Analysis of intracellular expressed proteins of *Mycobacterium tuberculosis* clinical isolates

Neelja Singhal¹, Prashant Sharma¹, Manish Kumar², Beenu Joshi³ and Deepa Bisht¹∗

**Abstract**

**Background:** Tuberculosis (TB) is the most threatening infectious disease globally. Although progress has been made to reduce global incidence of TB, emergence of multidrug resistant (MDR) TB threatens to undermine these advances. To combat the disease, novel intervention strategies effective against drug resistant and sensitive subpopulations of *M. tuberculosis* are urgently required as adducts in the present treatment regimen. Using THP-1 cells we have analyzed and compared the global protein expression profile of broth-cultured and intraphagosomally grown drug resistant and sensitive *M.tuberculosis* clinical isolates.

**Results:** On comparing the two dimensional (2-DE) gels, many proteins were found to be upregulated/expressed during intracellular state which were identified by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Four proteins (adenosylhomocysteinase, aspartate carbamoyltransferase, putatitive thiosulfate sulfurtransferase and universal stress protein) were present in both intracellular MDR and sensitive isolates and three of these belonged to intermediary metabolism and respiration category. Two proteins (alanine dehydrogenase and adenosine kinase) of intracellular MDR isolate and two (glucose-6-phosphate isomerase and ATP synthase epsilon chain) of intracellular sensitive isolate belonged to intermediary metabolism and respiration category. One protein (Peroxidase/Catalase) of intracellular MDR and three (HSPX, 14 kDa antigen and 10 kDa chaperonin) of sensitive isolate belonged to virulence, detoxification and adaptation category. ESAT-6 of intracellular MDR belonged to cell wall and cell processes category. Two proteins (Antigen 85-C and Antigen 85-A) of intracellular sensitive isolate were involved in lipid metabolism while probable peptidyl-prolyl cis-trans isomerase A was involved in information pathways. Four (Rv0635, Rv1827, Rv0036c and Rv2032) of intracellular MDR and two proteins (Rv2896c and Rv2558c) of sensitive isolate were hypothetical proteins which were functionally characterized using bioinformatic tools. Bioinformatic findings revealed that the proteins encoded by Rv0036, Rv2032c, Rv0635, Rv1827 and Rv2896c genes are involved in cellular metabolism and help in intracellular survival.

**Conclusions:** Mass spectrometry and bioinformatic analysis of both MDR and sensitive isolates of *M. tuberculosis* during intraphagosomal growth showed that majority of commonly upregulated/expressed proteins belonged to the cellular metabolism and respiration category. Inhibitors of the metabolic enzymes/intermediate can therefore serve as suitable drug targets against drug-resistant and sensitive subpopulations of *M. tuberculosis*.

**Background**

Despite more than a century of research, tuberculosis (TB) as a disease claims more deaths than any other infectious agent making its causative organism *Mycobacterium tuberculosis*, one of the most successful human pathogens. Inappropriate treatment regimen and patient poor-compliance have led to the appearance of drug resistant TB. Multi Drug Resistant TB (MDR-TB) is caused by bacteria that are resistant to the most effective anti-TB drugs (Isoniazid and Rifampicin) with or without resistance to other drugs. 50% of MDR-TB cases in world are estimated in India and China [1]. In 2010, the largest WHO MDR-TB survey reported the highest rates of MDR-TB, with 28% of new TB cases in some settings of the former Soviet Union [1]. This is an alarming situation which calls for exploring therapeutics equally effective against drug sensitive and resistant population of *M. tuberculosis*. A major impetus of TB...
drug development process is to develop chemical compounds capable to cure TB patients, regardless of whether the disease is caused by *M. tuberculosis* which is drug sensitive or resistant to the current first and second line drugs [2]. Identification and development of improved intervention strategies requires better understanding of host-pathogen interactions. Different approaches have been used to study mycobacterial genes that play a role in the interaction with host cells and thus in virulence. These include *in vivo* induced antigen technology, subtractive hybridization, *in vivo* expression technology etc. [3-5].

Proteomics is a powerful tool to study complex biological samples and its application has greatly contributed to a better understanding of the biology of *M. tuberculosis* and other pathogenic bacteria. Putative drug targets, vaccine candidates, and diagnostic markers for TB have also been identified using this approach [6-8]. Identification of mycobacterial proteins of drug resistant and sensitive isolates by two-dimensional electrophoresis (2-DE) and mass spectrometry has largely been applied to broth grown cultures, because abundant amounts of protein are available here for analysis and comparison. Such studies have been carried out by us [9] and other researchers [10,11]. Difficulty in recovery of sufficient amounts of protein from intracellular state accounts for the existence of only a few such studies [12,13]. To date, our knowledge regarding the proteomic profiles of drug resistant and sensitive *M. tuberculosis* during intracellular growth have been fragmentary.

In the present work, we have analyzed the protein expression profile of *M. tuberculosis* MDR and sensitive isolates while infecting THP-1 cells to study the gene expression changes that actually affect survival and growth of resistant or sensitive isolates while growing inside the host macrophage cells to identify proteins or protein-class which are upregulated/expressed inside macrophages and could be used as a common drug target for both types of microbial population.

Results
The goal of this study was to identify mycobacterial proteins upregulated/expressed during growth in macrophages by comparative proteome analysis of broth-cultured and intraphagosomally grown mycobacteria. To elucidate protein spots unique to intraphagosomal mycobacteria the generated 2-DE gels (Figure 1) were analyzed using the software program PDQuest (Biorad, USA) and spots which were upregulated/expressed with at least 2.5 intensity were selected.

2-DE of intracellular *M. tuberculosis* drug sensitive isolate proteins and their MALDI-MS analysis
Comparison of composite 2-DE gels of broth-cultured *M. tuberculosis* MDR and sensitive isolates with intraphagosomally grown *M. tuberculosis* MDR isolate showed sixteen protein spots to be upregulated/expressed by the intraphagosomal mycobacteria (Figure 1B). Spots I1 and I2 (Rv1908c) were identified as peroxidase/catalase. Spots I3, I4, I5, I6 (Rv3248c) were identified as isoforms of Adenosylhomocysteinase. I7 (Rv2623) was identified as a Universal stress protein. Spots I8 (Rv2780c) and I9 (Rv2202c) were identified as Alanine dehydrogenase and Adenosine kinase respectively. Spots I10 (Rv2032), I11 (Rv0036c), I12 (Rv1827) and I15 (Rv0635) were identified as hypothetical proteins. Spot I12 (Rv1380) was identified as Aspartate carbamoyltransferase. Spot I11 (Rv0815c) was identified as Putative thiosulfate sulfurtransferase. Spot I16 (Rv2347c) was identified as a putative ESAT-6 like protein. Table 1 shows details of upregulated/expressed proteins identified by mass spectrometry.

2-DE of intracellular *M. tuberculosis* drug sensitive isolate proteins and their MALDI-MS analysis
Comparison of composite 2-DE gels of broth-cultured *M. tuberculosis* MDR and sensitive isolates with intraphagosomally grown *M. tuberculosis* sensitive isolate showed twenty one protein spots which were upregulated/expressed by the intraphagosomal mycobacteria (Figure 1C). Spot D1 (Rv0946c) was identified as Glucose-6-phosphate isomerase. Spots D2, D3, D4, D5, D6, (Rv3248c) were identified as isoforms of Adenosylhomocysteinase. Spots D7 (Rv2896c) and D13 (Rv2558) were identified as a hypothetical proteins. D8 (Rv2623) was identified as a Universal stress protein. Spots D9 (Rv0129c) and D12 (Rv3804c) were identified as Antigen 85-C and Antigen 85-A mycolyl transferases. Spot D10 (Rv1380) was identified as Aspartate carbamoyltransferase. Spot D11 (Rv0815c) was identified as Putative thiosulfate sulfurtransferase. Spots D14 and D15 were Probable peptidyl-prolyl cis-trans isomerase A (Rv0009). Spots D16 and D17 (Rv1311) were identified as ATP synthase epsilon chain. Spots D18, D20 (Rv2031c) and D19 (Rv0251) were identified as 14 kDa antigen, heat shock protein HSPX. Spot D21 (Rv3418c) was 10 kDa chaperonin. Table 2 shows details of differentially expressed proteins identified by mass spectrometry.

Functional characterization of hypothetical proteins employing bioinformatic tools
(a) BLAST Analysis
Prediction of probable function of hypothetical proteins was done using BLASTp search performed at NCBI server http://blast.ncbi.nlm.nih. The top five BLAST hits of all six mycobacterial proteins showed statistically significant alignment with 90-100% sequence coverage. Rv0036, Rv2032c, Rv2896c and Rv2558 though found to be highly conserved in mycobacterial species could not
be assigned any function because the hits were identified as hypothetical proteins. BLAST hits of Rv0635 were found to be highly conserved in different mycobacterial species where it functions like 3R-hydroxyacyl-ACP dehydratase subunit HadA. BLAST hits of Rv1827 were highly conserved in mycobacterial sp. but attributed it diverse functional annotations like hypothetical protein sequences, Chain A PknB-Phosphorylated protein, FHA (Forkhead-associated) domain containing protein. FHA domain is a phosphopeptide recognition domain found in many regulatory proteins. As BLAST analysis could not reveal probable function of many proteins, we also searched for the presence of motif in these proteins using MotifScan.

(b) MotifScan Analysis
MotifScan run was performed for all the hypothetical proteins, but no motif was found except in Rv0635. A motif YNNN_MF_00799 http://myhits.isb-sib.ch/cgi-bin/view_mot_entry?name=hamap:YNNN_MF_00799 was detected in Rv0635. This motif is conserved in UPF0336 family of proteins. The proteins of this family comprise M. leprae, M. paratuberculosis, M. tuberculosis and M. bovis in addition to many other species of bacteria. This motif is termed as MaoC_dehydratase domain which is a member of HotDog superfamily and is involved in oxidoreductase activity.

(c) Domain Analysis
We selected top five BLAST hits for probing into the conserved domains and hence annotating the function of each query protein http://www.ncbi.nlm.nih.gov/cdd. Rv0635 had a putative R_hydratase like conserved domain http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=4803 which is found in the proteins belonging to HotDog superfamily. Analysis of conserved
Table 1 Details of over-expressed proteins of *M.tuberculosis* MDR isolate identified by mass spectrometry

| Spot No. | Protein identified                                      | MASCOT Score | Nominal Mass (Da) | pI  | Sequence Coverage % | Accession Number |
|----------|---------------------------------------------------------|--------------|-------------------|-----|---------------------|-----------------|
| I1       | Peroxidase/catalase T                                   | 144          | 80725             | 5.09| 31%                 | Rv 1908c        |
| I2       | Peroxidase/catalase T                                   | 64           | 80725             | 5.09| 9%                  | Rv 1908c        |
| I3       | Adenosylhomocysteinase (AdoHcyase)                     | 105          | 54461             | 5.07| 23%                 | Rv 3248c        |
| I4       | Adenosylhomocysteinase (AdoHcyase)                     | 120          | 54461             | 5.07| 25%                 | Rv 3248c        |
| I5       | Adenosylhomocysteinase (AdoHcyase)                     | 88           | 54461             | 5.07| 19%                 | Rv 3248c        |
| I6       | Adenosylhomocysteinase (AdoHcyase)                     | 80           | 54461             | 5.07| 17%                 | Rv 3248c        |
| I7       | Universal stress protein                               | 162          | 31747             | 5.46| 57%                 | Rv2623          |
| I8       | Alanine dehydrogenase                                  | 90           | 38974             | 5.81| 31%                 | Rv2780c         |
| I9       | Adenosine kinase                                        | 108          | 34621             | 5.14| 31%                 | Rv2202c         |
| I10      | Hypothetical protein                                   | 82           | 36765             | 5.28| 23%                 | Rv2032          |
| I11      | Putative thiosulfate sulfurtransferase                 | 37           | 31110             | 5.14| 24%                 | Rv0815c         |
| I12      | Aspartate carbamoyltransferase (ATCase)                 | 50           | 33798             | 6.60| 22%                 | Rv1380          |
| I13      | Hypothetical protein                                   | 95           | 27586             | 4.84| 44%                 | Rv0036c         |
| I14      | Hypothetical protein                                   | 64           | 17240             | 4.29| 34%                 | Rv1827          |
| I15      | Hypothetical protein                                   | 85           | 17584             | 4.51| 51%                 | Rv0635          |
| I16      | Putative ESAT-6 like protein                            | 57           | 10970             | 5.17| 29%                 | Rv2347c         |

Table 2 Details of over-expressed proteins of *M.tuberculosis* drug sensitive isolate identified by mass spectrometry

| Spot No. | Protein identified                                      | MASCOT Score | Nominal Mass (Da) | pI  | Sequence Coverage % | Accession Number |
|----------|---------------------------------------------------------|--------------|-------------------|-----|---------------------|-----------------|
| D1       | Glucose-6-phosphate isomerase (GPI)                     | 136          | 59937             | 5.40| 29%                 | Rv0946c         |
| D2       | Adenosylhomocysteinase (AdoHcyase)                     | 87           | 54461             | 5.07| 19%                 | Rv 3248c        |
| D3       | Adenosylhomocysteinase (AdoHcyase)                     | 177          | 54461             | 5.07| 38%                 | Rv 3248c        |
| D4       | Adenosylhomocysteinase (AdoHcyase)                     | 188          | 54461             | 5.07| 41%                 | Rv 3248c        |
| D5       | Adenosylhomocysteinase (AdoHcyase)                     | 58           | 54461             | 5.07| 13%                 | Rv 3248c        |
| D6       | Adenosylhomocysteinase (AdoHcyase)                     | 60           | 54461             | 5.07| 11%                 | Rv 3248c        |
| D7       | Hypothetical protein                                   | 20           | 40376             | 6.61| 12%                 | Rv2890c         |
| D8       | Universal stress protein                               | 113          | 31747             | 5.46| 57%                 | Rv2623          |
| D9       | Antigen 85-C, Mycolyl Transferase 85c                 | 85           | 36771             | 5.1 | 30%                 | Rv0129c         |
| D10      | Aspartate carbamoyltransferase (ATCase)                 | 60           | 33798             | 6.60| 24%                 | Rv1380          |
| D11      | Putative thiosulfate sulfurtransferase                 | 37           | 31110             | 5.14| 24%                 | Rv0815c         |
| D12      | Antigen 85-A FBPA (Mycolyl transferase 85A)             | 80           | 28634             | 5.3 | 25%                 | Rv3804c         |
| D13      | Hypothetical protein                                   | 21           | 26044             | 6.18| 6%                  | Rv2558          |
| D14      | Probable peptidyl-prolyl cis-trans isomerase A         | 30           | 19285             | 5.80| 20%                 | Rv0009          |
| D15      | Probable peptidyl-prolyl cis-trans isomerase A         | 110          | 19285             | 5.80| 54%                 | Rv0009          |
| D16      | ATP synthase epsilon chain ATPC                        | 51           | 13127             | 4.55| 25%                 | Rv1311          |
| D17      | ATP synthase epsilon chain                              | 38           | 13127             | 4.55| 26%                 | Rv1311          |
| D18      | Heat shock protein HSPX (alpha-crystallin homolog)     | 108          | 16217             | 5.0 | 59%                 | Rv2031c         |
| D19      | 14 kDa antigen (16 kDa antigen)                        | 74           | 16217             | 5.00| 38%                 | Rv0251c         |
| D20      | Heat shock protein HSPX (alpha-crystallin homolog)     | 79           | 16217             | 5.0 | 37%                 | Rv2031c         |
| D21      | 10 kDa chaperonin                                       | 67           | 10798             | 4.62| 35%                 | Rv3418c         |
regions of BLAST hits of Rv1827 revealed the presence of FHA http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=28942 and a phosphopeptide binding site and analysis of Rv0036 revealed presence of two conserved domains, of MDMPI_N Superfamily and Radical_SAM superfamily http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=174256. A Lysine_decarboxylase conserved domain was found to be present in the top five BLAST hits of Rv2896c http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?RID=7FEA98PB016 while no conserved domains were found to be present in the top five BLAST hits of Rv2558.

Discussion

In the present study we have analyzed the protein profiles of M.tuberculosis MDR and sensitive clinical isolates while infecting human macrophage-like THP-1 cells as a means of analyzing the environmental conditions faced by the pathogen during infection. This may contribute significantly in understanding whether mechanisms adopted by resistant and sensitive mycobacteria to survive and grow inside the host macrophages are similar or different and would help in finding new drug targets. The use of human THP-1 macrophage cell line, rather than human peripheral blood monocyte derived or alveolar macrophages, was necessary to provide sufficient cells to recover intracellular mycobacteria.

MALDI-MS identification of the intracellular MDR and sensitive isolates revealed that majority of the common proteins upregulated/expressed in the intracellular state belonged to intermediary metabolism and respiration category. Four proteins (adenosylhomocysteinase, aspartate carbamoyltransferase, putative thiosulfate sulfurtransferase and universal stress protein) were present in both isolates and three of these belonged to intermediary metabolism and respiration category. Two proteins (alanine dehydrogenase and adenosine kinase) of intracellular MDR isolate and two (glucose-6-phosphate dehydrogenase and universal stress protein) were present as a means of analyzing the environmental conditions faced by the pathogen during infection. This may contribute significantly in understanding whether mechanisms adopted by resistant and sensitive mycobacteria to survive and grow inside the host macrophages are similar or different and would help in finding new drug targets. The use of human THP-1 macrophage cell line, rather than human peripheral blood monocyte derived or alveolar macrophages, was necessary to provide sufficient cells to recover intracellular mycobacteria.

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Two proteins (Antigen 85-C and Antigen 85-A) of intracellular sensitive isolate were involved in lipid metabolism while probable peptidyl-prolyl cis-trans isomerase A was involved in information pathways. Four (Rv0635, Rv1827, Rv0036c and Rv2032) of intracellular MDR and two proteins (Rv2896c and Rv2558c) of sensitive isolate were hypothetical proteins which were functionally characterized using bioinformatic tools.

Adenosylhomocysteinase (SAHH) catalyzes the reversible hydrolysis of S-adenosylhomocysteine (SAH) into free adenosine (ADO) and L-homocysteine (HCY). SAH is produced from S-adenosylmethionine (SAM) as a by-product of SAM-dependent methyltransferase reactions. Methylation plays a role in a wide range of cellular processes, including DNA replication and repair, methionine metabolism, polyamine and phospholipid biosynthesis. SAHH is also considered “druggable” [14], has been shown to be essential for growth in vitro and appears to be upregulated in infected mouse lung tissue [15]. Aspartate carbamoyltransferase (also known as aspartate transcarbamoylase or ATCase) catalyzes the first step in the pyrimidine biosynthetic pathway. Thiosulfate sulfurtransferase is a Rhodanese-like protein which catalyzes transfer of sulfane sulfur from substrate to enzyme active site and then to a thiophilic acceptor. Much information on the functional role of rhodanese is not available however lack of rhodanase encoding gene has shown to affect the sensitivity of Azotobacter vinelandii to oxidative damages [16]. M. tuberculosis has universal stress proteins, whose function is unknown. Proteomic and transcriptomic analysis have shown that a number of these genes are significantly upregulated under hypoxic conditions and in response to nitric oxide and carbon monoxide, as well as during M. tuberculosis infection of macrophage cell lines, suggesting their probable role in persistence and/or intracellular survival [17].

NAD (H)-dependent L-alanine dehydrogenase catalyzes the oxidative deamination of L-alanine and reductive amination of pyruvate to generate alanine for protein and peptidoglycan synthesis and plays a key role in cell wall synthesis. Feng et al. [18] have shown its important role in alanine utilization and anaerobic growth in mycobacteria. Huttera and Dicka [19] using M. smegmatis reported its upregulated during oxygen depletion-induced dormancy. This indicates that during intracellular state bacilli are not metabolically inactive but maintain a low level metabolism to tide over the unfavorable condition. Adenosine kinase which catalyzes the phosphorylation of adenosine is important for the regulation of cellular levels of adenosine and its nucleotides and was found to be expressed by MDR isolate during intracellular state. Purine metabolism has been proposed as a drug target [20]. Glucose-6-phosphate isomerase (PGI) plays a central role in glycolysis and gluconeogenesis. Interruption of PGI gene resulted in glucose auxotrophy [21]. During mycobacterium infection in macrophages, a metabolic shift from a strict
aerobic respiratory mode to anaerobic metabolism occurs, with a significant increase in the levels of glycolytic enzymes. During this stage, 70% of the organism’s energy is derived from glycolysis. Thus, being central to the organism’s survival, the enzymes involved in glycolysis are an attractive target for drug design. *M. tuberculosis* sensitive isolate showed an increased expression of ATP synthase epsilon chain. ATP synthase epsilon chain produces ATP in presence of a proton gradient across the membrane. Tran and Cook [22] showed that it is an essential gene in *M. smegmatis* during growth on non-fermentable and fermentable carbon sources. Gengenbacher et al. [23] showed that nutrient-starved, non-replicating *M. tuberculosis* requires respiration, ATP synthase and isocitrate lyase for maintenance of ATP homeostasis and viability.

Intracellular MDR isolate showed an increased expression of Catalase/peroxidase protein. Encoded by the katG gene it is an important virulence determinant of *M. tuberculosis* which protects against oxidative stress. Catalase/peroxidase activity has been shown necessary for growth and persistence in mice and guinea pigs [24] and in human peripheral blood monocyte [25]. Isoniazid, a widely used frontline antimycobacterial agent requires activation by catalase-peroxidase KatG before exerting a lethal effect. KatG couples the isonicotinic acyl with NADH to form the isonicotinic acyl-NADH complex that ultimately confers antitubercular activity [26]. HSPX is a 14kDa antigenic stress protein induced by anoxia and has a proposed role in maintenance of long-term viability during latent, asymptomatic infections, in replication during initial infection of macrophages [12,27].

6 kDa early secreted antigenic targets (ESAT-6) which forms a heterodimeric complex with culture filtrate protein (CFP-10) was exclusively detected during intracellular growth of MDR isolate and is involved in host-pathogen interactions. They induce a strong T cell mediated response, involved in membrane/host-cell lysis and represent key virulence factors [28]. ELISA-IGRA test uses ESAT-6 and CFP-10 as stimulating antigens for detecting tuberculosis infection [29] and has the ability to discriminate between tuberculosis infection and previous exposure to BCG vaccine or atypical mycobacteria reactivity.

Antigen 85-C and Antigen 85-A mycolyl transferases constitute a major fraction of the secreted proteins of *M. tuberculosis* culture filtrate. Gene disruption studies encoding the three Ag85 components of *M. tuberculosis* suggests that Ag85A may be the most essential component for bacterial survival within macrophages. Ag85A and Ag85B have been reported to be ideal vaccine candidates in a number of studies [30,31]. Peptidyl-prolyl cis-trans isomerases catalyzes the interconversion of cis and trans peptide bonds and are therefore considered to be important for protein folding. They are also thought to participate in processes such as signalling, cell surface recognition, chaperoning and heat shock response [32]. 14 kDa antigen (16 kDa antigen) is believed to be involved in the initiation step of translation at high temperature and is possibly a molecular chaperone. Sharma et al. found that the expression of this protein was upregulated in drug -pressure of streptomycin [33]. Chaperonins form a sub-group of molecular chaperones and 10-kDa antigen has homology with the GroES or chaperonin-10 (Cpn 10) family of heat shock proteins [34]. 10-kDa antigen has been shown to be an important T-cell antigen in tuberculosis patients.

Further, intracellular resistant/sensitive *M. tuberculosis* hypothetical proteins were functionally characterized using bioinformatics tools. Rv0036, Rv2032c, Rv2896c and Rv2558 though found to be highly conserved in *Mycobacterium* species could not be assigned any function through BLASTp analysis. Motif Scan webserver also didn’t find any motifs in these proteins while CDD found conserved domains in these except Rv2558. Top five BLAST hits of Rv0036 revealed the presence of two conserved domains, MDMPI_N and Radical_SAM superfamily. No information is available on the function of proteins belonging to MDMPI_N Superfamily. As enzymes of Radical SAM superfamily catalyze steps in metabolism, DNA repair etc. [35], it can be inferred that Rv0036 may participate in such activities. Rv2032c showed a conserved domain belonging to Nitro_FMN_reductase superfamily. Proteins of this superfamily catalyze the reduction of flavin or nitrocompounds. Lysine_decarbox conserved domain was found in Rv2896c. This family includes proteins annotated as lysine decarboxylases, whose synthesis is enhanced on exposure to fluoroquinolones [36]. Rv0635 was found highly conserved in different *Mycobacterium* species where it functions like 3R-hydroxyacyl-ACP dehydratase subunit HadA. MotifScan analysis predicted the presence of YNNN_MF_00799 motif which is conserved in UPF0336 family of proteins. This motif is termed as MaoC_dehydratase domain which is a member of Hot-Dog superfamily and is involved in oxidoreductase activity. CDD investigation also revealed the presence of R_hydratase like conserved domain in Rv2032c. Rv0635 was found conserved domains in these except Rv2558 though found to be highly conserved in drug-pressure of streptomycin through BLASTp analysis. Motif Scan webserver also didn’t find any motifs in these proteins while CDD found conserved domains in these except Rv2558. Top five BLAST hits of Rv0036 revealed the presence of two conserved domains, MDMPI_N and Radical_SAM superfamily. No information is available on the function of proteins belonging to MDMPI_N Superfamily. As enzymes of Radical SAM superfamily catalyze steps in metabolism, DNA repair etc. [35], it can be inferred that Rv0036 may participate in such activities. Rv2032c showed a conserved domain belonging to Nitro_FMN_reductase superfamily. Proteins of this superfamily catalyze the reduction of flavin or nitrocompounds. Lysine_decarbox conserved domain was found in Rv2896c. This family includes proteins annotated as lysine decarboxylases, whose synthesis is enhanced on exposure to fluoroquinolones [36]. Rv0635 was found highly conserved in different *Mycobacterium* species where it functions like 3R-hydroxyacyl-ACP dehydratase subunit HadA. MotifScan analysis predicted the presence of YNNN_MF_00799 motif which is conserved in UPF0336 family of proteins. This motif is termed as MaoC_dehydratase domain which is a member of Hot-Dog superfamily and is involved in oxidoreductase activity. CDD investigation also revealed the presence of R_hydratase like conserved domain in Rv0635. Dhillon and Bateman [37] unified numerous prokaryotic, archaeal and eukaryotic proteins and found that hotdog domain containing proteins wrap up a superfamily of thioestersases and dehydratases. Combining the results of BLASTp, MotifScan and CDD it can be inferred that Rv0635 codes for a metabolic enzyme which may be involved in dehydratase activities. BLAST hits of Rv1827 found it to be highly conserved in *Mycobacterium* species but attributed diverse functional annotations to it.
like hypothetical protein sequences, Chain A PknB-
Phosphorylated protein, FHA (Forkhead-associated-
domain containing protein. MotifScan reported no
motifs in Rv1827 while CDD analysis revealed the
presence of FHA domain. It is a putative nuclear signalling
domain and is known to mediate phosphorylation-
dependent protein-protein interactions. Diverse func-
tional attributes associated with FHA-domain containing
proteins like cell shape regulation, signal transduction,
etambutol resistance etc. [38] and the expression of
Rv1827 by MDR isolate during intraphagosomal state
highlights the importance of this protein in cellular phy-
siology of bacteria and its intracellular survival. Based
on the bioinformatics findings we can predict that pro-
teins encoded by Rv0036, Rv2032c, Rv0635, Rv1827 and
Rv2896c genes are involved in cellular metabolism and
help in intracellular survival of drug resistant/sensitive
M.tuberculosis.

Conclusions
Taken together, the identified proteins unique to intra-
phagosomal M.tuberculosis are likely to contribute to
the adaptation of the bacteria to the milieu within pha-
gosomes. The deduced functions of identified proteins
are consistent with the conditions encountered within
the macrophages. The induction of their expression
observed in vitro may therefore represent a strategy
employed by M.tuberculosis to promote the infection
and to enhance intracellular survival. Identification of
the exact role of these proteins will allow direct interfer-
ence with their function to control intracellular growth
of mycobacteria. Mass spectrometry and bioinformatic characterization of both drug resistant and sensitive isolates of M.tuberculosis during intraphagosomal growth showed that majority of commonly expressed/upregulated proteins belonged to the cellular metabolism and respiration category. Though both the clinical isolates were from different clades, some proteins common in them were found overexpressed. It represents that some common mechanisms is adopted by sensitive/resistant mycobacteria for their survival within macrophages and thus could serve as important drug targets. The substantive response of the host immune system that includes both oxidative and nitrosative stress provokes M.tuberculosis to adopt metabolic enzymes to catabolize these toxic compounds and undergo fundamental physiological and metabolic changes to facilitate intracellular survival. Matteelli et al. [39] reported that TMC207 is a first-in-
class anti-TB diarylquinoline with activity against drug-
sensitive and resistant TB and appears to be safe and
well tolerated. It inhibits the proton pump of mycobac-
terial ATP synthase, a critical enzyme in the synthesis of
ATP for M. tuberculosis. Though knowledge is meagre
regarding the metabolism M. tuberculosis during its resi-
dence in host cells but studies have shown that M. tuberculosis faces a hypoxic, carbon-poor, oxidative, and
nitrosative environment in the host cells and organs. A
close intersection exists between core intermediary
metabolism and resistance to host-imposed biochemical
stress in M. tuberculosis which has suitably adapted its
metabolism for persistence in the human macrophage. Inhibitors of the metabolic enzymes therefore warrant exploration as suitable drug targets against drug-resis-
tant and sensitive subpopulations of M.tuberculosis.

Methods
Source of culture
M.tuberculosis drug sensitive (to five first line drugs)
(ST11/EA13_IND family) and MDR (resistant to rifampi-
cin, isoniazid and streptomycin) (ST288/CAS2 family)
clinical isolates were procured from Mycobacterial
Repository Center at National JALMA Institute for
Leprosy and other Mycobacterial Diseases, Agra. Sus-
sceptibility testing was performed by conventional LJ
proportion method [40]. Cultures were grown in Sau-
ton’s liquid medium at 37°C and harvested in late expo-
nential phase (3 weeks) of growth [41,42]. These
cultures were used for macrophage infection.

Culture of cell line and infection with mycobacteria
The human leukemic macrophage like cell line THP-1
(kind gift from National Centre for Cell Science, Pune,
India) was routinely maintained as suspended cells in
RPMI 1640 media (Sigma, USA), supplemented with
10% v/v heat-inactivated foetal calf serum, 2 mM glu-
tamine and 100 µg/ml antibiotic-antimycotic cocktail
from Sigma (penicillin, streptomycin, amphotericin B) at
37°C in a CO2 humidified incubator. Cells were grown
by washing twice in warm RPMI 1640 (at 37°C) and the
cells to adhere [12]. Non-adherent cells were removed
with 2-5 × 10⁶ cells/ml in 25 cm² flat-bottom
tissue culture flasks and passaged every third day. Prior
to infection with M.tuberculosis isolates the THP-1 cells
were passaged at least three times in supplemented anti-
biotic-free RPMI 1640 growth medium before expansion
into 75 cm² flat bottom tissue culture flasks containing
30 ml RPMI 1640 growth medium. The cells grown to a
density of 2-5 × 10⁶/ml, were stimulated with 20 nM
(12 ng/ml) phorbol 12-myristate13 acetate (PMA;
Sigma-Aldrich, USA) for 24 hours so as to allow the
cells to adhere [12]. Non-adherent cells were removed
by washing twice in warm RPMI 1640 (at 37°C) and the
resulting monolayers (approx:3-5 × 10⁶ cells per flask)
were covered with 30 ml supplemented RPMI 1640
growth medium. Mid-exponential phase mycobacterial
cells (5 × 10⁸ bacilli) recovered from Sauton’s liquid
medium by centrifugation at 5000 g for 10 minutes
were resuspended in 1 ml RPMI 1640 growth medium
and added to the adhered macrophage monolayer at an
infection ratio of 10 bacilli per macrophage [43] and left to phagocytose for 12 hours (to ensure that sufficient mycobacteria are taken up by macrophages) at 37°C in a 5% humidified CO₂ incubator. After 12 hours extracellular mycobacteria were removed by decanting the supernatant and extensively washing the adhered cells twice in warm RPMI 1640. The infected macrophages were replenished with 30 ml complete RPMI 1640 medium containing gentamycin (10 μg/ml) to prevent extracellular replication of mycobacteria. Macrophage viability (never fell below 80%) was assessed by trypan blue exclusion. Incubations were carried out for further 5 days at 37°C in a humidified CO₂ incubator. After 5 days incubation, the supernatant was discarded and macrophages were washed and scrapped off in chilled Phosphate buffered saline (PBS) solution. The macrophages were then lysed by incubating for 5 minutes at 37°C in 0.05% SDS/PBS (w/v). Lysed cell suspension was transferred to 15 ml centrifuge tubes and centrifuged at 5000 g for 20 minutes. To remove any contaminating macrophage proteins, and ensure the recovery of intracellular mycobacteria only, pelleted mycobacteria were pooled and washed further twice in PBS containing 0.1% Tween 80 (w/v) and 0.1% (w/v) SDS. The washed mycobacterial pellet containing only the intracellular mycobacteria was finally collected.

Mycobacterial cell lysate proteins

Cell lysate proteins were prepared according to the recommended protocol [44] with slight modification. Intracellular mycobacterial pellet was suspended in appropriate volume of protein precipitation mixture with trichloro acetic acid (TCA)-acetone precipitation procedure [45]. 10% TCA was added to the cell lysate, the mixture was incubated at -20°C overnight and then precipitated protein was collected by centrifugation at 18,000 g at 4°C for 15 minutes. It was again washed twice with 100% ice cold acetone and allowed to air dry. The protein pellet was suspended in appropriate volume of two-dimensional rehydration buffer (BIO-RAD, Hercules, CA, USA). Protein concentration was estimated using the Bradford assay [46].

Two-dimensional gel electrophoresis (2-DE)

Isoelectric focusing (IEF) was carried out using the method of ‘in gel rehydration’ [47] with slight modifications. Immobilized pH gradient (IPG) strips of pH 4-7 and length 17 cm (BIO-RAD, Hercules, CA, USA) were rehydrated overnight at 20°C with 500 μg protein mixed with rehydration buffer. Strips were then focused on an IEF unit PROTEAN IEF Cell (BIO-RAD, Hercules, CA, USA) using the following four step programme: a) 0-250 V for 2 hours in linear mode; b) 250 V constant for 2 hours in rapid mode; c) 250-5000 V for 4 hours in linear mode; and d) 5000 V constant until 35 kVh reached. The current limit was set at 50 μA per strip. After IEF, IPG strips were equilibrated for 15 minutes in equilibration buffer I (6 M urea, 2% SDS, 0.375 M Tris; pH 8.8, 20% glycerol) containing 130 mM dithiothreitol (DTT) followed by equilibration buffer II containing 135 mM iodoacetamide instead of DTT for 15 minutes. Proteins were separated in second dimension on 12% SDS-PAGE [48] in a vertical electrophoretic dual gel unit PROTEAN II XI (BIO-RAD, Hercules, CA, USA) at constant voltage of 250 V for 5-6 hours. Gels were stained with Coomassie Brilliant Blue R250 to visualize proteins. Images of gels were acquired by Chemidoc (BIO-RAD) using Quantity One software (BIO-RAD, Hercules, CA, USA). 2 D gels were analysed using PDQuest Advanced software (BIO-RAD, Hercules, CA, USA). Gel images were also manually checked for artifactual spots, merged spots, and missed spots. Equal amount of protein was loaded in all gels and experiments were repeated at least three times.

In-gel digestion with trypsin

Method of Shevchenko et al. [49] was followed with slight modifications. Protein spots of interest were excised from gels using spot picker ‘Investigator ProPic’ (Genomic Solutions, Huntingdon, UK) and collected in 96 well PCR plate. Digest of proteins and spotting of peptides on MALDI-TOF target plate was carried out using protein digester ‘Investigator ProPrep’ (Genomic Solutions, Huntingdon, UK). The gel plugs were destained and dehydrated by washing three times (~10 minutes) with 25 mM NH₄HCO₃-50% acetonitrile (ACN) (1:1). Dry gel plugs were treated with freshly prepared 10 mM DTT in 50 mM NH₄HCO₃ for 45 minutes at 56°C. After incubation, the DTT was replaced quickly by the same volume of freshly prepared 55 mM iodoacetamide in 50 mM NH₄HCO₃ for 30 minutes and then dehydrated with 100% ACN. The dried gel pieces were incubated for 12 hours at 37°C with 25 mM NH₄HCO₃ containing 0.02 μg/μl of mass spectrometry grade trypsin (Promega, Madison, WI, USA). The resulting peptides were extracted twice from the gel pieces, using peptide extraction buffer [1:1 mixture of 70% ACN and 0.1% trifluoroacetic acid (TFA)].
Mass spectrometry

Digested samples were desalted and concentrated on C-18 ZipTips (Millipore, Billerica, MA, USA) using the manufacturer’s protocol. ZipTips were eluted on MTP 384 target plate with 2 μl of a-cyano-4-hydroxycinnamic acid (HCCA) (Sigma-Aldrich, USA) saturated solution dissolved in 50% ACN and 0.2% TFA. Mass spectra of digested proteins were acquired using Autoflex II TOF/TOF 50 (Bruker Daltonik GmbH, Leipzig, Germany) in positive reflectron mode, in the detection range of 500-3000 m/z. External calibration to a spectrum, acquired for a mixture of peptides with masses ranging from 1046 to 2465 Da, was done prior to acquisition. The proteolytic masses obtained were then processed through Flex Analysis v.2.4 programme for peak detection of proteins. Peak detection in MALDI spectra and submission of peak lists to the Peptide mass fingerprint (PMF) Mascot server were done using the Mascot Wizard program (Matrix Science, U.K.). Peptide mass tolerance was set to 50 ppm with carbamidomethylcystein set as fixed modification, oxidation of methionine as variable modification and 1 missed cleavage site was allowed.

Computational analysis of hypothetical proteins employing bioinformatics tools

Computational analysis of the differentially expressed hypothetical proteins was carried out using softwares and web-servers like BLASTp, MotifScan and Conserved Domain Database (CDD). Protein sequences of all hypothetical proteins were retrieved from Tuberculist server http://genolist.pasteur.fr/Tuberculist/ hosted by Pasteur Institute, Paris for whole annotated genome of H37Rv.

(a) BLAST Analysis

The prediction of probable function of hypothetical proteins was done using BLASTp search [50] performed at NCBI server http://blast.ncbi.nlm.nih. Protein sequence of hypothetical proteins were retrieved from Tuberculist server http://genolist.pasteur.fr/Tuberculist/. Top five BLAST hits were selected for annotating the function. BLAST runs were performed at NCBI server using the default threshold E-value of 10 and inclusion of threshold value of 0.005.

(b) Motif Scan Analysis

The motif search was done using HAMAP profiles and PROSITE patterns motif databases [51].

(c) Domain Analysis

The Conserved Domain Database (CDD) of NCBI which is a database of multiple sequence alignment [52] was used to predict functions of hypothetical proteins. Top five BLAST hits were selected for probing into the conserved domains and functional annotation http://www.ncbi.nlm.nih.gov/cdd.

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Author details

1. Department of Biochemistry, National JALMA Institute for Leprosy and other Mycobacterial Diseases, Tajganj, Agra 282001, India. 2. Department of Biophysics, University of Delhi, South Campus, Benito Juarez Road, New Delhi 110021, India. 3. Department of Immunology, National JALMA Institute for Leprosy and other Mycobacterial Diseases, Tajganj, Agra 282001, India.

Authors’ contributions

NS carried out the experiments, participated in the data analysis and drafted the manuscript. PS helped in mass spectrometric experiments and MK in bioinformatic analysis. BJ helped in carrying out cell line experiments and critical review of the manuscript. DB conceived and designed the study, interpreted the experiment data and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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