Comparing isogenic strains of Beijing genotype Mycobacterium tuberculosis after acquisition of Isoniazid resistance: A proteomics approach

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We determined differences in the protein abundance among two isogenic strains of Mycobacterium tuberculosis (Mtb) with different Isoniazid (INH) susceptibility profiles. The strains were isolated from a pulmonary tuberculosis patient before and after drug treatment. LC-MS/MS analysis identified 46 Mtb proteins with altered abundance after INH resistance acquisition. Protein abundance comparisons were done evaluating the different bacterial cellular fractions (membrane, cytosol, cell wall and secreted proteins). MS data have been deposited to the ProteomeXchange with identifier PXD002986.

Keywords:
Isogenic strains / Isoniazid / Microbiology / Resistance / Tuberculosis

In United States, the tuberculosis (TB) rate has been decreasing since 1992, having a reported rate of three cases per 100,000 population in 2014 [1]. However, this country began to experience a severe interruption in the supply of isoniazid (INH) in 2012 [2]. INH is one of the most effective drugs to treat TB and to prevent active TB in persons with latent TB infection [3,4]. The INH scarcity affected the US TB programs and created incomplete treatment regimens that may lead to higher INH resistance rates over time.

Despite the proven success of INH against Mycobacterium tuberculosis (Mtb, the causing agent of TB), the understanding of its mechanism of action and development of resistance has been a slow process. INH is a prodrug that needs the bacterial enzyme KatG (catalase-peroxidase) to become active. Activated INH inhibits mycolic acids biosynthesis, cell division, nucleic acid synthesis and electron transport, among other bacterial processes [5, 6]. INH resistance mechanisms include mutations in multiple genes, most often in the katG gene.

Simultaneous to INH resistance development, Mtb can undergo further variations including changes in protein levels which in turn may counteract the potential fitness loss due to the new phenotype. A previous proteomic analysis compared INH susceptible (INHs) and resistant (INHr) strains of Mtb and found five proteins overexpressed in the INHr strains. These proteins were not related to any of the known INH resistance mechanisms [7].

In the present study, we worked with clinical isogenic pairs of Mtb, to evaluate the variation in the protein levels after development of INH resistance. Clinical isogenic pairs are strains with the same genotype which can be obtained from the same patient before and after treatment. Although rare, isogenic pairs provide a unique setting to study drug resistance mechanisms and potential loss in fitness due to mutations conferring drug resistance without confounding effects due to intrinsic genotype differences.

We compared the global protein abundance levels of a clinical isogenic pair of Mtb and classified the proteome changes according with their functional category. Two isogenic strains of Mtb were isolated from a HIV positive patient, alcoholic, and intravenous drug user diagnosed in 1994 with pulmonary TB at University General Hospital of Gran Canaria Doctor Negrín, Las Palmas, Spain. The isolate obtained after drug treatment failure, was INHr to both concentrations tested (0.2
and 1.0 μg/mL). Both strains belong to the Beijing genotype, tested by restriction fragment length polymorphism RFLP - IS6110 [8] and spoligotyping [9]. Drug susceptibility profiles were confirmed for both strains using the agar proportion method [10] by National Jewish Hospital, Denver, CO. After INHr, the strain developed MDR (multidrug-resistance) phenotype (resistance to i and rifampicin) and was successfully treated with second line drugs.

Bacteria culture condition, Culture Filtrate Protein (CFP) preparation, subcellular fractionation and proteomic analysis were performed as previously described with minor modifications [11]. Briefly, three biological replicates of each strain were cultured in one liter Glycerol-Alanine-salts media. The bacteria culture condition, Culture Filtrate Protein (CFP) preparation, subcellular fractionation and proteomic analysis were performed as previously described with minor modifications [11]. Briefly, three biological replicates of each strain were cultured in one liter Glycerol-Alanine-salts media. The culture filtrates were harvested by centrifugation at 13,770 × g for 10 min. Culture filtrates were previously described with minor modifications [11]. Briefly, three biological replicates of each strain were cultured in one liter Glycerol-Alanine-salts media. The culture filtrates were harvested by centrifugation at 13,770 × g for 10 min. Culture filtrates were concentrated to a final volume of approximately 20 mL using a Millipore Amicon 10-kDa Bioseparations Stirred Cell with a 3-kDa mass cutoff membrane (Millipore). Concentrated CFP and CYP fraction were subjected to buffer exchange with 10 mM ammonium bicarbonate, using Amicon Ultra-15 centrifugal filter units with a 3-kDa molecular mass cutoff. The CW and MEM pellets were resuspended in 10 mM ammonium bicarbonate.

After the separation of CFP and mycobacterial cell fractions, protein was quantified by the bicinchoninic acid method (Thermo Scientific Pierce). 30 μg of MEM, CYP and CFP were subjected to acetone precipitation, solubilization, reduction with dithiothreitol, alkylation with iodoacetamide, and trypsin digestion (using a mix of 1% ProteaseMaxTM Surfactant (Promega) and trypsin (Roche)) as described previously [11]. Following digestion, samples were desalted with Pierce® PepClean C18 columns (Thermo Scientific) following the manufacturer instructions. CW proteins had a delipidation process [11] before to the protein digestion protocol described above.

One microgram of digested cellular fractions and CFP for all the three biological replicates were randomly analyzed in triplicate using LC-MS/MS as described previously [11]. Resulting raw data were converted into mzXML files using ProteoWizard [12]. LC-MS/MS spectra were then compared against Mtb genomic database (MtbReverse041712) using SORCERER (Sage-N Research, version 5.0.1). The parameters used for the analysis were: trypsin digestion, a maximum of two missed cleavages, a precursor mass range of 400 to 4500 amu, peptide mass tolerance of 1.5 amu, reduction and alkylation of cysteine residues (resulting in the addition of a carbamidomethyl group, 15.99 amu) and the oxidation of methionine (57.02 amu).

For each cellular fraction, peptide identifications from the MS/MS spectra previously searched were combined in the proteomic software Scaffold (version Scaffold 4.3.2, Proteome Software Inc., Portland, OR) summing all the technical replicates results for each biological sample. Normalized spectral abundance factor (NSAF) analysis was performed to measure the relative protein abundance [13]. Additional parameters required for the Scaffold algorithm for protein identification included a maximum of 5% of false discovery rate for peptide threshold as well as for protein threshold and at least of two peptides.

The MS proteomics data have been deposited to the ProteomeXchange Consortium [14] via the PRIDE partner repository with the dataset identifier PXD002986 and 10.6019/PXD002986. Differences between protein abundances among the two different susceptibility profiles were tested by two tailed Student’s t-test.

We found 46 proteins either more or less abundant after acquisition of INHr (with p < 0.05) that were grouped in seven different categories (Fig. 1). These protein differences were mostly observed in the CFP (39.6%) and MEM (35.4%) fractions (Fig. 1, Table 1).

In our quantitative analysis, we particularly found low levels of KatG in the INHr isolate potentially explaining the resistance phenotype. The reduced levels of KatG were observed in all cellular fractions, except in the cell wall (Table 1). In addition to the role of activating INH, KatG is also involved in the Mtb response to reactive oxygen intermediates produced by phagocytes during intracellular infections [15], making this protein a well-studied virulence factor.

The category “Intermediary metabolism and respiration (IMR)” presented the highest number of proteins (n = 20) with variable abundance among the strains. In this category the enzymes from the tricarboxylic acid (TCA) cycle SucC, SucD (located in the same operon), Mdh, Acm and AceE were all decreased in the INHr strain (Fig. 2). AceE belongs to the aerobic oxidative TCA cycle. Additionally, two enzymes of the pentose phosphate pathway Gnd2 and Tal were also significantly different in this analysis but with higher and lower levels in the INHr strain, respectively (Table 1).

Among lipid metabolism, we detected differences in proteins involved in lipid biosynthesis and degradation pathways. For the former, FabG4 and Fas were increased in the INHr strain. FabG4 participates in the elongation of saturated fatty acids while Fas is a structurally integrated type I fatty acid synthase (FAS-I), similar to those found in eukaryotes. This particular enzyme has all catalytic domains contained within a single protein chain [16]. There were also enzymes identified in this study that belong to the FAS-II system, Rv0241 (HtdX) and Rv3389 (HtdY), but with different behavior. While HtdX had higher, HtdY had lower abundance levels in the INHr strain. Due to their sequence and structure, both enzymes are considered 3-hydroxyacyl thioester dehydratases, but HtdX has a particular high capacity to produce lipoic acid and an increased preference for its substrate (3-hydroxyacylacyl carrier protein, ACP) [17]. In our study, HtdX trend was similar to the other enzymes involved in lipid biosynthesis. The different levels observed between HtdX and HtdY may...
Table 1. Description of significantly different proteins in the INHr vs INHs Beijing strain comparison (t-test, \( p < 0.05 \))

| Proteins significantly different (t-test, \( p < 0.05 \)) | Gene name | Rv number | Functional category | Fold change (INHs/INHr) |
|----------------------------------------------------------|-----------|-----------|---------------------|------------------------|
| **CFP (n = 19)**                                         |           |           |                     |                        |
| Iron-regulated peptidyl-prolyl-cis-trans-isomerase A     | ppiA      | Rv0009    | IMR                 | 1.6                    |
| Chaperone protein DnaK                                   | dnaK      | Rv0350    | V                   | 0.5                    |
| Succinyl-CoA synthetase beta chain                       | succC     | Rv0961    | IMR                 | 4.1                    |
| Succinyl-CoA synthetase alpha chain                      | succD     | Rv0852    | IMR                 | 3.9                    |
| Enoyl-CoA hydratase EchA9                                | echA9     | Rv1071c   | L                   | 1.8                    |
| 6-phosphogluconate dehydrogenase, decarboxylating Gnd2   | gnd2      | Rv1122    | IMR                 | 2.9                    |
| Integration host factor MihF                            | mihF      | Rv1388    | IP                  | 1.7                    |
| Transaldolase                                            | tal       | Rv1448c   | IMR                 | 0.5                    |
| Catalase-peroxidase-peroxynitrilase T KatG               | katG      | Rv1908c   | V                   | 14                     |
| Conserved protein                                        | Rv2204c   | Rv2204c   | C                   | 0.5                    |
| Trigger factor protein                                   | tig       | Rv2462c   | CW                  | 3.5                    |
| Conserved protein                                        | Rv2699c   | Rv2699c   | C                   | 3.9                    |
| Adenosylhomocysteinase                                   | sahH      | Rv3248c   | IMR                 | 0.4                    |
| Thiosulfate sulfurtransferase                            | sseA      | Rv3283    | IMR                 | 3.6                    |
| 3-hydroxyacyl-thioester dehydratase HtdY                 | htdY      | Rv3389c   | L                   | 3.9                    |
| 10 kDa chaperonin (protein CPN10), MPT57                 | groES     | Rv3418c   | V                   | 0.8                    |
| Conserved protein                                        | Rv3433c   | Rv3433c   | C                   | 0.2                    |
| Conserved membrane protein                               | Rv3587c   | Rv3587c   | CW                  | 0.6                    |
| Secreted fibronectin-binding protein antigen protein      | fbpD      | Rv3803c   | L                   | 0.4                    |
| **CW (n = 6)**                                           |           |           |                     |                        |
| 3-oxoacyl-[acyl-carrier protein] reductase FabG4          | fabG4     | Rv0242c   | L                   | 0.4                    |
| Acetyl-CoA acyltransferase FadA2                         | fadA2     | Rv0243    | L                   | 0.5                    |
| Immunogenic protein MPT63                                | mpt63     | Rv1926c   | CW                  | 2.9                    |
| ATP-dependent clp protease proteolytic subunit 2         | clpP2     | Rv2460c   | IMR                 | 0.5                    |
| Fatty-acid synthase (FAS)                                | fas       | Rv2524c   | L                   | 0.3                    |
| Transcriptional regulator, crp/fmr-family                | crp       | Rv3676    | R                   | 0.1                    |
| **CYT (n = 6)**                                          |           |           |                     |                        |
| Two component system transcriptional regulator PrrA      | prrA      | Rv0903c   | R                   | 0.3                    |
| 5-methyltetrahydropyrolylglutamate-homocysteine methyltransferase MetE | metE | Rv1133c | IMR | 5.2 |
| Malate dehydrogenase                                     | mdh       | Rv1240    | IMR                 | 1.3                    |
| Phosphoglycerate kinase                                  | pgk       | Rv1437    | IMR                 | 1.8                    |
| Catalase-peroxidase-peroxynitrilase T KatG               | katG      | Rv1908c   | V                   | 61                     |
| Aminomethyltransferase                                   | gcvT      | Rv2211c   | IMR                 | 0.2                    |
| **MEM (n = 17)**                                         |           |           |                     |                        |
| 3-hydroxyacyl-thioester dehydratase Hdx                  | hdx       | Rv0241c   | L                   | 0.09                   |
| Transport protein SecE2                                  | secE2     | Rv0379    | CW                  | 1.3                    |
| Poly-preynyl-diphosphate synthase                        | gpcC1     | Rv0562    | IMR                 | 1.3 \(\text{INF}^{b}\) |
| 50S ribosomal protein L23, RplW                            | rplW      | Rv0703    | IP                  | 0.5                    |
| Phosphoribosylformylglycinamidine synthase II            | purL      | Rv0803    | IMR                 | 1.7                    |
| Transcription termination factor Rho                     | rho       | Rv1297    | IP                  | 1.9                    |
| Thioredoxin                                             | Rv1324    | Rv1324    | IMR                 | 3.2                    |
| Iron-regulated aconitate hydratase                       | acn       | Rv1475c   | IMR                 | 1.3                    |
| Glycine dehydrogenase                                    | gcvB      | Rv1832    | IMR                 | 3.6                    |
| Catalase-peroxidase-peroxynitrilase T KatG               | katG      | Rv1908c   | V                   | 2.6                    |
| Monophosphatase                                          | cysQ      | Rv2131c   | IMR                 | 7.5                    |
| Pyruvate dehydrogenase E1 component                      | aceE      | Rv2241    | IMR                 | 1.2                    |
| Conserved protein                                        | Rv2402    | Rv2402    | C                   | 1.6                    |
| Chorismate synthase                                      | arOF      | Rv2540c   | IMR                 | 0.4                    |
| Acetyl-CoA dehydrogenase, EadE2                         | fadE2     | Rv3061c   | L                   | 0.5                    |
| Acetyl-CoA dehydrogenase, FadE32                        | fadE32    | Rv3563    | L                   | 0.1                    |
| Enoyl-CoA hydratase EchA21                               | echA21    | Rv3774    | L                   | 2.7                    |

\(a\) The quantitative method chosen for the statistical analysis and \(p\) value calculation was NSAF.  
\(b\) INF: NSAF in INHr strain was zero. IMR: Intermediary metabolism and respiration, V: Virulence, detoxification, adaptation, IP: Information pathways, L: Lipid metabolism, R: Regulatory protein, CW: Cell wall and cell wall processes, C: Conserved Hypothetical.
be in line with results elsewhere indicating that HdtY may not be part of the ACP-dependent FAS-II system [17].

For fatty acid β oxidation, the dehydrogenases FadE22 and FadE32 and the acetyl-CoA acyltransferase FadA2 were increased while the crotonases EchA9 and EchA21 were decreased in the INHr strain (Table 1).

In summary, we demonstrated that acquisition of INH resistance can result in significant changes in the mycobacteria proteome, particularly in pathways related to respiration and lipid metabolism, both of which may result as a compensatory mechanism to the decrease in KatG abundance and its consequent impact on mycobacterial physiology and fitness.

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Figure 1. Functional categories of the Mtb proteins showing different levels among the INHs and INHr isogenic strains (p value <0.05). All categories are listed according to Tuberculist (version 2.6, Release 27 - March 2013, http://tuberculist.epfl.ch/).

Figure 2. TCA cycle in Mtb. The enzymes in the boxes are reduced in the Beijing INHr strain. Adapted from http://biocyc.org/MTBRV.
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The proteomics MS data in this paper have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [18]: dataset identifier PXD002986.

The authors have declared no conflict of interest.

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