

## Streptomycin induced protein expression analysis in *Mycobacterium tuberculosis* by two-dimensional gel electrophoresis & mass spectrometry

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Received July 30, 2009

**Background & objectives:** The resistance of *Mycobacterium tuberculosis* to streptomycin, a core drug for treatment of category II tuberculosis (TB) has posed a major challenge to the health providers as well as research workers worldwide and has severely compromised the therapeutic options. A significant proportion of streptomycin resistant *M. tuberculosis* isolates failed to show mutations in conventional targets like *rpsL* and *rrs*. Although efflux, permeability, etc. are also known to contribute, yet a substantial proportion of isolates remains resistant suggesting involvement of other unknown mechanism. A resistant isolate may show altered gene as well as protein expression under drug induced conditions and a whole cell proteome analysis under induced conditions might help in further understanding the mechanisms of drug resistance. The present study was therefore designed with the objective to identify proteins related to streptomycin resistance in *M. tuberculosis* isolate grown in presence and absence of streptomycin (SM).

**Methods:** A clinical isolate of *M. tuberculosis* from Mycobacterial Repository Centre at the Institute (NJIL & OMD), Agra was grown in Sauton's medium for 36 h with/without subinhibitory concentration of the drug (2 µg/ml) and the cell lysate of isolates was prepared by sonication and centrifugation. Two-dimensional (2D) gel electrophoresis was employed to study the protein profile. The selected proteins were finally identified by MALDI-TOF mass spectrometry.

**Results:** Our study revealed eight inducible proteins (DnaK, fabG4, DNA-binding, hypothetical, two 14 kDa antigen and two 10 kDa chaperonin) that were upregulated in the presence of drug.

**Interpretation & conclusion:** This preliminary study has thrown light on whether or not and how the resistant isolate responds to streptomycin at its non-toxic but sub-inhibitory concentration. An in-depth study of the upregulated proteins will give an insight into probable sites of drug action other than established primary sites.

**Key words** 2D gel electrophoresis - mass spectrometry - mycobacteria - streptomycin - tuberculosis

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Tuberculosis (TB) is the most common cause of infectious disease related mortality worldwide. World Health Organization (WHO) estimates that 2 billion people have latent TB while another 3 million people worldwide die of TB each year<sup>1</sup>. Emergence of HIV-TB co-infection, increase in multi-drug resistant (MDR) cases of TB and variations in BCG efficacy have increased the severity and magnitude of TB epidemic. MDR-TB is posing a serious threat in global TB control and newly identified TB threat which leaves patients virtually untreatable using currently available drugs is extensive drug-resistant (XDR) TB.

It is well accepted that rapid and early detection of drug resistance and a therapeutic choice based on resistance profile are important parameters for handling MDR-TB. An in-depth study of the protein profile of a resistant isolate in the presence of sub-inhibitory concentrations of a drug could help in determining the chemotherapeutic potential of the drug in resistant cases and also in attempts to develop newer drugs based on explored resistance mechanisms. Using this approach, the potential benefit of treating isoniazid (INH)-resistant bacteria with INH at a level that is effective and safe, has been suggested<sup>2</sup>. However, a few reports exist in this regard and most of the studies have been carried out using genomics approach.

Nucleic acid-based systems offer rapid and sensitive methods to detect resistance and play critical role in the elucidation of resistance mechanisms. However, proteomics not only provides the opportunity to determine the functional genome but also facilitates the identification of proteins that have not been predicted by genome analysis<sup>3,4</sup>. Two-dimensional (2D) gel electrophoresis of proteins is currently the highest resolution analytical technique available for the study of protein profiles. 2D gel electrophoresis along with mass spectrometry and bioinformatics is emerging as a powerful tool to study the total proteins. Streptomycin (SM) is a broad spectrum antibiotic, and an important first line anti-tuberculosis drug. It inhibits protein synthesis in susceptible bacteria by interacting with several steps of the translation process<sup>5</sup>. Mutations in *rpsL* and *rrs* have been shown to be responsible for SM resistance in only 65-70 per cent of *Mycobacterium tuberculosis* isolates<sup>6,7</sup>. In the remaining isolates mechanisms are not clear, though efflux pump, permeability, structural changes, *etc.* has been suggested to contribute.

The resistance of *M. tuberculosis* to SM which is a core drug for treatment of category II TB, has

posed a major challenge to the health providers as well as research workers worldwide and has severely compromised the therapeutic options. A resistant isolate may show altered gene as well as protein expression under drug induced conditions and a whole cell proteome analysis under induced conditions might help in further understanding the mechanisms of drug resistance and in modifying the SM dosage in such resistant cases as well as in developing new agents based on inducible protein profile of the SM mono-resistant *M. tuberculosis* clinical isolate under the pressure of sub-inhibitory drug concentrations. Such an approach has been found fairly successful in the case of INH<sup>2</sup>. The present study was therefore designed to identify proteins related to SM resistance in *M. tuberculosis* clinical isolate grown in presence and absence of SM.

### Material & Methods

*M. tuberculosis* clinical isolates & drug susceptibility testing: One total sensitive (sensitive to first line drugs isoniazid, rifampicin, pyrazinamide, ethambutol and SM) and one SM mono-resistant (sensitive to other first line drugs) *M. tuberculosis* clinical isolate was obtained from Mycobacterial Repository Centre of National JALMA Institute for Leprosy & Other Mycobacterial Diseases, Agra, India. Susceptibility testing for all five first line drugs was performed by LJ proportion method<sup>8</sup> and minimum inhibitory concentration (MIC) of the resistant isolate was determined by resazurin microtitre assay (REMA) plate method<sup>9,10</sup>.

*MIC determination by REMA plate method*: REMA was performed to determine the MIC for mycobacterial isolate against SM. Briefly, 100  $\mu$ l volume of Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD, USA) supplemented with 0.2 per cent (v/v) glycerol, 10 per cent oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson, Sparks, MD, USA) was dispensed in each well of a 96-well cell culture plate (Nunc, Roskilde, Denmark). SM concentrations prepared directly in the medium were: 0.2, 0.5, 1, 2, 4, 8, 16, and 32  $\mu$ g/ml. Perimeter wells of the plate were filled with sterile water to avoid dehydration of medium during incubation. A bacterial suspension of no.1 McFarland standard was prepared and diluted 1:20 in 7H9 broth, 100  $\mu$ l inoculum was used to inoculate each well of the plate. A growth control containing no SM and a sterile control without inoculum were also included for each isolate. Plates were sealed and incubated at 37°C for one wk; 25  $\mu$ l of 0.02 per cent resazurin (Sigma-Aldrich, St. Louis,

USA) solution was added to each well and plates were reincubated for additional two days. A change in colour from blue to pink indicated the growth of bacteria, and the MIC was read as the minimum concentration of drug that prevented the colour change in resazurin solution.

**Culture and drug treatment:** *M. tuberculosis* SM mono-resistant clinical isolate was cultured in two flasks containing 170 ml of Sauton's medium in each and incubated at 37°C. After four weeks (late log phase) SM (Sigma-Aldrich, St. Louis, USA) was added in one flask at the working concentration of 2 µg/ml which was the sub-inhibitory concentration and in another flask drug was not added. The cells were harvested from both flasks after 36 h. As the generation time of *M. tuberculosis* is from 24 to 36 h, sufficient time was given so that all proteins of mycobacteria are expressed.

**Preparation of mycobacterial cell lysate:** The mycobacterial cell lysate was prepared according to the method of Brodie *et al*<sup>11</sup>. Cells were washed with normal saline and then suspended in sonication buffer [50mM Tris-HCl containing 10 mM MgCl<sub>2</sub>, 0.1% sodium azide, 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 1 mM ethylene glycol tetraacetic acid (EGTA); pH 7.4] at a concentration of 1 g wet cell mass per 5 ml and then broken by intermittent sonication for 15 min at 4°C. The homogenate was centrifuged at 12,000 g for 20 min at 4°C. Pellets were discarded and supernatant was stored at -20°C until used. Protein extractions were done for biological and technical replicas.

**Protein precipitation with trichloroacetic acid (TCA)-acetone:** Cell lysate proteins were precipitated using published protocol<sup>12</sup>. Cell lysates were treated with 1 per cent sodium dodecyl sulphate (SDS) and then subjected to TCA-acetone precipitation procedure. TCA ( 10% v/v) was added to the cell lysate, mixture was incubated at -20°C overnight and precipitated protein was collected by centrifugation at 18,000 g, 15 min, 4°C. It was again washed with 100 per cent ice cold acetone and allowed to air dry. The protein pellet was suspended in appropriate volume of 2D rehydration buffer [8M urea, 2% CHAPS (wt/vol), 50 mM dithiothreitol (DTT), 0.2% Bio-Lyte 3/10 ampholyte, 0.001% bromophenol blue] (BIO-RAD, Hercules, CA, USA) and the protein concentration was estimated by Bradford method<sup>13</sup> using bovine serum albumin as standard.

**Two-dimensional gel electrophoresis:** Isoelectric focusing (IEF) was carried out using the method of

“in gel rehydration” with slight modifications<sup>14</sup>. An immobilized pH gradient (IPG) strip of pH 4-7 and length 17 cm (BIO-RAD, Hercules, CA, USA) was rehydrated overnight at 20°C with 500 µg protein which was mixed with 2D rehydration buffer. Strips were then focused on an IEF unit PROTEAN IEF Cell (BIO-RAD, Hercules, CA, USA) at 20°C using the following four-step program: (i) 0-250 V for 2 h in linear mode; (ii) 250 V constant for 2 h in rapid mode; (iii) 250-5000 V for 4 h in linear mode; and (iv) 5000 V constant until 35 kVh reached. The current limit was set at 50 µA per strip. After IEF, strip was equilibrated for 15 min in equilibration buffer I [6 M urea, 2% SDS (wt/vol), 0.375 M Tris; pH 8.8, 20% glycerol (v/v)] containing 130 mM DTT followed by equilibration buffer II containing 135 mM iodoacetamide instead of DTT for 15 min. Proteins were separated in second dimension on 12 per cent SDS-polyacrylamide gels<sup>15</sup> in a vertical electrophoretic unit PROTEAN II XI (BIO-RAD, Hercules, CA, USA) at a constant voltage of 250 V for 5-6 h at 100 W and gels were stained with coomassie brilliant blue R250 to visualize proteins. Images of gels were acquired by Chemidoc [BIO-RAD, Segrate (Milan), Italy] using Quantity One software (BIO-RAD, Hercules, CA, USA) and upregulated proteins were analysed using PDQuest software (Bio-Rad, Hercules, CA, USA).

Student t-test was used for the statistical analysis by PDQuest software. The system picks up the spots with differential intensity of significant levels built in the system. Equal amount of protein (500 µg) was loaded on all gels and experiment was repeated three times.

**In-gel digestion of protein spots with trypsin:** Protocol of Shevchenko and co-workers<sup>16</sup> was followed. Protein spots of interest were excised from the coomassie blue stained 2D gels using spot picker Investigator ProPic (Genomic Solutions Ltd., Huntingdon, UK) and collected in 96 well PCR plate. Digestion of proteins and spotting of peptides on matrix assisted LASER desorption/ionization-time of flight (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 (for 10 min) with 25 mM NH<sub>4</sub>HCO<sub>3</sub>-50 per cent acetonitrile (ACN) (1:1 v/v). Dried gel plugs were treated with freshly prepared 10 mM DTT in 50 mM NH<sub>4</sub>HCO<sub>3</sub> at 56°C for 45 min. After incubation, the DTT was replaced quickly by the same volume of freshly prepared 55

mM iodoacetamide in 50 mM NH<sub>4</sub>HCO<sub>3</sub> for 30 min and then dehydrated with 100 per cent ACN. The dried gel pieces were incubated for 12 h at 37°C with 25 mM NH<sub>4</sub>HCO<sub>3</sub> 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 v/v mixture of 70% ACN and 1% trifluoroacetic acid (TFA) (wt/vol)] and transferred to a new 96 well PCR plate and reduced to 20 µl in the vacuum centrifuge CentriVap Concentrator (LABCONCO, Kansas City, Missouri, USA).

**Mass spectrometric analysis:** Prior to mass spectrometric analysis, 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 α-cyano-4-hydroxycinnamic acid (HCCA) saturated solution dissolved in 50 per cent ACN (v/v), 0.2 per cent TFA. Mass spectra of digested protein 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.

The proteolytic masses obtained were then evaluated using Mascot, a peptide mass fingerprinting tool. Peak detection in MALDI spectra and submission of peak lists to the database were done using the Mascot wizard (Matrix Science, UK). Peptide mass tolerance was set to 50 ppm with carbamidomethyl-cysteine set as fixed modification, oxidation of methionine

as variable modification and 1 missed cleavage site allowed.

## Results

This study was focused on the proteins upregulated in the SM mono-resistant *M. tuberculosis* isolate grown in presence and absence of drug SM. The MIC of SM for resistant isolate was 4 µg/ml by REMA plate method. Fig. 1(a, b and c) shows the 2D profile of *M. tuberculosis* total sensitive, SM mono-resistant isolate grown without drug and with drug respectively. On comparing 2D gel profile of sensitive with resistant isolate grown in absence of drug, five proteins (spot 1-5) were found upregulated in case of resistant isolate. The difference observed in resistant isolate compared to susceptible isolate may not be differing on account of lineage as both isolates are the classical representatives of various clinical isolates studied (data not shown). When 2D profile of resistant isolate (absence of drug) was compared with resistant isolate grown in presence of drug (SM), 69 protein spots were identified as differentially expressed. However, visualization of individual spots with naked eyes suggested that many spots would be on account of streaking and hence clear cut spots were selected. Of the 48 spots, only those spots which were upregulated with at least 2.5 fold intensity were selected. Also those proteins were selected which besides low expressed in SM mono-resistant isolate were also absent/low expressed in total sensitive isolate. Finally eight proteins (spot 6-13) were found

**Table I.** Details of upregulated constitutive proteins identified by mass spectrometry

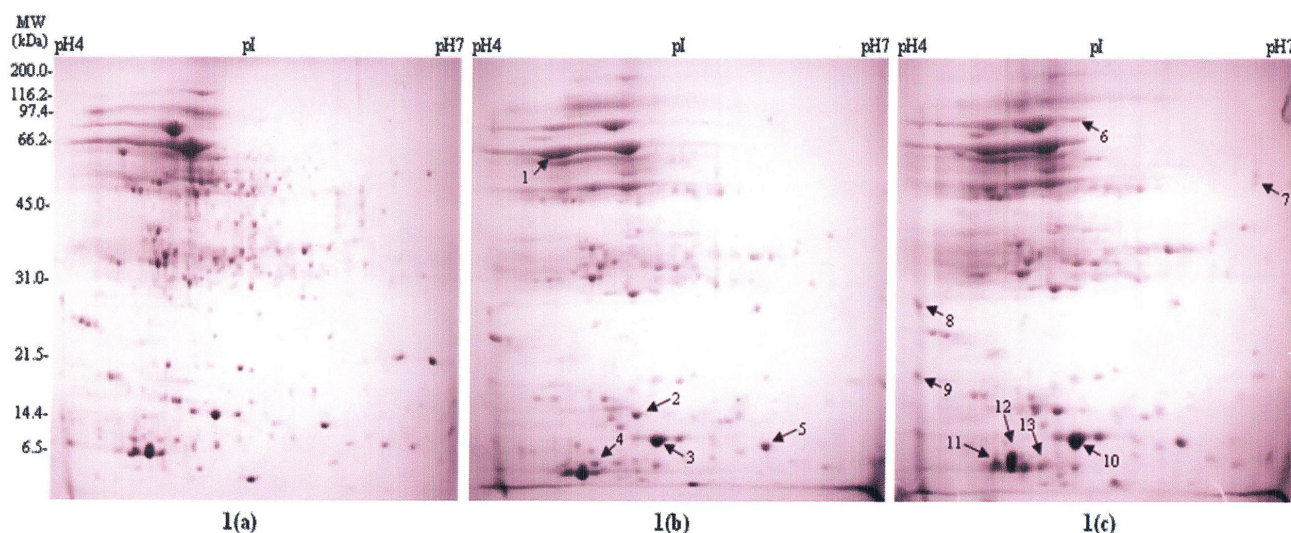
Protein spot no.	Protein identified	MASCOT score	Nominal mass (Da)	pI	Sequence coverage %	Accession number	Function
1	Trigger factor (TF)	154	50586	4.43	36	Rv2462c	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions
2	Transcription elongation factor greA	119	17844	4.90	68	Rv1080c	Necessary for efficient RNA polymerase transcription elongation past template-encoded arresting sites
3	14 kDa antigen	98	16217	5.00	59	Rv0251c	A molecular chaperone, thought to be involved in the initiation step of translation at high temperature. Bound to 30s ribosomal subunit
4	14 kDa antigen	50	16217	5.00	34	Rv0251c	A molecular chaperone, thought to be involved in the initiation step of translation at high temperature. Bound to 30s ribosomal subunit
5	Hypothetical protein Rv1636	147	15303	5.51	78	Rv1636	Function unknown

to be upregulated with SM induced isolate. Magnified region of these upregulated proteins are shown in Fig. 2. These protein spots were further identified by MALDI-TOF mass spectrometry (Tables I & II).

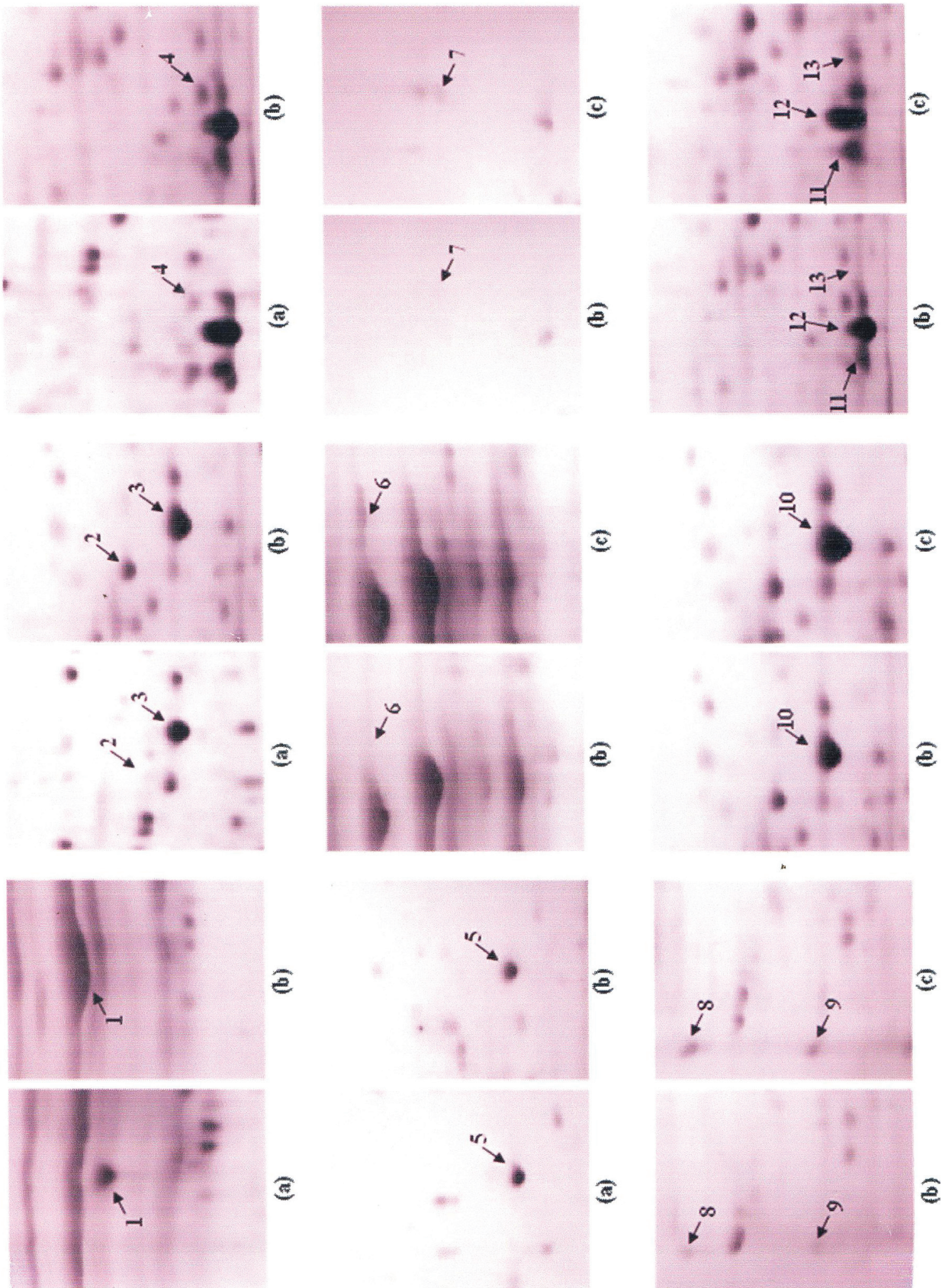
By mass spectrometry five constitutive proteins were identified as trigger factor, transcription elongation factor greA, two 14 kDa protein and one hypothetical protein. Eight induced proteins were identified as

**Table II.** Details of upregulated induced proteins identified by mass spectrometry

Protein spot no.	Protein identified	MASCOT score	Nominal mass (Da)	pI	Sequence coverage %	Accession number	Function
6	Chaperone protein dnaK (Heat shock protein 70)	69	66790	4.85	24	Rv0350	A chaperone. Involved in induction by stress conditions e.g. heat shock. Possibly has an ATPase activity
7	Probable fabG4 protein	98	46916	6.04	28	Rv0242c	Involved in the fatty acid biosynthesis pathway
8	Hypothetical protein	77	19933	3.91	44	Rv3867	Function unknown
9	Single-stranded DNA-binding protein	79	17400	5.12	36	Rv0054	Essential for replication, also involved in DNA recombination and repair
10	14 kDa antigen (16 kDa antigen)	98	16217	5.00	59	Rv0251c	A molecular chaperone, thought to be involved in the initiation step of translation at high temperature. Bound to 30s ribosomal subunit
11	10 kDa chaperonin	48	10798	4.62	43	Rv3418c	Binds to CPN60 in the presence of Mg-ATP and suppresses the ATPase activity of the latter
12	10 kDa chaperonin	67	10798	4.62	35	Rv3418c	Binds to CPN60 in the presence of Mg-ATP and suppresses the ATPase activity of the latter
13	14 kDa antigen (16 kDa antigen)	74	16217	5.00	38	Rv0251c	A molecular chaperone, thought to be involved in the initiation step of translation at high temperature. Bound to 30s ribosomal subunit



**Fig. 1.** Two-dimensional gel profile of *M. tuberculosis* clinical isolates: **(1a)** Total sensitive, **(1b)** SM mono-resistant, grown without drug, and **(1c)** SM mono-resistant, induced with SM.



**Fig. 2.** Magnified regions of the upregulated proteins: (a) Total sensitive, (b) SM mono-resistant, grown without drug, and (c) SM mono-resistant, induced with SM. Arrows indicate the position of protein spots in the gels and numbers indicate the protein spot number as mentioned in Tables I and II.

DnaK protein, fabG4 protein, DNA-binding protein, hypothetical protein, two 14 kDa antigen and two 10 kDa chaperonin.

### Discussion

The aim of present study was to analyze the protein profiles which will ultimately help in understanding unexplored novel mechanisms of streptomycin resistance as well as to get an idea of the protein(s) which could be considered for targeting while developing newer/alternate drugs. Therefore, the study included whole cell lysate proteins which will cover all proteins present in the cells as against the limited secretory proteins. The 2D gel profile of SM mono-resistant *M. tuberculosis* isolate whether uninduced or induced with drug exhibited many proteins that were upregulated. Interestingly, both constitutive and inducible proteins were found to be upregulated. The susceptible isolate was included to get a preliminary idea on the pattern of constitutive changes in the resistant isolate. Five constitutive proteins were found to be upregulated in SM mono-resistant isolate on comparing with total sensitive isolate. Protein spot 1 (Rv2462c) was identified as trigger factor (TF) which prevents misfolding and promotes refolding of polypeptides generated under stress conditions. Protein spot 2 (Rv1080c) encodes transcription elongation factor greA which is supposed to be necessary for efficient RNA polymerase transcription elongation. Two proteins (spots 3 and 4) were identified as 14 kDa antigen with accession number Rv0251c. They are believed to be involved in the initiation step of translation at high temperature and possibly a molecular chaperone. Spot 5 (Rv1636) which encodes hypothetical protein could not be assigned any function. These proteins might play some role in imparting resistance to the isolate.

Eight proteins were found to be upregulated in induced as compared to uninduced culture. However, those proteins were selected which besides low expressed in SM mono-resistant isolate were also absent/low expressed in total sensitive isolate. The concentration of streptomycin was decided after performing REMA assay (to determine the MIC of isolate). Sub-inhibitory concentration of streptomycin was used in the experiment and bacteria exhibited positive growth. Spot 6 with accession number Rv0350 was identified as chaperone protein DnaK (hsp70) and is involved in induction by stress conditions. Spot 7 (Rv0242c) is a probable fabG4 protein and is suggested to be involved in fatty acid biosynthesis pathway. Spot 8

(Rv3867) which encodes hypothetical protein could not be assigned any function. Spot 9 with accession number Rv0054 was identified as ss-DNA binding protein, which is essential for replication of chromosomes and is also involved in DNA recombination and repair. Two spots (10 and 13) were identified as 14 kDa antigen with accession number Rv0251c (same as 3 and 4). Further, spots 11 and 12 (Rv3418c) encodes 10 kDa chaperonin. 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<sup>17-19</sup>. Cpn10 are shown to exist as heptamers and dissociation to monomers takes place in dilute solutions<sup>20-22</sup>. 10-kDa antigen has been shown to be an important T-cell antigen in tuberculosis patients<sup>23</sup>.

Majority of the upregulated proteins appeared as molecular chaperone. Interestingly, Rv0251c was found to be upregulated in resistant isolate as compared with total sensitive but its expression further enhances on exposing the resistant isolate to the drug. As this protein appeared constitutive as well inducible, it is predicted that this protein might be playing a crucial role in the survival of bacteria during drug pressure. Molecular chaperones are a diverse set of proteins that mediate the correct folding, assembly, transport and degradation of other proteins. It has been shown to be induced on ribosomes under oxygen-deficient conditions. HspX and universal stress proteins (USP) Rv2623 have been found to be upregulated in stationary phase/hypoxic conditions in strain BCG and *M. tuberculosis*<sup>24</sup>. Its role in maintenance of long-term viability during latent, asymptomatic infections and in replication during initial infection has also been proposed. It has also been demonstrated that partial disruption of heat-shock regulation in *M. tuberculosis* has an important impact on virulence, impairing the ability of bacteria to establish chronic infection<sup>25</sup>. It is quite likely that where two spots are identified as similar proteins, one with lower molecular mass could be the proteolytic degradation product. Starck *et al*<sup>26</sup> have also found HspX and GroEL2 as proteolytic fragments. Two proteins (spots 7 and 9) are basically required for growth of the organism. Proteins involved in mycolic acid synthesis pathway are reported to be upregulated during INH treatment<sup>27</sup>. Finally, for one hypothetical protein (spot 8) that may be involved in survival, no clear-cut conclusion could be drawn. The role of these induced proteins may be broad-based and not specific. It may be possible that the ss-DNA

binding protein under streptomycin pressure might bind to the drug and its overexpression is required for regular DNA recombination and repair thus indicating the probable hitherto unexplored action of streptomycin. The probable fabG4 protein might play a role in altering the drug permeability in resistant isolate by changing the fatty acid composition of the cell envelope. Besides, the chaperones might play a secondary role by protecting the protein which is being affected by drug stress. This aspect needs to be further established by transformation studies and also studies on other anti-mycobacterial drugs. Genes for identified proteins can be cloned into a susceptible host or knockout strains for these genes can be used and the altered MICs can be checked to prove their role in streptomycin resistance. Further, expression in *Escherichia coli* would lead to high yields of purified protein for biochemical studies and drug binding assay can be performed with purified cloned proteins.

Because of the clinical importance, mechanism of resistance to SM has been extensively studied, especially in *M. tuberculosis*. While molecular mechanism of SM is not completely understood yet, high-level SM resistance is often linked to mutations within *rrs*, a 16S rRNA gene, or *rpsL*, which encodes the ribosomal protein S12. Mutations in *gidB* gene confers low-level SM resistance have been recently reported<sup>28</sup>. This does not hold true for all the isolates. Drug resistance is not an “all-or-none” phenomenon. An important question to be answered in this study is whether a resistant *M. tuberculosis* clinical isolate responds to drug SM. We found that the selected resistant isolate exhibited different protein profile when exposed to drug at a sub-inhibitory concentration. It is believed that these induced proteins are playing an important role in the survival of mycobacteria in presence of drug. This would ultimately lead to important information that can be used to make the decision of using SM in case of SM resistance at a level that is effective and safe. Finally, an in-depth study of the upregulated proteins will give an insight into the unexplored resistance mechanisms.

### Acknowledgment

This work was supported by grant from Department of Biotechnology (DBT), New Delhi (BT/PR7872/Med/14/1154/2006) and from LEPR, UK for selective chemicals. Authors thank Shri Ajeet Pratap Singh for technical help. The first author (PS) thanks ICMR, New Delhi, and the third author (NS) thanks CSIR-UGC, New Delhi for providing senior research fellowship. The second author (BK) was a Project Assistant in the DBT funded project.

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