA*, *katG*, *narX*, *ndh* and *tgs1* were designed using the Primer Express 1.0 software (Life Technologies, Carlsbad, USA) and are detailed further in Supplementary Table 3 (Available at www.future-science.com/toc/fmc/2/8). All probes were labeled with 5-carboxyfluorescein (FAM) at the 5′, and *N,N,M′,N′*-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3′. An internal control, *sigA* (labeled with VIC), was used to normalize mRNA levels. Mycobacterial RNA (5 μg) was reverse transcribed in a total volume of 25 μl using random primers (Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. A total of 1 μl of template cDNA was used for qRT-PCR with AmpliTaq Gold polymerase, alongside no template and no RT negative controls. qRT-PCR was performed using an ABI PRISM 7700 Sequence detector system (Life Technologies, Carlsbad, USA), in amplification conditions of 95°C for 10 min followed by 50 cycles at 95°C for 15 s and at 60°C for 60 s. Each reaction was performed in triplicate. The Ct method [41] was used to determine changes in relative gene expression.

Results & discussion

■ Gene-expression programs illustrate the transition from log-phase to nonreplicating persistence

To examine the relationship between growth rate and phenotypic drug tolerance, we used genome-wide microarray RNA profiling and qRT-PCR to map the transcriptional status of *M. tuberculosis* with and without exposure to INH in log-phase and in the Wayne model of NRP. We hypothesize that transcriptome patterns may reveal mechanisms that explain INH tolerance, such as changes in expression levels for genes involved in drug efflux, alteration in target levels (*inhA*) or reduced *katG* expression (activation of drug). We first compared drug-free NRP bacilli to log-phase bacilli (drug-free NRP2 vs drug-free log-phase) to describe the changes in gene expression that occur as oxygen and nutrients become limiting, as defined by Wayne and Hayes [31].

As mycobacterial growth rate slows and then plateaus, 944 genes were significantly differentially expressed in NRP1 (323 induced and 621 repressed) and 1451 genes differentially regulated in NRP2 (649 induced and 802 repressed) compared with aerobic log-phase growth (Supplementary Table 1). As expected, there were highly significant overlaps in gene expression patterns with previous studies characterizing the transcriptional response of bacilli to stationary phase growth [26], oxygen limitation [25], nutrient starvation [28] and the Wayne model itself [42], with hypergeometric p-values for enrichment of NRP2-induced genes of 7.17×10^{-38} , 3.44×10^{-18} , 4.23×10^{-16} and 9.36×10^{-31} , respectively. Genes with functional classifications such as aerobic respiration, ribosomal protein synthesis and ATP-proton motive force were repressed in NRP2 compared with aerobic axenic growth (hypergeometric p-values: 5.14×10^{-8} , 8.52×10^{-6} and 4.34×10^{-5} , respectively) [43]. Conversely, the *dosR* (hypoxia and NO-responsive dormancy regulon) [44], *mprA* (two-component response regulator) [45] and *kstR* (cholesterol metabolism) [46] regulons were significantly induced in NRP2 compared with log-phase growth (hypergeometric p-values: 5.85×10^{-28} , 6.10×10^{-5} and 1.20×10^{-4} , respectively); as

were the alternative sigma factors *sigB*, *sigE*, *sigF* and *sigG*, and the regulator of the stringent response, *relA*. The transition from rapidly multiplying to nonreplicating bacilli in this Wayne model of dormancy mirrors previously identified transcriptional changes, reflecting a change in respiratory state from aerobic to anaerobic as oxygen becomes limited, a switch to lipid-based carbon sources as nutrients become scarce and a modification of cell-wall composition and a slowing of metabolism as growth terminates [27,47].

■ Nonreplicating bacilli do not mount a transcriptional response to INH

We then asked whether the transcriptional responses to INH would differ between these two physiologically distinct populations of bacilli. The hypothesis being that drug-induced responses reveal genes involved in potential resistance mechanisms or compensatory responses that reduce the effect of the drug and, hence, may underpin tolerance. We defined genes differentially expressed after 4 h exposure to 0.2 µg/ml INH in log-phase and NRP2 bacilli (in INH-treated log-phase vs drug-free log-phase, and INH-treated NRP2 vs drug-free NRP2 comparisons).

In rapidly multiplying log-phase bacilli, 100 genes were significantly differentially regulated after 4 h INH treatment; with 71 genes induced and 29 genes repressed relative to drug-free log-phase bacilli (Supplementary Table 2). There was a high degree of correlation between INH-responsive genes identified in this investigation and previous studies examining INH-mediated transcriptional programs; hypergeometric p-values: 2.15×10^{-24} [48], 5.79×10^{-16} [22], 3.54×10^{-12} [23] and 5.20×10^{-10} [49]. Genes indicative of cell wall inhibitors such as *accA3*, *accD4*, *ahpC*, *ahpD*, *efpA*, *fadD32*, *fbpC*, *htdX*, *iniA*, *iniB*, *lprJ*, *pks16* and *Rv3717* were significantly induced [23]. By comparison, no genes were significantly differentially expressed after INH treatment of NRP2 bacilli relative to drug-free NRP2 bacilli. To ensure that the absence of differentially regulated genes after INH treatment in NRP2 bacilli was not an artifact of significance testing, we plotted the expression pattern of log-phase INH-responsive genes in both log-phase and NRP conditions (Figures 1 & 2). Figure 1 shows a heat map of six replicates of all genes significantly upregulated by INH in log-phase bacilli and the gradual diminution of transcriptional response as transition proceeds through NRP1 towards NRP2. This confirmed that genes comprising the INH transcriptional signature of log-phase bacilli were not differentially regulated after INH treatment in NRP2 bacilli. These observations were verified by quantitative RT-PCR using a panel of genes selected to be indicative of mycobacterial growth state and implicated in INH resistance (Figure 3). Thus, increased tolerance of NRP2 bacilli to INH seems not to be dependent on the initiation of a transcriptional program after exposure to INH that renders the drug ineffective.

The absence of a transcriptional response to INH indicates no activity of the drug in NRP2 bacilli. This could be explained in several ways:

- Isoniazid has not entered the cell due to alterations in cell wall structure, or has been exported rapidly;
- INH is not converted to the toxic product by KatG, or there is reduced availability of NAD to form the INH–NAD adduct;

- The enzyme target(s) of INH–NAD outnumber the toxic adducts generated, or the inhibition of pathways affected by INH are no longer functionally significant for bacilli in NRP2.

We have utilized the gene-expression signatures of NRP2 and INH as a framework to explore these hypotheses further.

■ Differential regulation of genes identified to harbor mutations in INH-resistant *M. tuberculosis* strains do not explain INH tolerance in NRP2

Following the hypothesis that differences in transcript abundance between log-phase and NRP2 bacilli might influence INH tolerance, we asked whether the differential regulation of genes associated with INH resistance might explain INH tolerance in NRP2 bacilli. We used the assumption that differential expression of these genes (drug-free NRP2 vs drug-free log-phase) may relate to changes in protein (or drug) activity resulting in tolerance, even though these genes may not be directly involved in the mechanism of drug action. Therefore, we compared the expression profiles of 18 genes with mutations in INH drug-resistant strains, collated on the TB drug-resistance mutation database [19] and present in the filtered microarray dataset used in this study (Figure 4). Mutations that result in the overexpression of *inhA* (increased target) lead to INH resistance by overwhelming the active INH–NAD adduct present in exposed bacilli; however, both *inhA* and the upstream gene *fabG1* were repressed in NRP2 relative to log-phase bacilli [14]. An alternative mechanism of drug resistance might be caused by the increased activity of arylamine *N*-acetyltransferase, which inactivates INH by acetylation leading to resistance. However, the induction of *nat* was not observed in NRP2 compared with log-phase bacilli. The catalase–peroxidase activity encoded by *katG* is required for the formation of the INH–NAD adduct; mutations in this gene result in INH resistance. Therefore, a downregulation of *katG* could be hypothesized to increase INH tolerance. However, *katG* expression was induced in NRP2 relative to log-phase bacilli, which might be predicted to increase rather than decrease the susceptibility of NRP2 bacilli to killing by INH. Conversely, the enzyme activity of this catalase–peroxidase is reduced at low oxygen levels, which might result in the formation of less INH–NAD adduct and, thus, INH tolerance [50]. Notably, this reduction of KatG activity in hypoxic conditions might also be predicted to result, through compensatory feedback mechanisms, in the induction of *katG* gene expression in NRP2 compared with log-phase bacilli, as was observed. The repression of *ndh*, leading to a reduction in NDH-attributed NADH dehydrogenase activity and an accumulation of NADH that acts as a competitive inhibitor with INH–NAD for INHA binding [51], thereby increasing drug tolerance, was not evident.

Genes identified as potentially influencing INH activity because they are highly upregulated after INH treatment (such as *fbpC*, *iniBAC*, *Rv1592c*, *Rv1772* and *efpA*), were not induced in NRP2 compared with log-phase growth as might be the case if these gene products contributed to INH tolerance in NRP2 (Figure 4) [22,52]. Interestingly, *fadE24*, a probable acyl-CoA dehydrogenase likely involved in the β -oxidation of fatty acids that is induced after INH exposure, was marginally induced in NRP2 relative to log-phase bacilli. It has been previously proposed that the induction of genes involved in lipid degradation may be responsible for recycling fatty acids that accumulate after exposure to INH and the inhibition of the FASII cycle [22]. The differential regulation of genes encoding efflux pumps may

modify INH entry and exit kinetics affecting killing efficacy: *efpA*, *mmr*, *Rv1819c*, *Rv2459* and *Rv3728* have been demonstrated to be induced by INH in multidrug-resistant strains [53]. However, none of these genes were significantly induced in NRP2 relative to log-phase growth. Of the genes assigned by Cole *et al.* to the functional category III.A.6 efflux genes [43], three were induced in NRP2: *Rv1250* (a probable drug-transport integral membrane protein, a member of the major facilitator superfamily), *Rv1634* (a possible drug-efflux protein, also a member of the MFS family) and *Rv2209* (a conserved integral membrane protein); four genes were repressed relative to log-phase bacilli (*drrB*, *drrC*, *emrB* and *Rv2459*). This may be a possible explanation for increased efflux-based tolerance, but little is known about the specificity of each transporter. The expression profiles of a subset of these genes were verified by qRT-PCR (Figure 5); this demonstrated excellent correlation between ratios generated from microarray and qRT-PCR techniques.

■ Complex transcriptional responses reflecting the changing physiological state of bacilli in NRP2 likely affect INH efficacy

Unlike mutations to single genes that affect the primary target of INH or its modification into an active molecule that results in INH resistance, adaptations involving the differential expression of many genes involved in respiratory and metabolic pathways may influence INH tolerance in NRP2. For example, the maintenance of redox potential in NRP2 bacilli is very different to log-phase aerobically respiring bacilli. This may prohibit the conversion of INH to INH–NAD adduct (akin to the inactivation of KatG). Cytochrome P450 genes, and their putative redox partners [54], were differentially regulated in NRP2 relative to log-phase; with *cyp51*, *cyp124*, *cyp125*, *cyp130*, *cyp132*, *fdxA*, *fdxC*, *fdxD*, *fprB* and *fprD* induced and *cyp121*, *cyp135A1*, *cyp136*, *cyp140*, *cyp141* and *cyp143* repressed. In addition, the requirement for cytochrome F420 may differ [47], with the NADPH-dependent glucose-6-phosphate dehydrogenases (*zwf1* and *zwf2*) downregulated, and F420-requiring glucose-6-phosphate dehydrogenases (encoded by *fgd1* and *fgd2*) induced in NRP2. Mutations conferring INH resistance in genes coding for mycothiol biosynthesis – *mshA*, *mshC*, *mshD* in *Mycobacterium smegmatis* [55] and *aphC* [56] – have also been suggested to reduce the capacity for prodrug conversion, resulting in increased tolerance to INH. However, these genes were not significantly differentially expressed in NRP2 relative to log-phase bacilli. Most interestingly, recent evidence suggests that *M. tuberculosis* is able to maintain redox potential using the anabolism of propionate-derived lipids as a reductant sink [57]. A range of genes involved in fatty acid metabolism was differentially regulated in NRP2 relative to log-phase (Supplementary Table 1). Furthermore, modulation of lipid metabolism in NRP2 may transform the architecture of the mycobacterial cell, which has been observed to thicken in stationary-phase bacilli [58]. Changes to the cell wall structure affecting cell permeability (or the suppression of porin expression) may impact on a range of physiological processes [59]; not least in this scenario, limiting drug uptake that may result in increased tolerance to INH in nonreplicating bacilli.

Both *fabG1* (*mabA*) and *inhA* were repressed in NRP2 compared with log-phase growth; *inhA* was downregulated two- to three-fold as determined by microarray and qRT-PCR. This reduction in INH target (*inhA*) is perhaps consequent on a redundant metabolic role for FASII in NRP2 due to the absence of cell wall synthesis in the nonreplicating state. As such,

INH does not induce a compensatory response in NRP2 as cell wall biosynthesis is already homeostatically downregulated. In addition, genes involved in mycolic acid modification *cmaA2*, *cmrA*, *mmaA2*, *mmaA3*, *mmaA4* and *umaA1* were also repressed in NRP2 relative to log-phase bacilli. DNA replication encoding genes *dnaA*, *dnaB*, *dnaE1*, *dnaE2*, *dnaN*, *gyrA* and *ssb* were downregulated as expected in NRP2 relative to log-phase growth, as were *recA*, *recF* and *recR* [60]. The dihydrofolate reductase (product of *dhfrA*), an essential precursor to DNA replication and postulated to be a target for INH, was also repressed in NRP2 [17]. It is therefore likely that it is the reduced requirement for mycolic acids and the FASII cycle, necessary for *de novo* cell-wall biosynthesis, in NRP2 as replication ceases that confers phenotypic tolerance to INH.

■ Use of transcriptional profiling to screen for NRP and INH-responsive genes that may influence *M. tuberculosis* drug tolerance

Delineating the transcriptional response of *M. tuberculosis* to antimicrobial agents has led to the characterization of mechanisms affecting drug tolerance. For example, the *iniB/A/C* genes, encoding an MDR-like pump, were identified to be induced after INH treatment in *M. tuberculosis* [61]. While deletion mutants were more sensitive to killing by INH; overexpression conferred resistance to both INH and ethambutol [62]. This transmembrane complex likely acts as an efflux pump maintaining cellular functions after the perturbation of cell-wall biosynthesis. We reasoned that distinguishing genes that were differentially expressed in NRP2 that were also INH-responsive might reveal novel pathways that affect INH tolerance. We used the transcriptional program initiated by exposure to INH in log-phase bacilli (in an INH-treated log-phase drug-free vs log-phase comparison) to screen the 100s of genes differentially regulated in NRP2 relative to log-phase bacilli (drug-free NRP2 vs drug-free log-phase) (Figure 6).

We isolated 23 genes (Figure 6A) that were significantly induced by INH exposure and that were also upregulated in drug-free NRP2 relative to untreated logphase bacilli. A total of 22 genes (Figure 6B) repressed by INH were correspondingly repressed in NRP2 compared with log-phase bacilli (Supplementary Table 4 [available at www.future-science.com/toc/fmc/2/8]). Of the genes involved in fatty acid metabolism, seven were induced in both NRP2 and INH-treated log-phase bacilli (*fabG4*, *fadE5*, *fadA*, *fadB*, *prpC*, *fadE24* and *fadE23*), perhaps reflecting the redirection of lipid precursors, or metabolic shift to lipid degradation after environmental challenge. In addition, *fadD26*, *ppsA* and *ppsD*, part of the gene cluster responsible for the biosynthesis of phthiocerol dimycocerosate, were induced in both scenarios. Overexpression of this propionate-derived complex lipid may modify the redox status of bacilli and might also alter cell-wall permeability, thereby influencing INH tolerance. Furthermore, three genes that may also contribute to redox potential, *fprB*, an NADPH:adenodoxin oxidoreductase (and the adjacent conserved hypothetical *Rv0885*), together with possible oxidoreductase (*Rv1855c*) and putative monooxygenase (*Rv3049c*) were also induced. Other gene products that might influence drug kinetics, such as the predicted membrane proteins of unknown function, *Rv0446c*, *Rv3346c*, *Rv3355c* and *Rv3675*, were upregulated in NRP2 and after INH exposure. Interestingly, *pknG*, encoding a protein kinase associated with the regulation of *M. tuberculosis* metabolism [63] and the ability to survive intracellularly [64], was induced by

both INH and NRP2. The loss of *pknG* function has been previously implicated in the increased sensitivity of *M. smegmatis* to multiple antibiotics [65], perhaps due to a decrease in cell-wall hydrophobicity. In addition, quantitative RT-PCR (Figure 5) revealed that *icl1* (encoding isocitrate lyase, part of the glyoxylate bypass), *narX* (involved in nitrate reduction) and *tgsl* (a triacylglycerol synthase) were all NRP2 and INH-responsive. These three genes have all been associated with the ability of *M. tuberculosis* to persist *in vivo* [42, 66–68].

Conclusion

Understanding why drugs do and do not work during TB chemotherapy should advance novel drug-design strategies, and reveal a little about the *in vivo* physiological states of *M. tuberculosis* bacilli that clearly influence the efficacy of antibiotic regimens. Transcriptional profiling is a powerful tool in this respect, mapping the expression pattern of all annotated genes in a whole-genome approach to defining the adaptations necessary for *M. tuberculosis* survival. This strategy does not, however, take into account processes affecting protein activity that also influence drug action. In this article, we contrasted the *M. tuberculosis* transcriptional response to INH in log-phase and NRP2 bacilli as a model for INH tolerance. The Wayne model was selected to represent non-growing bacilli as this model is strictly defined, well characterized and likely results in a largely homogenous *M. tuberculosis* population as bacilli display features of synchronous growth on exit from NRP2 [31]. Thus, a comparison between log-phase, with the predominant gene-expression signature derived from rapidly multiplying bacilli, and NRP2 bacilli should model the differences between INH-sensitive and -tolerant bacterial populations. Exposure of NRP2 bacilli to INH did not elicit a transcriptional response, and the RNA abundance of genes inducible by INH in log-phase bacilli did not change after exposure of NRP2 bacilli to INH. This correlates with findings by Karakousis and colleagues, who found the INH-mediated transcriptional program greatly diminished in oxygen- and nutrient-depleted *in vitro* models and murine lung tissue after 6 weeks of aerosol infection [34].

Tolerance to INH in NRP2 bacilli cannot be correlated to the changing transcriptional profiles (comparing drug-free NRP2 to log-phase bacilli) of genes directly implicated in INH resistance through resistance-conferring mutations. However, it should be noted that factors affecting protein activity, such as the reduction of KatG catalase–peroxidase activity in hypoxic conditions, have not been characterized in this study [50]. Phenotypic tolerance in NRP2 is likely the result of complex multigene adaptations to limited oxygen and diminishing carbon resources as bacterial multiplication slows and stops. The reduced requirement for mycolic acids and the FASII cycle in nonreplicating bacilli renders bacilli tolerant to the effects of INH. Other factors such as redox state and cell wall permeability may also contribute to the abrogation of killing by INH in nongrowing *M. tuberculosis* bacilli.

We speculate that the induction of a subset of genes involved in lipid metabolism and capable of performing oxidative functions in both NRP2 and after exposure to INH suggest that specific pathways remodelling the metabolic and respiratory state of *M. tuberculosis* may also influence the efficacy of INH killing. Furthermore, many of these genes have been

identified to be upregulated after macrophage infection [69,70]. Could the induction of these pathways in replicating intracellular bacilli account for the significant decrease in INH sterilizing ability between axenic culture and after macrophage infection [71] or *in vivo* [72]? Finally, the upregulation of *tgsI*, involved in triacylglycerol lipid body formation, after INH exposure and in NRP2 bacilli suggests that the metabolic consequences of specific growth constraints that induce a drug-tolerant fat and lazy mycobacterial phenotype are also encountered in the human lung [42].

Future perspective

There are several technological advances that impact on our understanding of drug resistance mechanisms, which together may resolve the important phenomenon of tolerance in *M. tuberculosis*.

The application of high-throughput wholegenome DNA sequencing will transform *M. tuberculosis* resistance testing by mapping mutations to drug mode of action. Currently, in the absence of a comprehensive, genome-wide correlation of genotypic changes with phenotypic drug resistance, it is difficult to predict resistance simply by sequence analysis for single nucleotide polymorphisms (SNPs). Implementation of the soon-to-be-available, rapid and cost-effective, third-generation, highly parallel sequencing platforms will permit every *M. tuberculosis* isolate to be sequenced. The correlation of genomic sequence to phenotypic resistance profile of many thousands of isolates will result in complex predictive modeling of resistance-modifying SNPs. However, it would be unwise to abandon sensitivity testing completely. This SNP mapping together with consequent gene-specific studies will expose multiple primary and secondary drug targets, further elucidating mechanisms of antibiotic killing. It may also reveal new classes of mutation conferring low-level resistance, affecting features such as cell permeability or growth rate. Furthermore, such genome-wide analyses will begin to uncover linkage between resistance mutations, as well as a large number of mutations that have no effect on antibiotic resistance at all. The resulting payoff will provide clinicians with a resistance profile for problem isolates, enabling chemotherapy to be tailored effectively. Although tolerance is an adaptive process, genotypic changes revealed by sequencing will define a panel of genes and highlight complex pathways that when modified at the mRNA or protein level, may also influence antibiotic tolerance.

Predictive modeling of drug tolerance using transcriptional profiling and systems approaches to test *M. tuberculosis* drug-tolerant phenotypes will be instrumental in defining *in vitro* conditions that reflect the complex *in vivo* status. This will enable tolerance mechanisms to be investigated in models that accurately represent the antibiotic sensitivity profile of bacilli during natural infection and allow drug-screening programs to target persistent-like bacilli. Modeling the changing physiological state of bacilli exposed to different *in vivo* or intracellular environments, diverse carbon sources and varied oxidative scenarios (defining mRNA, protein or metabolite levels) will help to delineate the unique transcriptional profiles derived from human sputa or lung tissue sections.

Single cell technologies and microfluidics have enabled bacterial populations to be visualized and differentiated; further advances in these fields will allow responses of

individual bacteria to the changing microenvironment to be assayed. The identification and characterization of subpopulations of phenotypically diverse bacteria and the factors that influence their creation promises to reveal much about persisters, and their potential roles during infection. This, together with transcriptionally defined drug-screening targeted to specific bacterial metabolic and respiratory states, should pave the way for discovery of novel compounds that kill nonreplicating persistent bacteria. The continued support and patience of funding agencies will be required to smelt these silver bullets with the hope of eliminating the hidden monster of persistent infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Terms

Phenotypic drug tolerance

Mechanisms by which genetically susceptible bacilli are refractory to killing with antimicrobial agents. Tolerance may be dependent on pre-existing subpopulations of physiologically distinct bacilli, or may be initiated by changes in the microenvironment or drug exposure.

Persistence

Bacilli capable of causing active disease that survive prolonged chemotherapy, resulting in lengthy and expensive treatment for tuberculosis.

Isoniazid

Front-line anti-tuberculosis drug targeting fatty acid synthesis and affecting the mycobacterial cell wall; responsible for the majority of mycobacterial killing within the first 2 days of treatment with standard regimen.

Transcriptional profiling by microarray

Exploring the global responses of bacilli to the surrounding microenvironment by simultaneously quantifying mRNA abundance for all defined *Mycobacterium tuberculosis* genes.

Wayne model

In vitro model where primarily oxygen is limited to mimic *Mycobacterium tuberculosis* nonreplicating persistence.

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Executive summary

- Effective tuberculosis chemotherapy is hampered by prolonged treatment, which is necessary to remove persistent bacilli, most likely because most front-line antibiotics primarily kill replicating bacilli.
- An understanding of how and why *Mycobacterium tuberculosis* bacilli become phenotypically tolerant to some antibiotics would illuminate current drug-design programs, which are aimed at reducing duration of treatment from months to weeks.
- Transcriptional profiling may be used to reveal important metabolic and physiological adaptations associated with *M. tuberculosis* infection *in vivo* and underpinning phenotypic tolerance to antibiotics. This genome-wide approach is also valuable in highlighting genes of interest that have no predicted function and that may not have been identified by gene-specific assays. Novel insights into underlying mechanisms may thus be discovered.
- *M. tuberculosis* bacilli in nonreplicating persistence state 2 do not respond transcriptionally to isoniazid (INH) exposure and are tolerant to INH. Tolerance to INH in nongrowing bacilli is therefore not a result of INH-inducible adaptive responses, but rather a reflection of the underlying metabolic state in which INH has minimal effect.
- Altered transcript abundance of key genes implicated in INH resistance (involved in drug activation or target manipulation) does not explain why nonreplicating persistence state 2 bacilli become tolerant to INH.
- The reduced requirements for mycolic acids and disparate redox state of nongrowing bacilli probably result in tolerance to the effects of INH exposure.
- Genes involved in lipid metabolism and alternative redox pathways may play functionally significant roles in INH-tolerant *M. tuberculosis* bacilli that persist through chemotherapy.

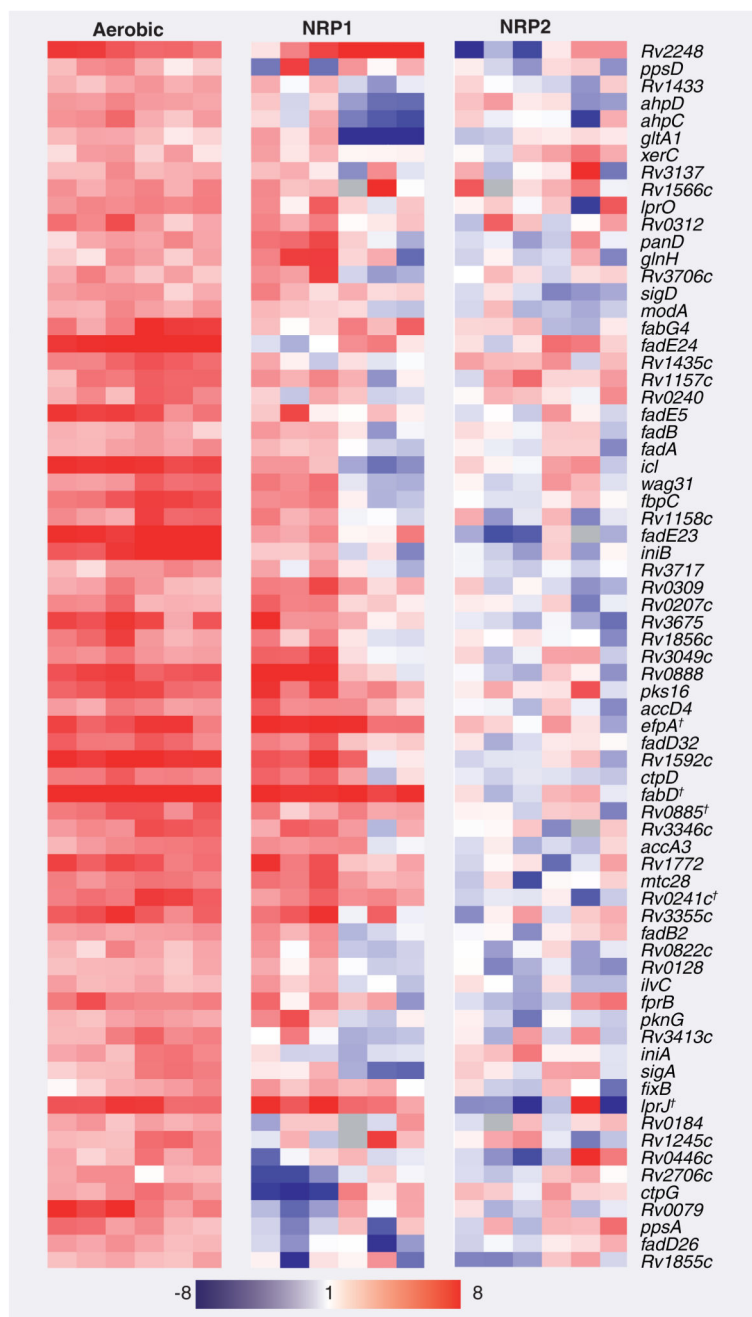


Figure 1. Transcriptional programs induced by isoniazid exposure in log-phase, NRP1 and NRP2 growth states.

The expression pattern of 71 genes induced by isoniazid (INH) treatment in aerobic conditions are clustered alongside the transcriptional activity of these genes after INH exposure in NRP1 and NRP2. The genes are displayed as rows, growth conditions as columns. No genes were significantly differentially expressed in NRP2 after INH treatment. Red coloring signifies induction; blue denotes repression relative to the respective drug-free growth state controls.

[†]Genes that were also INH-responsive in NRP1. NRP: Nonreplicating persistence.

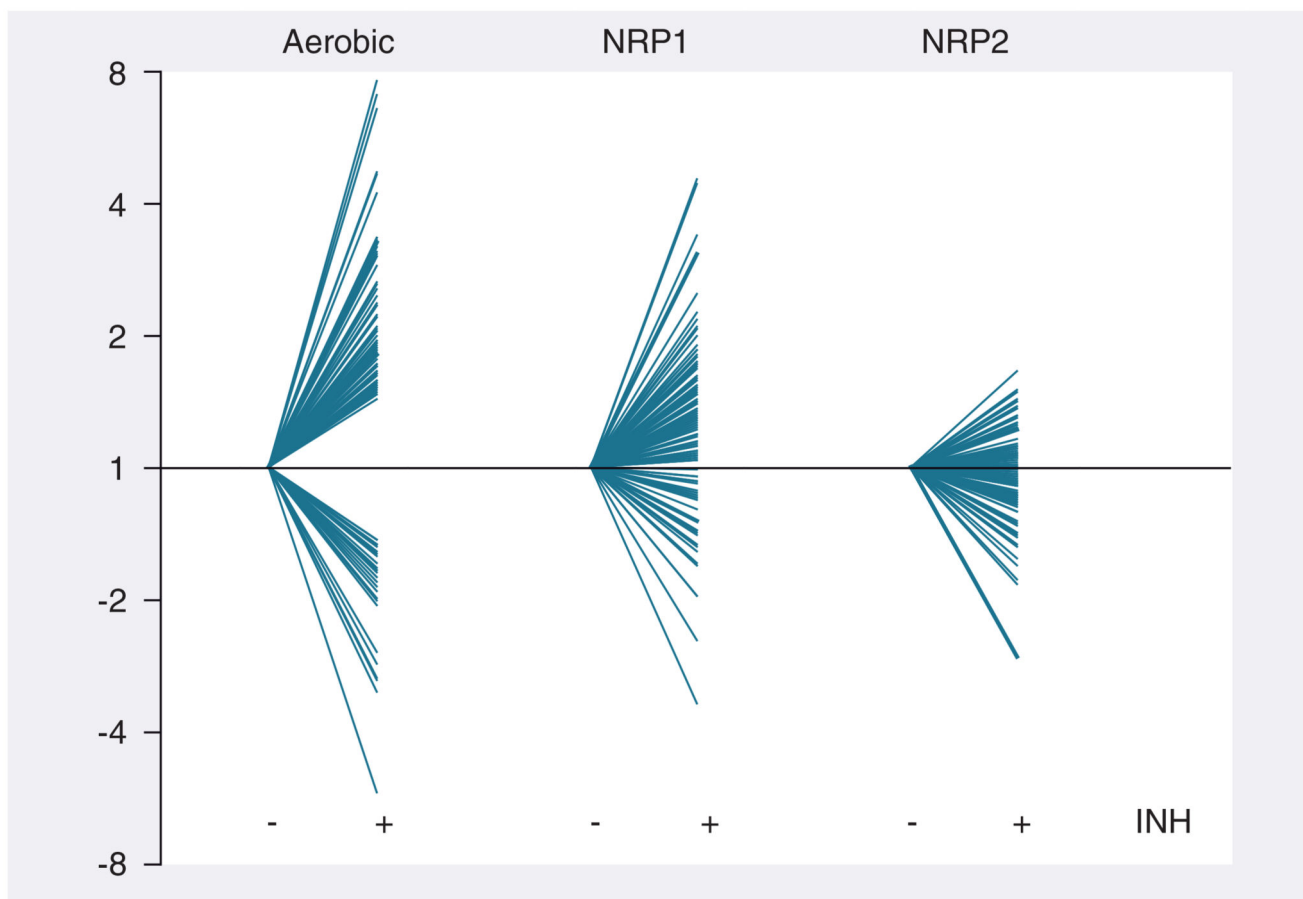


Figure 2. Diminished transcriptional response to isoniazid exposure in NRP2.

Gene expression profiles of 100 genes (71 induced, 29 repressed) significantly differentially expressed after INH treatment in aerobic conditions are plotted (\pm INH) alongside the expression pattern of these genes in NRP1 and NRP2. The expression ratios are presented as fold-change relative to drug-free growth state controls.

INH: Isoniazid; NRP: Nonreplicating persistence.

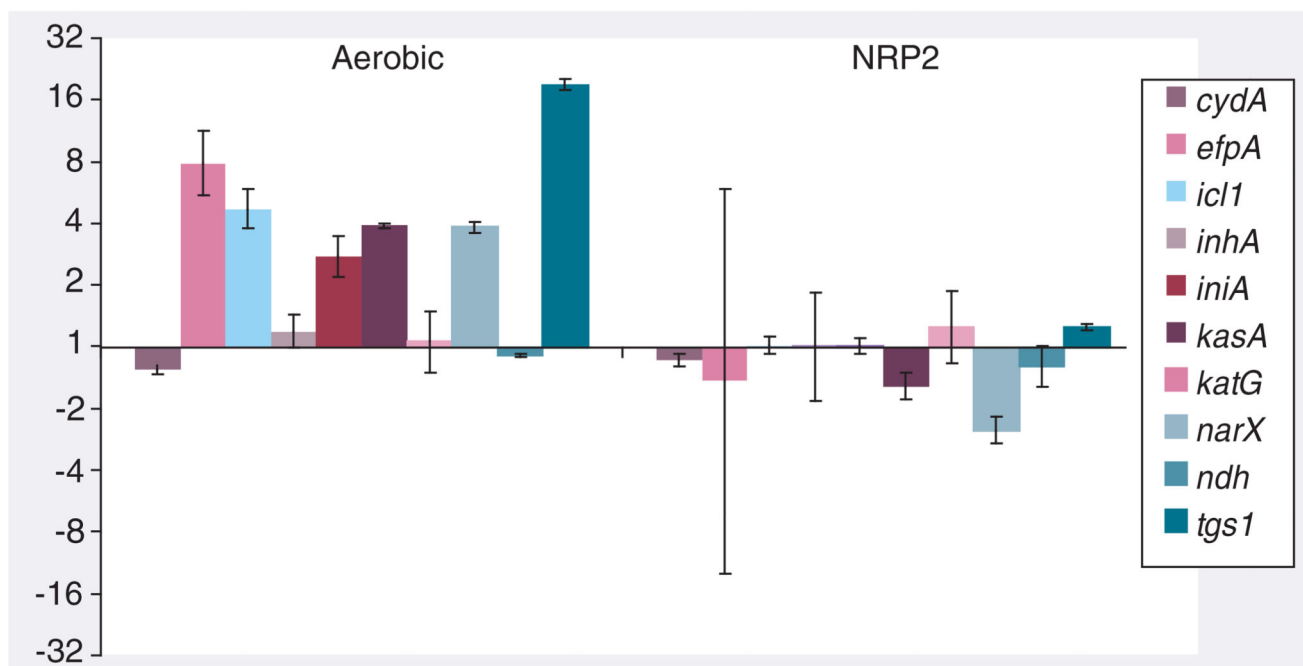


Figure 3. The absence of a transcriptional response to isoniazid in NRP2 bacilli, verified by quantitative RT-PCR with a panel of genes chosen to exemplify the isoniazid transcriptional signature and the physiological status of bacilli.

Expression ratios, marked in fold-change, were calculated relative to *sigA* and drug-free growth controls using the C_t method. Standard deviations from triplicate samples are marked. Red–pink coloring highlights genes selected to assay INH mechanism of action; blue coloring indicates genes chosen to illustrate the metabolic and respiratory state of bacilli.

INH: Isoniazid; NRP: Nonreplicating persistence.

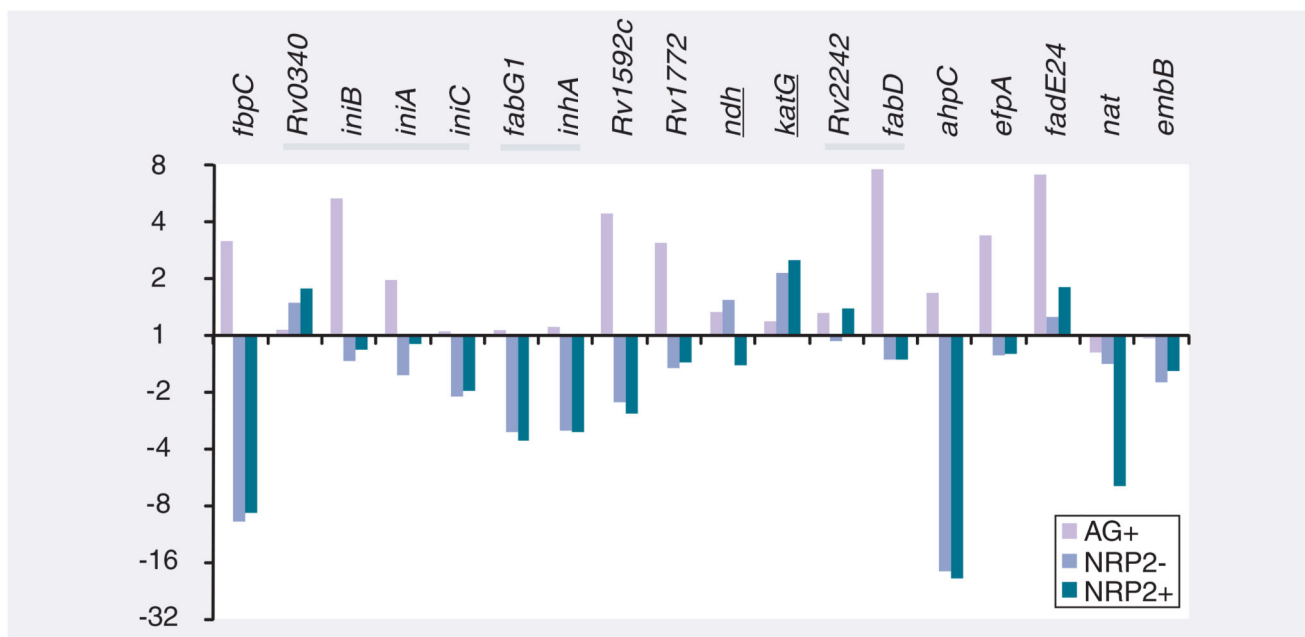


Figure 4. The changes in RNA abundance of genes associated with INH resistance in log-phase and NRP2 bacilli ± INH relative to drug-free log-phase bacilli.

Expression ratios, derived from the microarray dataset, are plotted in fold change for log-phase aerobic (AG) and NRP2 ± INH treatment. Genes are ordered by chromosome position, with the dark bars highlighting adjacent genes. Underlined gene annotations indicate that the repression of these gene products in NRP2 may be hypothesized to increase INH tolerance. Conversely, the over-expression of the remaining genes might be expected to increase INH tolerance in NRP2.

AG: Aerobic; INH: Isoniazid; NRP: Nonreplicating persistence.

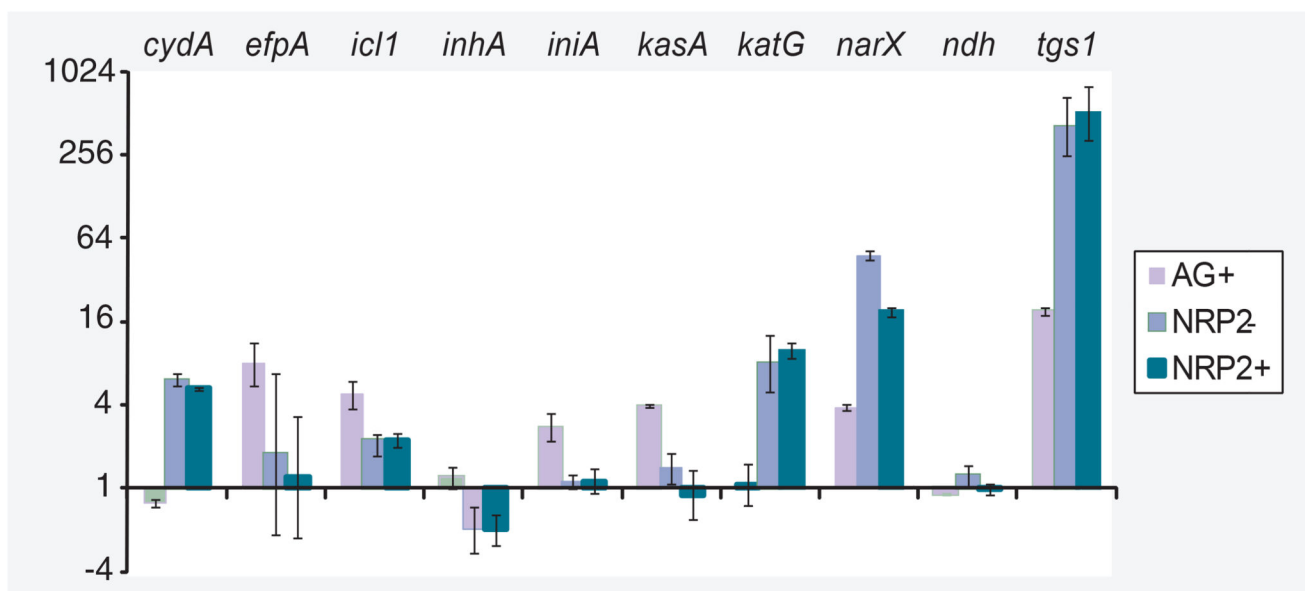


Figure 5. Quantitative RT-PCR confirming the differential regulation of isoniazid responsive genes and genes indicative of bacterial metabolic state in log-phase and NRP2 relative to drug-free log-phase bacilli.

Expression ratios \pm isoniazid (INH), measured in fold-change, were calculated relative to *sigA* and untreated log-phase control using the Ct method. Standard deviations from triplicate samples are marked.

AG: Aerobic; NRP: Nonreplicating persistence.

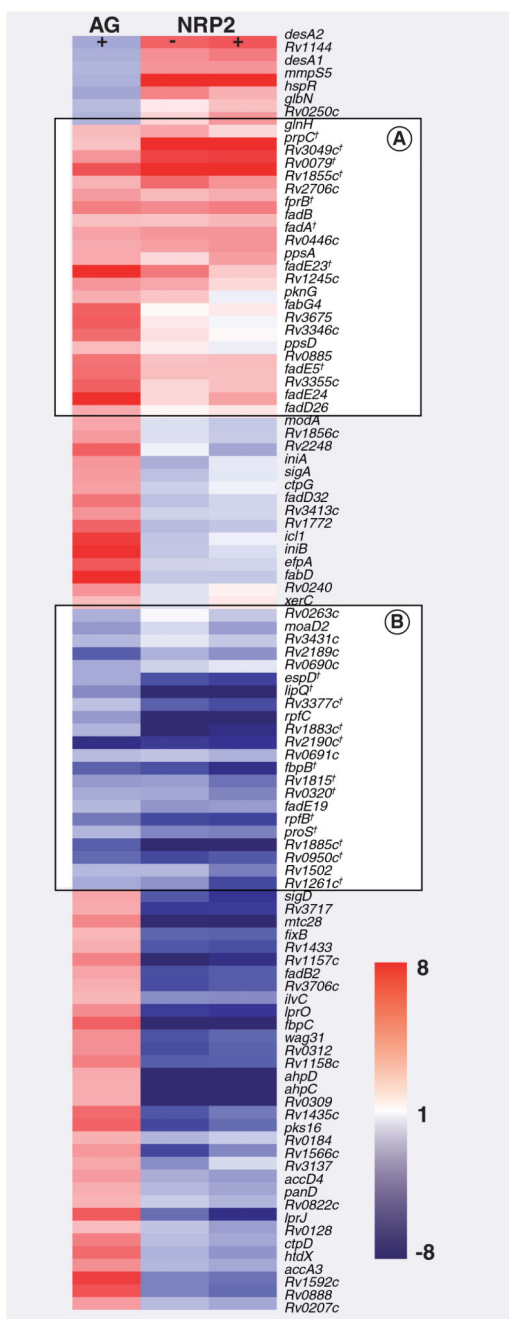


Figure 6. Expression pattern of isoniazid-responsive genes in log-phase and NRP2 bacilli ± isoniazid relative to drug-free log-phase bacilli.

A total of 100 genes, identified as significantly differentially regulated after isoniazid (INH) exposure in AG conditions, were clustered alongside the NRP2 transcriptional profiles. (A & B) show genes induced or repressed after INH exposure and transition to NRP2 compared with untreated log-phase bacilli. Genes are displayed as rows and growth conditions ± INH as columns. Red: induction; blue: repression relative to log-phase drug-free control.

†Genes significantly differentially expressed in both drug-free NRP2 and INH-treated AG bacilli compared with untreated log-phase bacilli.

AG: Aerobic; NRP: Nonreplicating persistence.