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Exploring drug action on *Mycobacterium tuberculosis* using affymetrix oligonucleotide genechips

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Summary

DNA microarrays have rapidly emerged as an important tool for *Mycobacterium tuberculosis* research. While the microarray approach has generated valuable information, a recent survey has found a lack of correlation among the microarray data produced by different laboratories on related issues, raising a concern about the credibility of research findings. The Affymetrix oligonucleotide array has been shown to be more reliable for interrogating changes in gene expression than other platforms. However, this type of array system has not been applied to the pharmacogenomic study of *M. tuberculosis*. The goal here was to explore the strength of the Affymetrix array system for monitoring drug-induced gene expression in *M. tuberculosis*, compare with other related studies, and conduct cross-platform analysis. The genome-wide gene expression profiles of *M. tuberculosis* in response to drug treatments including INH (isoniazid) and ethionamide were obtained using the Affymetrix array system. Up-regulated or down-regulated genes were identified through bioinformatic analysis of the microarray data derived from the hybridization of RNA samples and gene probes. Based on the Affymetrix system, our method identified all drug-induced genes reported in the original reference work as well as some other genes that have not been recognized previously under the same drug treatment. For instance, the Affymetrix system revealed that Rv2524c (*fas*) was induced by both INH and ethionamide under the given levels of concentration, as suggested by most of the probe sets implementing this gene sequence. This finding is contradictory to previous observations that the expression of *fas* is not changed by INH treatment. This example illustrates that the determination of expression change for certain genes is probe-dependent, and the appropriate use of multiple probe-set representation is an advantage with the Affymetrix system. Our data also suggest that whereas the up-regulated gene expression pattern reflects the drug's mode of action, the down-regulated pattern is largely non-specific. According to our analysis, the Affymetrix array system is a reliable tool for studying the pharmacogenomics of *M. tuberculosis* and lends itself well in the research and development of anti-TB drugs.

Keywords

Tuberculosis; Drug; Microarray; Genome

Introduction

The availability of the complete genome sequence of *Mycobacterium tuberculosis*[1] combined with the rapidly emerging microarray technology[2] has catalyzed the process of understanding the biology and pathogenicity of the organism and developing new diagnostics and therapeutics for tuberculosis (TB) (i.e. the disease caused by the organism). Based on the concept of simultaneously studying the expression of a large number of genes, a DNA microarray is a

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chip on which numerous probes are placed for hybridization with a tissue sample. Important applications of this technology include discovery of biomarkers of therapeutic response, identification and validation of new molecular targets and modes of action, and elucidation of the genetic basis for drug sensitivity and resistance.

The feasibility of the microarray technology for investigating *M. tuberculosis* has been demonstrated. DNA microarrays have been used to study genome differences between *M. tuberculosis* and various BCG daughter strains[3] and within the species of *M. tuberculosis*.[4] The enhanced resolution in detecting deleted DNA regions offers a more precise view of molecular evolution and leads to improvements in diagnostics and vaccine development. The technology was also used to explore altered gene expression induced by isoniazid (INH)[5] and other drugs as well. In addition, microarrays can be applied to identify species and detect rifampin-resistant mutants.[6,7]

Recently, there is growing interest in studying the pharmacogenomics of *M. tuberculosis* by means of microarrays.[5,8–10] The functional-genomics approach will unveil genes physiologically or biochemically relevant to the drug's mode of action, genes linked to its toxic consequence, and genes related to its resistance if they are regulated at the transcriptional level. A genomic criterion based on microarray evidence may well provide a more reliable indicator for clinical drug efficacy. In our current research project, we take this approach to study important anti-TB drugs and promising new drug candidates.

While the microarray approach has generated valuable information, a recent survey has found a lack of correlation among the microarray data produced by different laboratories on related issues concerning *M. tuberculosis*,[11] raising a serious question about the credibility of research findings. Thus, repeating the same experiment using a different platform could yield new insight into the problem. Using a chemical stimulus in a study unrelated to TB, the Affymetrix oligonucleotide array is shown to be more reliable for interrogating changes in gene expression than another major microarray format, called the cDNA array.[12] However, in studying *M. tuberculosis* exposed to drug treatment, none of the existing research uses the Affymetrix array. Here, we explored the drug-induced gene expression patterns associated with INH and ethionamide in *M. tuberculosis* using the Affymetrix system. We demonstrate that this array system can identify all genes recognized previously as well as some other important genes that have been omitted in previous studies. The results would encourage more use of such array system in the future investigation of new anti-TB drugs.

Materials and methods

Drug treatment of *M. tuberculosis*

M. tuberculosis strain H37Rv frozen stock was inoculated in 5 ml of the Middlebrook 7H9 media at 37 °C for 5 days. Then the culture was transferred into 50 ml of 7H9 media and incubated at 37 °C with 50 rpm shaking until the OD600 reached 0.7. The cells were harvested by centrifugation for RNA preparation.

We experimented with two drugs: INH and ethionamide. The selected H37Rv strain is sensitive to both drugs. The sensitivity of H37Rv to INH was tested on Petri plates, resulting in five colonies at 1 μg/ml, six colonies at 2 μg/ml, four colonies at 4 μg/ml, and two colonies at 5 μg/ml. H37Rv exhibited sensitivity (more than 99% inhibition of bacterial growth) to ethionamide at 10 μg/ml.

Drug treatment was conducted by adding the filtered stock solution of each drug to achieve the final concentration in the media: 5 μg/ml for INH and 10 μg/ml for ethionamide. Upon completion of the predefined duration (4 h) of drug treatment, the bacteria were harvested by

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centrifugation and then stored for RNA extraction. The RNA sample extracted before drug treatment was used as a control.

RNA isolation

M. tuberculosis has a complex cell wall and is refractory to disruption. Bacterial lysis and RNA isolation were performed following the procedure of Fisher et al.[13] at the CDC lab (Atlanta). The process started with aliquoting cells 25 ml/tube in Oak Ridge tubes and used the reagents including Trizol, chloroform: isoamyl alcohol, isopropyl alcohol, and ethanol. DNA was removed by treating with DNAse I. The RNA sample was precipitated and washed in ethanol, and redissolved to make a final concentration of 1 μg/μl. The purity of RNA was estimated by the ratio of the readings at 260 and 280 nm (A_{260}/A_{280}). RNA samples 20 μl were sent to the University of California, Irvine (UCI) DNA core and further checked through a quality and quantity test based on electrophoresis before microarray hybridization.

Microarray hybridization and analysis

In this project, we use the anti-sense Affymetrix *M. tuberculosis* genome array (GeneChip). The probe selection was based on the genome sequence of *M. tuberculosis* H37Rv.[1] Each annotated Open Reading Frame (ORF) and Intergenic Region (IG) was interrogated with oligonucleotide probe pairs. The gene chip represents all 3924 ORFs and 738 IGs of H37Rv. Twenty 25-mer probes were selected within each ORF or IG. These probes are called Perfect-Match (PM) probes. The sequence of each PM probe is perturbed with a single substitution at the middle base. They are called Mismatch (MM) probes. A PM probe and its respective MM probe constitute a probe pair. The MM probe serves as a negative control for the PM probe in hybridization. Upon hybridization, the GeneChip software compares PM and MM signal intensities across probe pairs for each gene. The corresponding RNA is considered as absent if the sum of the PM intensities minus the sum of the MM intensities is close to zero; otherwise it is considered as present (or marginal). The difference call is assigned by the Affymetrix GeneChip software and is a proprietary function of four experimentally measured variables.

Microarray hybridization followed the Affymetrix protocol. In brief, the assay utilized reverse transcriptase and random hexamer primers to produce DNA complementary to the RNA. The cDNA products were then fragmented by DNAase I and labeled with terminal transferase and biotinylated GeneChip DNA Labeling Reagent at the 3′ terminal.

Results

The microarray approach to the pharmacogenomic study of *M. tuberculosis* has generated interesting results,[5,8–10] but cross-experimental inconsistency is noted.[11] For research along this line, previous studies used a type of array system referred to as cDNA arrays. Such a system uses long cDNAs derived from PCR products, ESTs, or cloned cDNAs. In contrast, the Affymetrix arrays (GeneChips) use in situ synthesis of oligonucleotides. In this work, we adopted the Affymetrix system as an alternative microarray platform for comparison with the cDNA system on similar tasks. The main reference work for this study was that of Wilson et al.[5] although other related work was also analyzed. We chose INH and ethionamide as the experimental drugs because they were investigated before, and so cross-platform analysis is possible.

Global gene expression patterns in response to INH

The drug-induced genetic response was monitored by using Affymetrix arrays that measure the level of expression of each gene in the genome in terms of mRNA abundance in drug-treated bacterial cells. The RNA collected from the cells before drug treatment was the baseline sample to which the drug-treated sample was compared. The difference call computed by the

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Affymetrix system between the two samples indicates increase (I), decrease (D), marginal increase (MI), marginal decrease (MD), and no change (NC). The marginal cases were not considered here because they were somewhat uncertain and represented a much smaller set of potentially affected genes. The increase call means up-regulation of gene activity from the baseline to test condition, whereas the decrease call means down-regulation. The difference call is statistically based on four system parameters reflecting differences across multiple (40) probes implementing each gene between two conditions.

The Affymetrix system identified 154 up-regulated (I call) and 186 down-regulated (D call) genes when an INH-sensitive *M. tuberculosis* strain was exposed to the drug. These are genes whose activities are affected by INH and controlled at the transcriptional level. Since, however, the study is aimed at the drug's mode of action, it is important to narrow down the differentially expressed genes to those representing the characteristic drug response. To distinguish, a popular and acceptable criterion is based on fold change. In conjunction with other standards, this criterion works well in practice. So we concentrated on the most affected genes exhibiting at least two-fold increase or decrease in their expression intensity, and associated with I or D difference calls, respectively.

The above criteria led to the identification of 42 induced genes (Table 1) and 21 repressed genes (Table 2) in response to INH. Most importantly, an operon cluster of five genes encoding type-II fatty acid synthase (FAS-II) enzymes and *fbpC* were induced by INH, in consistency with an earlier report.[5] These genes encode enzymes that reside in the main pathway acted upon by the drug. The expression patterns of these genes constitute the drug-characteristic gene expression signature reflecting the drug's mode of action. While this finding is a reconfirmation of the previous work, it provides evidence for the validity of the Affymetrix system in this application. Some other genes were also induced by INH possibly as a result of the drug's toxic or stress effect. The results confirm the targets like *kasA* and *acpM* that have been previously identified on the basis of proteomics,[14] present a potentially more complete and coherent account about drug action, and perhaps suggest novel drug targets.

The present study based on the Affymetrix system recognized 42 INH-induced genes, compared with 14 genes based on the cDNA system.[5] Upon a close look, the former set is actually a superset of the latter set of genes. This implies that the first system will not miss out any discovery made by the second system but not vice versa. However, equally important is the balance between how sensitively the system is able to pick up genes in pathways related to drug action and how specifically the system does not make false discovery. Among the 42 genes found by the Affymetrix-based system, several are not included in the 14 gene set but reported by another independent research group.[8] Furthermore, all the 42 genes are associated with very high change *p*-values calculated by the Affymetrix system. Taken together, the data suggest that the Affymetrix-based system achieves a good balance between sensitivity and specificity for differential gene expression analysis, as also observed in other non-TB domain.[12] However, because of different experimental conditions used, cross-study comparison should emphasize on the quality and significance rather than the number of discovered genes.

The genes induced by INH in this work fall into the following functional categories (Table 3): lipid metabolism (10 genes), information pathways (1 gene), cell wall and cell processes (14 genes), intermediary metabolism and respiration (5 genes), regulatory proteins (2 genes), and conserved hypotheticals (10 genes). The basic pathways affected by INH can be deduced from the fact that most genes induced belong to the categories of cell wall and lipid metabolism. Specifically, the gene expression pattern reflects an already-known fact that INH disrupts fatty acid and mycolic acid synthesis. In this case, the most dominant gene-functional classes among induced genes imply the drug's mode of action. This line of reasoning appears to be a good way for interpreting drug-induced gene expression data in general.

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Despite apparent benefits microarray analysis can offer, it does not identify genes that are not subject to transcriptional regulation. For instance, the *inhA* gene showed no change in response to INH treatment, as was also found previously.[5] This observation suggests that the transcription of *inhA* is not sensitive to the drug challenge, though *inhA* protein is a target of INH as cloning from resistant organisms indicates.[14] It is thus clear from this example that gene expression data alone do not necessarily identify the actual drug targets but may well reveal affected pathways involving the targets and offer insight into drug action.

Mycolic acid is an important element in *M. tuberculosis* cell wall. Mycolic acid synthesis is related to lipid metabolism involving both FAS-I and FAS-II. FAS-I is encoded by *fas* (Rv2524c) while FAS-II encoded by a 5-gene operon. FAS-I catalyzes the formation of fatty acids from acetyl-CoA, malonyl-CoA, and probably creates precursors for elongation of all of the other fatty acid and polyketide systems.[1] FAS-II is not involved in de novo fatty acid synthesis but instead elongates fatty acid chains.[1] Up-regulation of five FAS-II genes under INH treatment is attributed to the feedback or compensatory mechanism that senses the disruption of the FAS-II cycle caused by the drug. This finding has been recognized previously and was also confirmed in our study. However, as to FAS-I, the present study produced a different result than that previously reported by Wilson et al.[5] In their work, the expression of *fas* was not changed by INH treatment. We found, however, the hybridization result depended on the probe set selection and drug concentration. Normally, each gene is implemented using one single probe set (20 PM and 20 MM probes), but *fas* was implemented using eight different probe sets on the Affymetrix array. The hybridization result was a mix, five up and three no change. Overall, the gene activity is considered up-regulated by INH treatment following the majority consensus in our setting. In contrast, the cDNA array system, which uses a single PCR-generated probe sequence for each gene, arrived at a different result. The situation here is complicated perhaps because *fas* has a long sequence (9210 bp, 3069 aa). Despite the inconsistency, the finding about up-regulation of *fas* makes sense in terms of the biological feedback mechanism.

Except for conserved hypotheticals, the functional class with the most genes down-regulated by INH treatment was information pathways (Table 3), which had little to do with the drug's mechanism. It is probable that down-regulated gene activities result from non-specific effect of or response to the drug. In contrast, the patterns of up-regulated gene activities carry far more information characteristic of the drug's mode of action.

Global gene expression patterns in response to ethionamide

Ethionamide inhibits mycolic acid synthesis by targeting a FAS-II enzyme called acyl carrier protein reductase (InhA). Thus, ethionamide had a similar mode of action to that of INH. We evaluated the response of the H37Rv strain to ethionamide under the same experimental design, and then used the same criteria for identifying the gene expression signature. The results showed that ethionamide induced the same characteristic gene expression pattern of FAS-II inhibition as was induced by INH. However, ethionamide also induced some genes that were not seen with INH treatment. Among the 56 genes induced by ethionamide (Table 4), 31 genes were also induced by INH, and 8 out of 14 genes repressed by ethionamide (Table 5) overlapped with those repressed by INH. The similar response pattern suggests that both drugs act on the same pathway (i.e. the FAS-II cycle), but the differences reflect the fact that they are different chemical compounds. Clinically, INH is a first-line drug whereas ethionamide is a second-line drug against TB. Notice that two drugs sharing a similar mode of action can still be distinguished by their associated gene expression profiles.[8]

As pointed out earlier, there is a discrepancy between our data and previously reported data about whether the activity of *fas* is changed in response to a FAS-II inhibitor. For ethionamide, it was again demonstrated that this gene was up-regulated in six out of eight probe sets encoding

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the gene, suggesting its role in general lipid metabolism. Combined evidence from both cases (INH and ethionamide) offers more credence about this finding. For ethionamide, the discrepancy about *fas* induction cannot be explained by drug concentration alone since the level used in this study is comparable with that used in the reference work.

Functional classification of induced genes showed 15 genes belonging to lipid metabolism and 18 genes in the class of cell wall and cell processes (Table 3). As in the case of INH, more than half of induced genes fall into these two categories. This fact immediately hints at the relevance of ethionamide to cell wall biosynthesis. In contrast, the profile of repressed gene activities by-and-large represents non-specific drug response.

Discussion

The cross-hybridization of related or overlapping gene sequences can contribute to false positive signals, especially in the case when long cDNA sequences are used as probes. To deal with the issue of cross-hybridization, the Affymetrix array uses the design features of unique oligonucleotide probes and the pair of PM (Perfect-Match) and MM (Mismatch) probes. A recent study indicated that Affymetrix microarrays produced more reliable results in detecting changes in gene expression than cDNA microarrays.[12] 50 or 70-mer oligonucleotide microarrays may also achieve a good compromise between sensitivity and specificity, but the latter option may increase the formation of secondary structure.[12] The Affymetrix array also comes with statistical software for discerning genes with expression changes. For performance and quality reasons, the choice of this type of array is justified.

There is considerable variation among different reports concerning INH-induced genes based on cDNA arrays: for instance, 14 genes,[5] 9–38 genes[8], and 155 genes,[10] respectively, in three independent studies. The results appear to be dependent on the statistical threshold for gene selection, drug concentration, exposure time, and quality control. Cross-laboratory inconsistency is found to be a potential problem in research about *M. tuberculosis* using microarrays.[11] The validity of the method adopted in each laboratory can be evaluated using a benchmark organism (like H37Rv) and drug (such as INH) whose consensus gene expression signature has been recognized. In this work, we demonstrate that the majority (more than half) of genes induced by INH belong to the classes of lipid metabolism and cell wall. This result indicates that our method based on the Affymetrix system has correctly confirmed the mode of action of INH as related to mycolic acid synthesis. As for the cDNA array system, one reported result[5] meets the same standard, but the other two results do not because the genes identified in the classes of lipid metabolism and cell wall do not represent a majority. In comparing results from different studies, qualitative statistics such as the relative distribution of drug-induced genes across functional classes should be emphasized more than quantitative statistics like the absolute number of genes induced since the experimental conditions often vary from study to study.

The Affymetrix GeneChip has certain advantages. For a long gene sequence, the system allows the implementation of multiple probe sets for improving the sensitivity and specificity of micro-array hybridization, whereas the cDNA array system lacks this feature. For example, the INH- and ethionamide-induced expression of *fas* was found by the Affymetrix system but not by the cDNA array system. Another strength is that the Affymetrix system provides both relative and absolute levels of gene expression intensity. The ratio data alone may be misleading in that it may not truly reflect the organism's behavioral change. For example, the state transition from 1 to 2 could be quite different compared with that from 100 to 200. In the first case, the beginning and ending levels could both lie within the normal variation of the signal for the same state. Thus, taking into account the absolute level of gene expression may change the biological interpretation based on the ratio data alone.

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Biological interpretation of the drug-induced genes could lead to hypotheses about drug targets. In the present study, those INH-induced genes related to cell wall synthesis have been analyzed in related work. Here we examine the potential implications of the remaining genes, highlighting more important ones that are not addressed before. The most highly induced genes were Rv0341-2, both currently classified to the category of cell wall and cell processes. They are found to be non-essential genes[15] and their functions remain unknown.

The second most highly induced gene was Rv1773c. This is a probable transcriptional regulatory gene belonging to the IclR family and is non-essential for growth in vitro, but the mutant is more virulent in SCID mice.[16] It is similar to *iclR* in *E. coli* that regulates the acetate operon constituted by three genes, *aceB*, *aceA* and *aceK*, which encode malate synthase, isocitrate lyase and isocitrate dehydrogenase kinase/phosphatase, respectively.[17] Although Rv1773c was highly up-regulated by the fold-change criterion, it was computed as non-expressed (A call) in both pre-and post-INH-treated cells. It is possible that a regulatory gene is active even if it is minimally expressed. Rv1773c supposedly regulated *icl* (Rv0467), which was also induced by INH. *icl* encoding isocitrate lyase is involved in the glyoxylate shunt, and found to be required for mycobacterial persistence in macrophages.[18] It is plausible that INH treatment imposes a stress similar to the intra-macrophage environment. The up-regulation of Rv0467 was not reported by Wilson et al.[5] but it was confirmed as strong in another related work.[10]

INH treatment induced another regulatory gene, *pknG*, which is an essential gene for *M. tuberculosis*. The gene encodes a serine/threonine protein kinase involved in signal transduction (via phosphorylation) and thought to regulate amino-acid uptake and stationary-phase metabolism.[19] An important question can be raised of whether the induction of this gene is secondary to the inhibition of cell wall synthesis or suggests a new drug mechanism.

For microarray validation, a high correlation of genome-wide gene expression between duplicate experiments has been demonstrated in the study. For example, the duplicates of the control samples showed a correlation of 0.98. In addition, all RNA samples in this study have passed a strict quality test before microarray hybridization. The FAS-II related drug-characteristic gene expression signatures obtained for the drugs, INH and ethionamide, on a sensitive strain have been confirmed by RT-PCR in previous work. Biological investigation will be carried out on genes that can potentially become new drug targets or suggest a new mechanism of drug action to be addressed in future work.

The Affymetrix array system has a built-in mechanism for dealing with the issue of cross-hybridization through unique probe set representation of each gene sequence. Research has shown that such a system has the advantage of precision and accuracy relative to other platforms. In *M. tuberculosis* research, we have demonstrated that the method based on the Affymetrix array system can correctly identify genes in the pathway characteristic of the drug's mode of action as well as important genes that provide additional insight in a broad context of drug action. The system is well poised for research in this area.

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Table 1

M. tuberculosis genes induced two-fold or greater by INH treatment. The change *p*-value is computed by the Affymetrix system.

ORF	Gene	Gene product	Fold change	Change *p*-value
Rv0063		Possible oxidoreductase	2.1	0.000001
Rv0129c	*fbpC2*	Mycolyl transferase	2.5	0.000000
Rv0179c	*lprO*	Possible lipoprotein	2	0.000000
Rv0206c	*mmpL3*	Possible conserved transmembrane transport protein	2.3	0.000000
Rv0207c		Conserved hypothetical protein	2.1	0.000000
Rv0208c		Hypothetical methlytransferase methylase	2.8	0.000001
Rv0312		Conserved hypothetical proline and threonine rich protein	2	0.00032
Rv0341		Isoniazid inductible gene protein	17.1	0.000000
Rv0342		Isoniazid inductible gene protein	16	0.000000
Rv0343		Isoniazid inductible gene protein	6.5	0.000001
Rv0381c		Hypothetical protein	2	0.000054
Rv0410c	*pknG*	Serine/threonine-protein kinase	3	0.000014
Rv0411c	*glnH*	Probable glutamine-binding lipoprotein	2	0.000008
Rv0412c		Possible conserved membrane protein	3	0.000000
Rv0467	*icl*	Probable isocitrate lyase	2.5	0.000000
Rv0677c	*mmpS5*	Probable conserved membrane protein	2	0.000000
Rv0888		Probable exported protein	2.3	0.000000
Rv1013	*pks16*	Putative polyketide synthase	2.3	0.000000
Rv1434		Hypothetical protein	2.1	0.00045
Rv1497	*lipL*	Probable esterase	2.6	0.000000
Rv1592c		Conserved hypothetical protein	6.1	0.000000
Rv1690	*lprJ*	Probable lipoprotein	2.6	0.000001
Rv1772		Hypothetical protein	4.9	0.000000
Rv1773c		Probable transcriptional regulatory protein	10.6	0.000472
Rv1774		Probable oxidoreductase	2.6	0.000000
Rv1775		Conserved hypothetical protein	2.3	0.000007
Rv2243	*fabD*	Malonyl CoA-acyl carrier protein transacylase	4.3	0.000000
Rv2244	*acpM*	Meromycolate extension acyl carrier protein	2.3	0.000000
Rv2245	*kasA*	β-Ketoacyl-ACP synthase	4	0.000000
Rv2246	*kasB*	β-Ketoacyl-ACP synthase	4.3	0.000000
Rv2247	*accD6*	Acetyl/propionyl-CoA carboxylase	3	0.000000
Rv2248		Conserved hypothetical protein	2.8	0.000008
Rv2293c		Conserved hypothetical protein	2.3	0.000000
Rv2524c	*fas*	Probable fatty acid synthase	2.6	0.000000
Rv2845c	*proS*	Probable prolyl-tRNA synthetase	4	0.000000
Rv2846c	*efpA*	Possible integral membrane efflux protein	4	0.000000
Rv2932	*ppsB*	Phenolpthiocerol synthesis type-I polyketide synthase	3	0.000658
Rv3140	*fadE23*	Probable acyl-CoA dehydrogenase	2.6	0.000000
Rv3310		Possible acid phosphatase acid phosphomonoesterase	2	0.000002
Rv3354		Conserved hypothetical protein	3	0.000001
Rv3645		Probable conserved transmembrane protein	2	0.000000
Rv3675		Possible membrane protein	3	0.000000

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Table 2

M. tuberculosis genes repressed two-fold or greater by INH treatment.

ORF	Gene	Gene product	Fold change	Change *p*-value
Rv0263c		Conserved hypothetical protein	3.2	0.000409
Rv0320		Possible conserved exported protein	5.7	0.000122
Rv0516c		Conserved hypothetical protein	52	0.000000
Rv0950c		Conserved hypothetical protein	4	0.000002
Rv1009	*rpfB*	Probable resuscitation-promoting factor	2.3	0.000004
Rv1010	*ksgA*	Probable dimethyladenosine transferase	2.1	0.000000
Rv1251c		Conserved hypothetical protein	2.3	0.000000
Rv1365c	*rsfA*	Anti-anti-sigma factor	5.7	0.001423
Rv1555	*frdD*	Probable fumarate reductase	3.5	0.002189
Rv1815		Conserved hypothetical protein	2.5	0.000005
Rv1885c		Conserved hypothetical protein	5.3	0.000004
Rv1886c	*fbpB*	Secreted antigen 85-B	5.3	0.000000
Rv1892		Probable membrane protein	2.1	0.002189
Rv2190c		Conserved hypothetical protein	4.9	0.000000
Rv2482c	*plsB2*	Probable glycerol-3-phosphate acyltransferase	2	0.000002
Rv2626c		Conserved hypothetical protein	2.1	0.000002
Rv2943		Probable transposase for insertion sequence element is1533	2.1	0.000000
Rv3166c		Conserved hypothetical protein	8.6	0.000996
Rv3263		Probable DNA methylase	2.3	0.001927
Rv3362c		Probable ATP/GTP-binding protein	2.3	0.000122
Rv3603c		Conserved hypothetical alanine and leucine rich protein	3.5	0.000429

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Table 3

Functional classes of genes induced or repressed by INH (isoniazid) and ETH (ethionamide) treatments.

	Functional class	INH		ETH	
		Up	Down	Up	Down
0	Virulence, detoxification, adaptation	0	1	1	1
1	Lipid metabolism	10	2	15	2
2	Information pathways	1	4	1	1
3	Cell wall and cell processes	14	2	18	2
4	Stable RNAs	0	0	0	0
5	Insertion seqs and phages	0	1	2	0
6	PE/PPE	0	0	0	0
7	Intermediary metabolism and respiration	5	1	7	2
8	Unknown	0	0	0	0
9	Regulatory proteins	2	0	2	0
10	Conserved hypotheticals	10	10	10	6
	Total	42	21	56	14

Functional classes are based on TubercuList (http://genolist.pasteur.fr/TubercuList/).

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Table 4

M. tuberculosis genes induced two-fold or greater by ethionamide treatment.

ORF	Gene	Gene product	Fold change	Change *p*-value
Rv0129c	*fbpC2*	Mycolyl transferase	2.1	0.000000
Rv0179c	*lprO*	Possible lipoprotein	2	0.000000
Rv0207c		Conserved hypothetical protein	2.1	0.000000
Rv0208c		Hypothetical methlytransferase methylase	2.6	0.000000
Rv0252	*nirB*	Probable nitrite reductase nadph large subunit fad flavoprotein	2.5	0.000014
Rv0341		Isoniazid inductible gene protein	16	0.000000
Rv0342		Isoniazid inductible gene protein	9.2	0.000000
Rv0343		Isoniazid inductible gene protein	4	0.000000
Rv0410c	*pknG*	Serine/threonine-protein kinase	2	0.000000
Rv0412c		Possible conserved membrane protein	2.3	0.000000
Rv0467	*icl*	Probable isocitrate lyase	2.6	0.000000
Rv0847	*lpqS*	Probable lipoprotein	2.5	0.000000
Rv0849		Probable conserved integral membrane transport protein	5.3	0.000015
Rv0850		Putative transposase fragment	4	0.000011
Rv0885		Conserved hypothetical protein	2.5	0.000000
Rv0886	*fprB*	Probable nadphadrenodoxin oxidoreductase	2	0.000000
Rv0888		Probable exported protein	2	0.000000
Rv1013	*pks16*	Putative polyketide synthase	2.1	0.000000
Rv1497	*lipL*	Probable esterase	2.3	0.000002
Rv1592c		Conserved hypothetical protein	5.7	0.000000
Rv1690	*lprJ*	Probable lipoprotein	2.5	0.000000
Rv1772		Hypothetical protein	3.7	0.000000
Rv1773c		Probable transcriptional regulatory protein	4.9	0.000869
Rv1779c		Hypothetical integral membrane protein	2.3	0.000002
Rv1993c		Conserved hypothetical protein	2	0.00052
Rv1995		Hypothetical protein	2.1	0.000000
Rv1999c		Probable conserved integral membrane protein	2	0.000011
Rv2243	*fabD*	Malonyl CoA-acyl carrier protein transacylase	3.7	0.000000
Rv2244	*acpM*	Meromycolate extension acyl carrier protein	2.3	0.000000
Rv2245	*kasA*	β-ketoacyl-ACP synthase	2.8	0.000000
Rv2246	*kasB*	β-ketoacyl-ACP synthase	3	0.000000
Rv2247	*accD6*	Acetyl/propionyl-CoA carboxylase β-subunit	2.6	0.000000
Rv2248		Conserved hypothetical protein	2.6	0.000000
Rv2524c	*fas*	Probable fatty acid synthase fas fatty acid synthetase	2.5	0.000000
Rv2721c		Possible conserved transmembrane alanine and glycine rich protein	2	0.000000
Rv2845c	*proS*	Probable prolyl-tRNA synthetase	3	0.000000
Rv2846c	*efpA*	Possible integral membrane efflux protein	3.7	0.000000
Rv2930	*fadD26*	Fatty-acid-CoA ligase	2.3	0.000000
Rv2931	*ppsA*	Phenolpthiocerol synthesis type-I polyketide synthase	2	0.000000
Rv2932	*ppsB*	Phenolpthiocerol synthesis type-I polyketide synthase	4	0.000000
Rv2963		Probable integral membrane protein	2	0.000000
Rv3049c		Probable monooxygenase	2	0.000000
Rv3139	*fadE24*	Probable acyl-CoA dehydrogenase	2	0.000000
Rv3140	*fadE23*	Probable acyl-CoA dehydrogenase	3	0.000000
Rv3252c	*alkB*	Probable transmembrane alkane 1-monooxygenase	2.1	0.000001
Rv3269		Conserved hypothetical protein	2.5	0.000000
Rv3270	*ctpC*	Probable metal cation-transporting p-type ATPase C	2.3	0.000000
Rv3289c		Possible transmembrane protein	2.5	0.000001
Rv3290c	*lat*	Probable l-lysine-epsilon aminotransferase	2.8	0.000005
Rv3337		Conserved hypothetical protein	2	0.000104
Rv3353c		Conserved hypothetical protein	2.5	0.001486
Rv3354		Conserved hypothetical protein	2.3	0.000000
Rv3466		Conserved hypothetical protein	39.4	0.00013
Rv3485c		Probable short-chain type dehydrogenase/reductase	7.5	0.00032

ORF	Gene	Gene product	Fold change	Change p-value
Rv3645		Probable conserved transmembrane protein	3	0.000000
Rv3675		Possible membrane protein	2.6	0.000000

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Table 5

M. tuberculosis genes repressed two-fold or greater by ethionamide treatment.

ORF	Gene	Gene Product	Fold change	Change *p*-value
Rv0263c		Conserved hypothetical protein	2.3	0.000000
Rv0320		Possible conserved exported protein	2	0.002189
Rv0516c		Conserved hypothetical protein	7.5	0.000000
Rv0950c		Conserved hypothetical protein	4	0.000000
Rv1365c	*rsfA*	Anti-anti-sigma factor	3.2	0.000084
Rv1550	*fadD11*	Probable fatty-acid-CoA ligase	2	0.000016
Rv1885c		Conserved hypothetical protein	4.6	0.000000
Rv1886c	*fbpB*	Secreted antigen 85-B	4.3	0.000000
Rv2190c		Conserved hypothetical protein	3	0.000000
Rv2485c	*lipQ*	Probable carboxylesterase	2.8	0.000000
Rv2560		Probable proline and glycine rich transmembrane protein	4	0.000016
Rv2633c		Hypothetical protein	2.1	0.000000
Rv2800		Possible hydrolase	2.3	0.001846
Rv3613c		Hypothetical protein	2.1	0.000000