

Differential Expression of Proteins of *Mycobacterium bovis* BCG during Adaptation to Anaerobic Non Replicating Persistence

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Abstract

The adaptive mechanisms involved in the establishment of a latent infection with *Mycobacterium tuberculosis* are not fully understood, but hypoxic condition in the central part of the granulomas is generally believed to be the environment encountered by the pathogen during persistence. In the present study, we followed the adaptive process of *Mycobacterium bovis* BCG during transition *in vitro* from activity aerobic growth to a state of anaerobic non-replicating persistence which was characterized by non dividing cells, low metabolic as assayed by luciferase reporter and >1% dissolved oxygen. The response of recombinant *M. bovis* BCG in aerobic replicating and anaerobic non replicating conditions was investigated. Proteome analysis of differentially expressed mycobacterial proteins during adaptation to anaerobic non-replicating persistence by two-dimensional (2-D) gel electrophoresis, mass spectrometry, and database searching, revealed 34 proteins, out of these 28 proteins showed either unique or increased expression and 6 proteins were observed to be downregulated under the conditions of hypoxia. These proteins are those involved in fatty acid oxidation, intermediary metabolism and respiratory pathways of mycobacteria, suggesting their role in persistence. Identification of proteins by this strategy will help elucidate the adaptive mechanisms in mycobacteria, provide insights into the physiology of latent bacilli and also serve as potential targets for development of new drugs for latent state of tuberculosis.

Keywords: *Mycobacterium bovis* BCG; Proteins; Latent state of tuberculosis; Anaerobic non replicating; 2-D gel electrophoresis; Mass spectrometry;

Introduction

Mycobacterium tuberculosis is an intracellular pathogen that can persist within an infected individual for extended periods of time, without causing overt clinical disease, in a state normally referred to as latent tuberculosis. The ability of the bacteria to persist in the form of a long term asymptomatic infection is central to the biology of the disease. It has been estimated that one third of the world's population is latently infected with *M. tuberculosis* and the latent carriers harbour a 2-23% life time risk of developing reactivation tuberculosis [15]. Latent *M. tuberculosis* infection presents one of the major obstacles in gaining control over tuberculosis worldwide.

Following initial infection mycobacteria replicate inside host macrophages until an effective immune response is mounted and the bacilli become restricted to the characteristic tuberculous lesions. The bacilli apparently cannot multiply because of oxygen deprivation and other factors [12]. Thus, granuloma is presumed to be the hypoxic environment which is responsible for holding bacterial replication in check and within this anaerobic environment of the caseous necrotic material, the mycobacterial dormancy probably establishes [63]. The ability of *M. tuberculosis* to persist in the human host may perhaps be due to adaptation to low oxygen environment and obligately aerobic tubercle bacilli are capable of adapting to survive hypoxia by developing into a non replicating or dormant form which maintain viability for extended periods [60, 47]. Furthermore, bacilli in the dormant form are resistant to anti-mycobacterial, thus dormancy might play a role in the persistence of tuberculosis infection despite prolonged chemotherapy.

The ability of *M. tuberculosis* to persist in a host is a complex process involving the coordinated expression of mycobacterial genes and metabolic pathways to maintain a persistent infection in an immunocompetent host. Defining these factors and their role in bacterial metabolism and physiology, will lead to understanding of biology of latency, drug targets and development of new drugs.

Lack of information about the state of the bacilli during latency hinders our ability to model latent tuberculosis in laboratory settings. However, both *in vitro* and *in vivo* systems have been developed which contribute to our current understanding of latency [3, 41, 42, 46, 61]. It was shown that *in vitro* oxygen depletion triggered a dormancy response with a metabolic downshift allowing survival of the bacilli with minimum metabolic activity and oxygen starved bacilli were sensitive to drug metronidazole [60, 61, 64]. Based on these observations investigators have used hypoxic culture condition to generate non replicating persistent mycobacterium as an *in vitro* surrogate of the metabolic state of the latent TB *in vivo*, and anaerobic and starved cultures are used as models to study the molecular basis of dormancy [11, 14, 29, 49, 61, 69]. Several attempts were made to examine the protein level response of *M. tuberculosis in vitro*

model systems [37, 43, 57]. The elevated expression of Rv 2031c, Rv 3133c, Rv 2623 and Rv 2626c have been reported during dormant state [5, 11, 68, 69]. The genetic factors responsible for dormancy are still lesser known.

It has been reported that *M. tuberculosis* and its close relative *M. bovis* BCG adapt and survive anaerobiosis by switching from growth to a state of non replicating persistence or dormancy [38, 60, 61]. Thus, in the present study *M. bovis* BCG was selected for understanding the adaptive behaviour of mycobacteria to oxygen deprivation conditions. Recombinant *M. bovis* BCG expressing firefly luciferase was constructed and the condition of non replicating persistent state was standardized. To analyse adaptive mechanisms initiated by *M. bovis* BCG in response to oxygen depletion, we have used two-dimensional (2-D) gel electrophoresis based protein profiling, which allowed high-resolution separation of several hundred proteins, as visualized by Coomassie or silver staining, with both MALDI and tandem electrospray MS. We have been able to identify 34 BCG proteins, the expressions of 28 proteins were either unique or up-regulated and 6 proteins were down regulated during condition of non replicating persistence.

Materials and Methods

Mycobacteria, media and growth conditions. *Mycobacterium bovis* BCG Pasteur strain (isolate 1173P2) and its isogenic recombinant derivative *M. bovis* (pCDlux) were employed in this study. Plasmid pCDlux (6.05 kb) has been described [13]. Mycobacteria were grown in liquid Sauton medium or Middlebrook 7H9 medium (Difco) supplemented with ADC (0.2% dextrose, 0.5% bovine serum albumin fraction V, 0.0003% beef catalase) and plated on solid Middlebrook 7H10/7H11 agar (Difco) + OADC enrichment (Becton Dickinson) [50]. All cultures were grown at 37°C with or without shaking. Antibiotics were added in the media when required at the following concentrations: kanamycin, 25 µg ml⁻¹; cycloheximide, 50 µg ml⁻¹. Chemicals were purchased from Sigma or GE Healthcare if not stated otherwise.

Construction of recombinant *M. bovis* BCG expressing firefly luciferase

pCDlux, an integrative vector expressing firefly luciferase and kanamycin resistance under the control of hsp60 promoter, was electroporated in *M. bovis* BCG and kanamycin resistant colonies were selected on MB7H10 plates. Kanamycin resistant colonies were checked for luciferase activity. Luminescence was measured by Berthold Autolumat LB953 tube luminometer [13].

in vitro conditions of anaerobic non replicating state

To establish anaerobic non replicating culture in dormant state, protocols described earlier were followed [61, 64]. The recombinant *M. bovis* BCG expressing firefly luciferase was grown in a Fermenter (Bio Console ADI 1025, applikon Holland) in Sauton's broth at 37°C without supply of air or nitrogen, with a head space ratio of 0.5 (0.5 HSR) and agitated at a stir rate of 100 rpm with non detectable perturbation of the surface of the

medium. Dissolved oxygen level, pH of medium and temperature were continuously monitored at various time points with the help of sensor probes linked to the Fermenter. The fermenter was operated and maintained within a Bio safety level 3 laboratory. Growth and viability was monitored by determining viable counts (CFU) plating on MB7H10 plates in triplicate and absorbance measuring optical density (A600). Luminescence (relative light units, RLU) was measured in Berthold luminometer at various time points which served to reflect the metabolic state of cells. Cells were harvested when viable counts, A600 and dissolved oxygen level have been achieved. Such cultures can be simulated in sealed bottles with methylene blue as indicator of dissolved oxygen. Comparison of drug susceptibility of *M. bovis* BCG under replicating and non replicating conditions was made. *M. bovis* BCG was grown in replicating (~ 6 days) and non replicating (~ 22 days) conditions in drug-free media in duplicate. Metronidazole (120 and 150 µg ml⁻¹) and rifampicin (1 and 3 µg ml⁻¹) were added to one set of cultures and the other set remained incubated without drugs. After 48 h of further incubation, CFU was determined by plating cultures of replicating and non replicating cells on MB7H10 plates and per cent inhibition in both conditions was calculated in comparison to cultures grown in drug-free media.

Sample preparation for two-dimensional gel electrophoresis

Bacilli were harvested at desired time points, washed twice in phosphate-buffered saline, and resuspended in lysis buffer containing 9 M urea, 4% 3-[[3-(cholamidopropyl)-dimethylammonio]¹⁻-propanesulfonate (CHAPS), 50 mM dithiothreitol, pefabloc at 1 mg ml⁻¹, pepstatin at 1 mg ml⁻¹, and leupeptin at 1 mg ml⁻¹. Lysis mixture was subjected to sonication four times for 30 s intermittently while keeping the tubes in ice bath. The cell lysates were centrifuged and the supernatant was removed and stored in small aliquots at -70°C until they were required. The protein content of each sample was estimated using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA).

Isoelectric focusing and two-dimensional gel electrophoresis

Isoelectric focusing (IEF) was carried out by two-dimensional gel electrophoresis using the sample-loading buffer (8 M urea, 4% (w/v) CHAPS, 40 mM Tris base, 65 mM dithiothreitol (DTT), 0.5- 2% IPG Buffer) in the IPGphor system (GE Healthcare Biosciences, Uppsala, Sweden) [5, 31]. IEF was performed with Immobline drystrips (GE Healthcare Biosciences, Sweden) of pH 4–7. Depending upon the length of strips (13 or 18 cm), 50–150 µg of total protein was applied to the strips and the strips were rehydrated for 12 h. The 13, 18 strips were focused for 32,000, 52,000 Volt hours (Vh), respectively. Second dimensional gel electrophoresis was carried out with a 12.5% SDS-polyacrylamide (Protean IIxi system; Bio-Rad) gel. IPG strips were equilibrated for 15 min in equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris, pH 8.8, 20% glycerol) first with 130 mM dithiothreitol and second with 135 mM iodoacetamide prior

to performing the second dimension gel electrophoresis. These strips were then overlaid onto vertical second dimension gels and sealed with 0.7% (w/v) agarose in SDS running buffer containing trace amounts of bromophenol blue. The gels were Coomassie or silver stained for protein spot comparisons according to previously published protocols. At least three different gels were run for each sample. For subsequent mass spectrometric analysis gels were rerun and Coomassie staining was performed. The gel pictures were scanned and then analyzed using the Image Master 2D Platinum (version 6.0) software analysis program (GE Healthcare Biosciences, Uppsala, Sweden).

MALDI-MS (Matrix assisted laser desorption-ionization mass Spectrometry)

The protein spots of interest were excised manually. The small gel pieces were washed three times with 400 µl of 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate buffer, pH 8.0 and dehydrated in 100% acetonitrile. After drying under vacuum, the small gel pieces were soaked in sequencing grade modified trypsin (Promega, Madison, WI) solution (10 mg ml⁻¹ in the above buffer). The trypsin-soaked gels were then incubated for 16 h at 37° C. The tryptic peptides were extracted twice with 50 µl 50% acetonitrile (v/v) with 5% trifluoroacetic acid (TFA), then dried completely in a Speed Vac evaporator. The dried tryptic peptides were re-dissolved in 3.0 µl 50% acetonitrile (v/v) with 0.1% TFA and the reconstituted extract was mixed with the same volume of 10 mg/ml a-cyano-4-hydroxycinnamic acid (CHCA) in 50% (v/v) acetonitrile. Five hundred nano liters of the mixture was applied to the sample plate for MS analysis. The peptides were extracted, concentrated and desalted using Ziptips (Millipore Corporation, Billerica, MA, USA). The spectra were internally calibrated using trypsin autolysis peaks (average mass of 2807 and 3000). The mass spectra were recorded on Voyager DE-Pro (Applied Biosystems, Foster City, CA). To assign a positive identification at least three peptides had to match the identified protein sequence with a search tolerance of 100 ppm. MASCOT Software (Matrix Science, United Kingdom) was used to interpret spectra and identify proteins.

Bioinformatic analysis

Data mining for the identified proteins was performed mainly from the databases of TubercuList (<http://genolist.pasteur.fr/TubercuList>) and SWISSPROT (<http://us.expasy.org/sprot>). Information on previously identified proteins was obtained from the databases of the Max-Planck-Institute (<http://www.mpiib-berlin.mpg.de/2D-PAGE>) as well as from the published literature.

Results and Discussion

Standardization of *in vitro* conditions of replicating and anaerobic non replicating states

For establishing non replicating persistent state, a recombinant *M. bovis* BCG Expressing firefly luciferase was used. Luciferase reporter system was used as an index of viability and determination of metabolic state of bacilli. Since ATP is present only in the live and metabolically active bacteria, the assay of

luciferase activity (RLU) allowed us the rapid test of cellular viability. Seed culture of recombinant BCG was uniformly derived from actively growing cultures in Sauton medium. Replicating cultures were obtained by growing in Sauton medium with aeration at 37° C. Cells were harvested after 140-150 h of incubation in mid-log phase (Figure1F). No change in the pH of the medium was observed.

To obtain anaerobic non replicating cultures in dormant state, recombinant BCG, was grown in Fermenter in which the culture self generated a gradual temporal oxygen gradient. With slow depletion of oxygen the culture of BCG was able to adapt and survive anaerobiosis by shifting to a state of non replicating persistence in 22 days which was characterized by stationary CFU at about 6×10^7 cells/ml, 0.4 absorbance at 600 nm, base level RLU, pH drop to 6.4 from 7.2 and <1% level of dissolved oxygen in the Fermenter (Figure1A-E). Thus, it was observed that with the slow depletion of oxygen recombinant BCG was able to shift to a state of non replicating persistence. Drug susceptibility of recombinant BCG against antimycobacterial compounds in replicating and non replicating states was determined to validate the respective culture conditions because it is known that aerobic replicating mycobacteria are sensitive to killing by rifampicin and not to metronidazole whereas the reverse is true for non replicating persistent bacilli. Non replicating anoxic cultures were found sensitive to killing by metronidazole (99.7% at 120 µg ml⁻¹ and 99.87% at 150 µg ml⁻¹) whereas 2% (120 µg ml⁻¹) and 4% (150 µg ml⁻¹) killing was observed for aerobic replicating cells (Figure 2A). On the other hand, rifampicin was found effective against actively replicating (aerobic) cells and non replicating anoxic cells were resistant to killing by rifampicin (Figure 2B).

Comparative proteome analysis and Protein identification

The continuous requirement of bacteria for adaptation to physiological and environmental stresses forced the development of very complex regulatory networks at the cellular level to cope up with stress conditions. In this investigation, comparative proteome analysis was carried out between replicating cells and anaerobically grown non replicating cells by 2-D gel electrophoresis (Figure 3 A, B). Three samples were prepared for each culture condition and per sample two 2-D gels were produced. To elucidate protein spots unique to hypoxia condition representative gels of both conditions were first compared visually. The observed qualitative and quantitative spot variations were subsequently verified by analyzing all gels using the image analysis software program and only variations confirmed stringently were accepted as specific differences. Spot detection and quantification were performed by fitting spot intensities with a 2-D Gaussian model. Corresponding spots in individual gels were matched using a distortion model that takes into account local gel running differences. Spot detection and matching were checked and corrected thoroughly. Protein spots found to be more intense or down regulated in response to hypoxia were identified by tryptic digestion of excised spots followed by MALDI TOF-MS analysis to determine the molecular

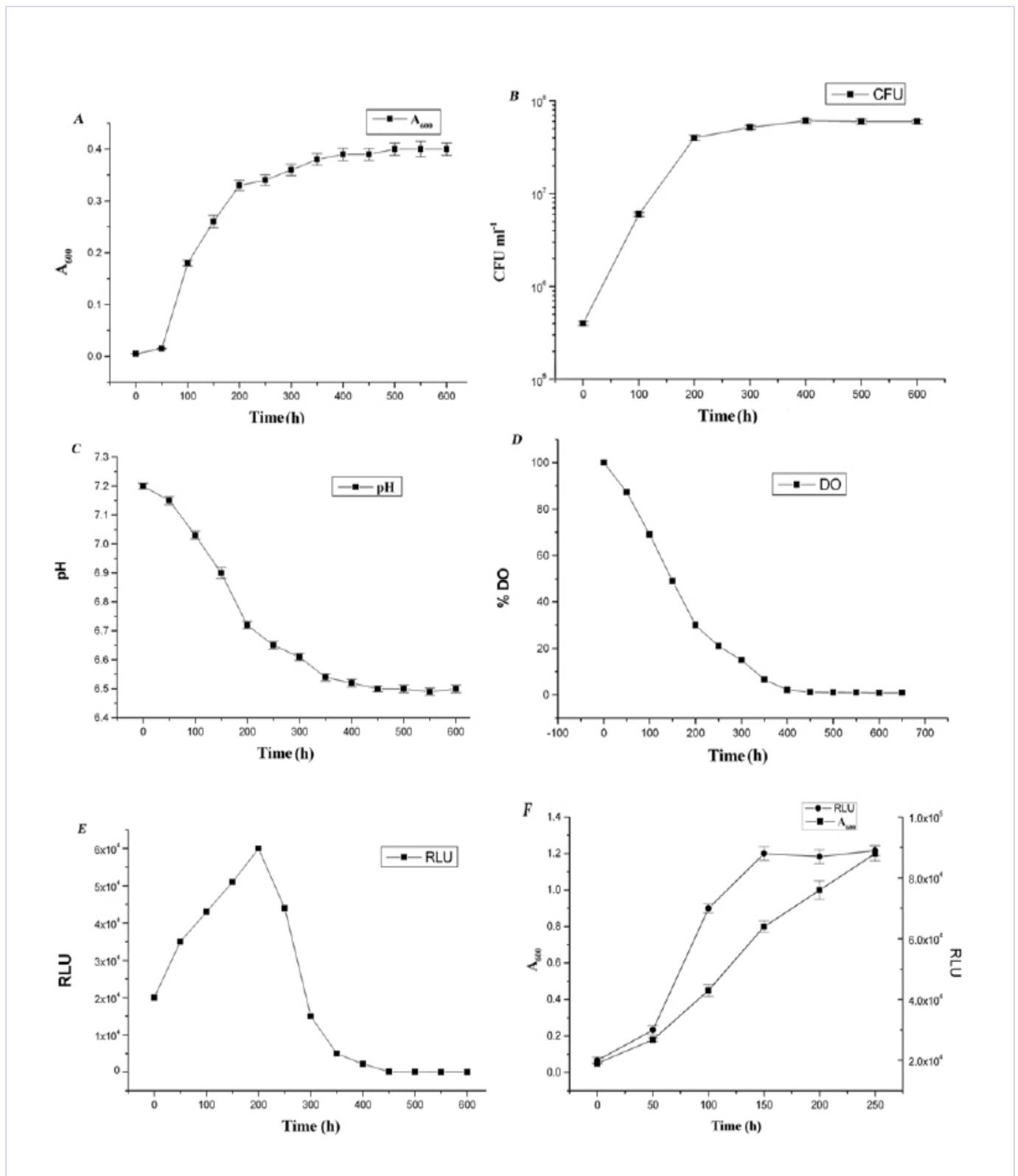


Figure 1: Growth and survival of *M. bovis* BCG during non replicating (A-E) and replicating (F) condition. (A) Optical density (B) Viable counts/colony forming units (CFU) (C) pH (D) % DO (E) RLU. Exponentially growing cells of *M. bovis* BCG were inoculated in Sauton's medium in a fermenter with declining oxygen level leading to viable non replicating persistent state. (F) Optical density and RLU of *M. bovis* BCG during replicating condition as described in Materials and Methods. The Experiments were carried out three times with duplicate cultures.

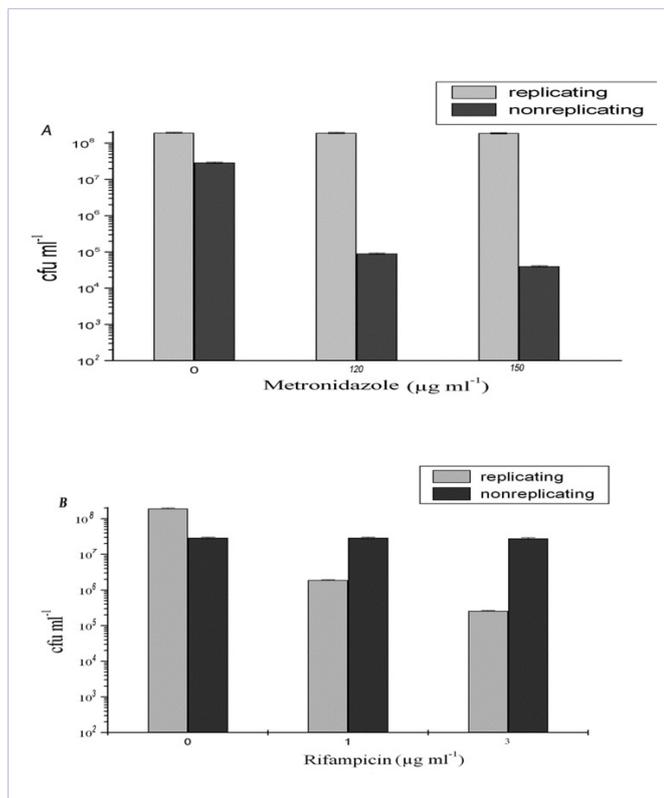


Figure 2: Drug susceptibility of *M. bovis* BCG against (A) metronidazole and (B) rifampicin after growth in replicating and non replicating conditions. Cells were grown in both conditions and then exposed to metronidazole and rifampicin for 48 h. CFU was determined by plating on MB7H10.

masses of the resulting tryptic peptides. Partial sequence data of some of the peptides were used to search computerized peptide mass and protein sequence databases for known proteins. Some proteins had no previous references in databases or publications pertaining to the *M. tuberculosis* proteome, hence considered as 'new' to the proteome (Table 1, 2). Details of the predicted pI, molecular masses, peptides matched, the percentage sequence coverage obtained for each protein and comparative protein spot intensity level are presented in Table 1 and 2. Discrepancy between observed and predicted protein pI values is a common feature of proteomic analysis using 2D gels. This is considered to be due to, amongst other things, conformational differences, post-translational modification and other processing events of proteins affecting their migration. The analysis revealed about 40 BCG proteins exclusively detected either unique, upregulated or downregulated in anaerobic condition, out of these 34 proteins could be identified by mass spectrometry. Among the identified proteins, 28 were up-regulated (Table 1) and 6 proteins were downregulated in response to hypoxia (Table 2).

Functional profiling of differentially expressed proteins

Functional profiling of differentially expressed proteins was based on the functional annotation developed at the Sanger

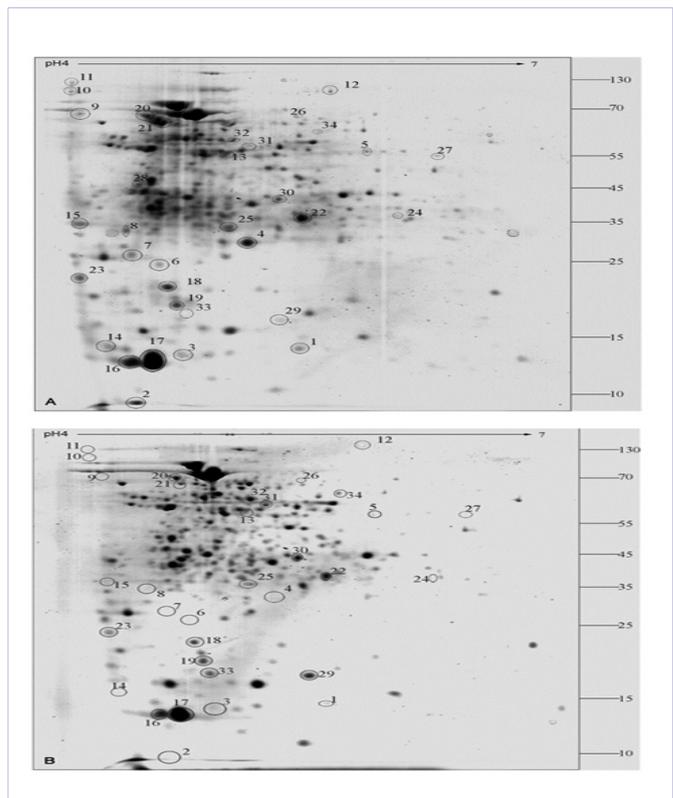


Figure 3: 2-D gel electrophoresis of proteins expressed during (A) anaerobic non replicating and (B) aerobic replicating conditions. Proteins were separated by IEF on pH 4-7 IPG strips in the first dimension and 12% SDS-PAGE in the second dimension. Protein spots are numbered and their identity listed in Table 1 and 2. Molecular masses (kDa) of prestained standard proteins run in parallel are indicated.

Institute (http://www.sanger.ac.uk/Projects/M_tuberculosis/Gene_list/). The majority of the identified differentially expressed proteins in response to hypoxia, belonged to the following functional categories: category 1-lipid metabolism (fatty acid biosynthesis and degradation (5 proteins), category 7 - intermediary metabolism and respiration (12 proteins), category 0- virulence, detoxification (1 proteins), adaptation (5 proteins) , category 2- information pathways (3 proteins), category 10- conserved hypotheticals (3 proteins). Another important observation was that functional classes that contain more significantly up-regulated genes include those involved with fatty acid degradation, anaerobic respiration and amino acid/ amine degradation. Conversely, functional classes that contain down-regulated genes include carbon degradation, and purine / pyrimidine synthesis. The relevance of these proteins as factors enabling survival of *M. tuberculosis* in anaerobic persistence is discussed.

Lipid metabolism

In the present study, we observed the induction of FadE5 and FadB, involved in fatty acid degradation and it is predicted that *M. tuberculosis* uses fatty acids as a carbon source during infection. FadE5 could be a probable acyl-coA dehydrogenase involved in lipid degradation, while FadB may be involved in beta-oxidation

Table 1: *M. bovis* BCG proteins up-regulated during hypoxia.

Protein name / Accession number (NCBI)	Spot No.	SWISS-PROT	Mass/PI	Function	MALDI- MS Score	No. of peptides matched	Sequence coverage (%)
Rv0148	15	P96825	29.8/5.12	Oxidoreductase	69	2	57.0
Rv0183	24	Q7DAB5	30.3/6.18	Possible lysophospholipase	128	2	46
FabG4/Rv0242c	5, 27	O53665	46.7/6.38	Probable 3-oxoacyl-reductase	69	7	25.1
FadE5/Rv0244c	9	O53666	66.0/5.19	Probable acyl-coA dehydrogenase	135	18	47.5
Acr2/Rv0251c	28	O53673	17.7/5.04	Heat shock protein	86	6	65.3
DnaK/Rv0350	7	P32723	66.8/4.59	Probable chaperone, heat shock protein 70	231	4	66.0
GroEL/Rv0440	4,8,20	P06806	56.5/4.5	60 kDa chaperonin 2	63	5	37.0
Tuf/Rv0685	13	P31501	43.5/5.12	Probable iron-regulated elongation factor	81	15	46.2
PurC/Rv0780	6	Q59566	32.9/4.95	Phosphoribosylaminoimidazole-succinocarboxamide synthase	64	8	37.0
FadB/Rv0860	12	Q7D952	76.1/5.21	Probable fatty oxidation protein	81	13	20.4
TrpC/Rv1611	25	O06129	28.1/4.86	Probable indole-3-glycerol phosphate synthase	80	9	38.2
TB16.3/Rv2185c	19	O53519	16.3/4.48	Conserved hypothetical protein	70	9	59
SucB/Rv2215	10,11	Q10381	57.1/4.64	Probable pyruvate dehydrogenase	90	12	28.4
AccD6/Rv2247	26	Q10506	56.5/5.0	Acetyl/propionyl-coA carboxylase	98	11	23.1
NdkA/Rv2445c	1	P71904	14.5/5.18	Probable nucleoside diphosphate kinase	65	6	53.7
ClpP1/Rv2461c	18	O53188	21.6/4.54	Probable ATP-dependent clp protease	151	2	81.0
Rv2626c	3	O06186	15.6/4.8	Conserved hypothetical protein	65	7	62.9
Mpt70/Rv2875	23	Q50769	19.1/4.54	Major secreted immunogenic protein	109	7	67.3
GroES/Rv3418c	2,16,17	P09621	10.7/4.5	Heat shock protein, chaperonin	67	6	42.4
IlvX/Rv3509c	21	G70806	52.1/4.53	Probable acetohydroxyacid synthase	71	12	26.8
Ppa/Rv3628	22	O06379	18.3/4.52	Inorganic pyrophosphatase	62	7	45.7
BfrB/Rv3841	14	F70653	20.4/4.23	Bacterioferritin	77	2	62

NOTE: Spot numbers refer to the proteins of interest on 2-D gels that were analyzed by

Table 2: *M. bovis* BCG proteins down-regulated during hypoxia.

Protein name/ Accession number (NCBI)	Spot No	SWISS-PROT	Mass/PI	Function	MALDI- MS Score	No. of peptides matched	Sequence coverage (%)
Icl/Rv0467	32	O53752	47.1/4.79	Isocitrate lyase	89	15	53.7
GreA/Rv1080c	33	O53428	17.8/4.63	Probable transcription elongation factor	104	10	73.8
AhpE/Rv2238c	29	Q10520	16.8/5.12	Probable peroxiredoxin	72	2	54.
KasA/Rv2245	31	Q10524	43.3/4.93	3-oxoacyl-synthase	78	10	39.4
Tsf/Rv2889c	30	Q10788	28.7/5.01	Probable elongation factor	82	10	44.6
LeuC/Rv2988c	34	O53237	50.2/5.29	Probable 3-isopropylmalate dehydratase	78	15	37.8

NOTE: Spot numbers refer to the proteins of interest on 2-D gels that were analyzed by MS.

During adaptation to stress conditions, mycolic acids in the cell wall of mycobacteria plays an important role as it becomes resistant to antibiotics and thickening of cell wall occurs in the present study FabG4, a probable 3-oxoacyl-reductase, involved in the fatty acid biosynthesis pathway and AccD6, involved in mycolic acid biosynthesis were found to be upregulated. AccD6 has shown to be induced by isoniazid or ethionamide treatment and nutrient starvation [11] [40] [20] [65] [3]. In contrast, decreased expression of KasA, a part of FasII system involved in mycolic acids synthesis and meromycolate extension was observed in our study[33]. KasA expression level was strongly reduced in cell extracts from *M. thermoresistibile* grown at 55 °C system and at low pH *in vitro* conditions indicating that it is not upregulated by all stress conditions [32] [17].

Proteins of information pathways

In the present study we observed up-regulation of EF-TU and down-regulation of EF-TS and GreA in response to hypoxia which suggested that expression of Tuf was required during anaerobic persistence. EF-TU a GTPase which promotes the binding of aminoacyl-tRNA to ribosomes, during protein biosynthesis showed about 6 fold up-regulation during hypoxia and when *M. tuberculosis* was exposed to high iron conditions *in vitro*[54, 55, 66]. EF-TU has been shown to be methylated and membrane associated when *E. coli* was starved for glutamate, or phosphate [67]. It was proposed that association of EF-TU with membrane allows its interaction with receptors or proteins that interact with nutrients in the environment that could regulate its methylation suggesting that EF-TU may also have role in regulation of cell growth and the organism's response to stress. Besides regulating translation through its interaction with tRNA and ribosomes, enabling it to stop the translation of unnecessary proteins and the synthesis of stress-induced proteins, EF-TU may also act as

a transcriptional activator in the presence of RNA polymerase and appropriate sigma factor [26, 59]. Therefore, it may be able to regulate both the translation and transcription of starvation-induced proteins.

During stress conditions the low metabolic activity of mycobacteria most probably leads to reduced RNA and protein synthesis. In our study, EF-TS, Probable elongation factor and GreA, a probable transcription elongation factor necessary for efficient RNA polymerase transcription elongation past template-encoded arresting sites, were found to be down regulated.

Intermediary metabolism and respiration

A number of proteins involved in intermediary metabolism and respiration were identified as differentially expressed in response to anaerobic condition. The up-regulated proteins Rv0183, ClpP, BfrP, TrpC, PurC, Ppa, IlvX, SucB, NdkA were involved in amino acids synthesis, TCA cycle and purine biosynthesis, whereas two proteins LeuC and Icl (glyoxylate shunt) were down-regulated. NdkA is a probable nucleoside diphosphate kinase, plays a major role in the synthesis of nucleoside triphosphates other than ATP. Indeed it has been shown that for some pathogenic bacteria, secretion of ATP-utilizing enzymes into the extracellular environment aids in pathogen survival [10]. SucB is a probable pyruvate dehydrogenase involved in tricarboxylic acid cycle and is considered an essential gene by Himar1-based transposon mutagenesis in H37Rv strain [50]. Rv0183, codes for a possible lysophospholipase probably involved in cellular metabolism but exact function is not known.

Rv0148 codes for NADP dependent dehydrogenases, the short-chain alcohol dehydrogenase superfamily. Members of this subgroup are known to act on a large variety of substrates, including sugars, steroids, aromatic hydrocarbons, antibiotics,

and compounds involved in nitrogen metabolism. In contrast to our results, it was downregulated in low-iron-concentration medium [66]. We observed induction of PurC, involved in de novo purine biosynthesis and TrpC (Rv1611) which is a probable indole-3-glycerol phosphate synthase involved in tryptophan biosynthesis [30]. TrpC was also upregulated in *M. tuberculosis* during oxygen depletion [9]. Ppa encoding inorganic pyrophosphatase, plays an important role in macromolecular biosynthesis and is considered essential for the viability of *E. coli* and yeast and induced during intracellular infection of *Legionella pneumophila* in U937 macrophage-like cells [8, 35, 39]. *ilvX* codes for a probable acetolactate synthase that catalyzes a reversible reaction to form pyruvate from acetolactate, and could be involved in valine and isoleucine biosynthesis. These are essential amino acids that are not available to mycobacteria during growth in host and the auxotrophic strain of mycobacteria could not proliferate in eukaryotic tissues thus could be a target for new drug development [24, 25].

ClpP proteases are known to degrade proteins that cannot be refolded in gram-positive bacteria and expressed in response to stress and required for survival [18, 19, 21, 22, 34, 36, 48]. Experiments revealed that ClpP is important for *S. typhimurium* to grow under various stressful conditions, such as low pH, elevated temperature and high salt concentrations [58]. BfrB, a bacterioferritin, involved in intracellular storage of iron and induced under at reduced oxygen atmosphere and elevated temperatures [53, 56]. Induction of iron storage gene during anaerobic persistence may be required to increase iron stores for use during long periods of dormancy. LeuC, involved in leucine biosynthesis, was down-regulated after nutrient starvation as observed in our study in response to hypoxia. The role of LeuC in relation to oxidative stress has not been elucidated but leucine auxotrophy is known to restrict the growth of *M. bovis* BCG in the macrophages [2, 3].

ICL, isocitrate lyase is one of the first *M. tuberculosis* proteins shown to be required for persistent infection an initial enzyme in the glyoxylate shunt, allows bacteria to utilize fatty acids as carbon sources when the availability of primary carbon sources is limiting [44]. Expression of ICL in human lung granulomas in cultured macrophages and in microaerophilic conditions has been reported [16, 23, 62, 49]. But in our study, the downregulation of ICL appears to match the reported decrease in transcription and expression of the enzyme during hypoxia in *M. bovis* BCG [28, 38].

Conserved hypothetical proteins

The increased levels of Rv2626c observed in the present study are in agreement with the studies in the *in vitro* Wayne model DNA microarray analysis of *M. Tuberculosis* and in human macrophage cell line THP-1 with *M. bovis* BCG suggesting that Rv2626c could be important in hypoxia and intracellular survival [4, 45, 49, 53]. The observation on upregulation of Rv2185c in our study was consistent with its increase in *M. tuberculosis* during anaerobic condition [55].

Adaptation and detoxification

During stress, abnormal or misfolded proteins accumulate in the cell due to denaturation and errors in biosynthesis. Cells respond to this accumulation by increasing the synthesis of molecular chaperones, which assist the proper folding or refolding of proteins [21]. In our study we identified up regulation of heat shock proteins or chaperones DnaK, Acr2, Gro ES, Gro EL in response to hypoxia. DnaK, a major immunodominant antigen found to be induced in stress conditions, such as heat shock at 45°C macrophage infection and showed enhanced protective immunity in mice over expressing *dnaK* [1, 27, 37, 56]. MPT70 is also a major secretory immunogenic protein which upregulated after 4h of nutrient starvation [3]. Acr2 a molecular chaperone and heat-stress-induced ribosome-binding protein A, shows induction at high temperatures nutrient starvation and during hypoxia and oxidative stress [3, 52, 56]. GroES (CPN10) upregulated at high temperatures and GroEL2 was observed up-regulated within the mouse IC-21 cell line and human THP-1 cell line [1, 37, 45, 56].

The downregulation of AhpE encoding probable peroxiredoxin, involved in detoxification of organic peroxides suggesting that this protein may not required during hypoxia [6, 7].

In conclusion, therefore, the differential protein expression described herein during non replicating persistence of *M. bovis* BCG involved in stress responses and metabolic pathways may suggest that the expression of preferred proteins is essential for survival. The non-replicating persistence phenotype of is assumed to be responsible for the maintenance of latent infection and the requirement of a long treatment duration for active tuberculosis. A better understanding of mycobacterial gene expression under anaerobic non replicating conditions may provide insight in to the mechanism of adaptation of mycobacteria inside the host and may represent biomarkers for identification and development of new drug targets. It is interesting that some of the proteins identified in our study have also been reported earlier with *M. tuberculosis* [3, 49, 53, 55]. To further enhance understanding of latent *M. tuberculosis* as well as BCG, the role of the identified proteins in response to hypoxia must be elucidated. 6.

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