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Thiol specific oxidative stress response in *Mycobacteria*

Nirpjit S. Dosanjh [a], Mamta Rawat [b], Ji-Hae Chung [a], Yossef Av-Gay [a,*]

[a] *Department of Medicine, Division of Infectious Diseases, University of British Columbia, Vancouver, BC, Canada V5Z 3J5*
[b] *Department of Biology, California State University-Fresno, Fresno, CA 93740, USA*

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Abstract

The cellular response of mycobacteria to thiol specific oxidative stress was studied in *Mycobacterium bovis* BCG cultures. Two-dimensional gel electrophoresis revealed that upon diamide treatment at least 60 proteins were upregulated. Fourteen of these proteins were identified by MALDI-MS; four proteins, AhpC, Tpx, GroEL2, and GroEL1 are functionally related to oxidative stress response; eight proteins, LeuC, LeuD, Rv0224c, Rv3029c, AsnB, Rv2971, PheA and HisH are classified as part of the bacterial intermediary metabolism and respiration pathways; protein EchA14 belong to lipid metabolism, and NrdE, belongs to the mycobacterial information pathway category. Reverse transcription followed by quantitative real time PCR in response to diamide stress demonstrated that protein expression is directly proportional to the corresponding gene transcription.

Keywords: Mycothiol; Diamide; Mycobacteria; Disulfide

1. Introduction

Tuberculosis caused by *Mycobacterium tuberculosis* is responsible for 2.2 million deaths annually and currently 45 million people worldwide suffer from it [1]. The ability to infect, survive and replicate within the human macrophages is one of the keys to success of *M. tuberculosis* as a pathogen. In the phagosome of infected host macrophages *M. tuberculosis* experiences endogenous stress as a result of incomplete reduction of molecular oxygen during aerobic respiration and exogenous stress as a result of the "oxidative burst" generated by NADPH oxidase in the phagosome. The reactive oxygen species that are created in these processes include peroxides, hydroxyl radicals and superoxide. In the macrophage, mycobacteria also face nitrosative stress from reactive nitrogen species that include nitric oxide, nitrite, nitrogen dioxide and nitrate. Pathogenic mycobacteria normally produce enzymes, such as peroxyredoxins, catalase, peroxidase, superoxide dismutase, and nitrosothiol reductase, to counteract the effect of reactive oxygen species and reactive nitrosative species and assist in intracellular survival and persistence in the host [2].

Thiols play an important role in the cell by maintaining a reducing environment for chemical reactions to occur and are also important in stabilizing three dimensional structure and catalytic activity of proteins. Thiol-disulfide interchange may also act as an in vivo molecular switch in signal transduction and is well documented in chloroplasts [3]. In addition, thiols may directly detoxify oxidants or act as coenzymes in reactions that detoxify oxidants. The cell maintains the redox-homeostasis of the cytosol by reversibly reducing oxidized thiols that are created in these reactions [4]. Diamide is a thiol specific probe, which has been used

* Corresponding author. Tel.: +604 7241439; fax: +604 875 4013.
E-mail address: yossi@interchange.ubc.ca (Y. Av-Gay).

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to elucidate changes in oxidation state of thiols [4]. It penetrates the cells readily and causes oxidative stress by specifically oxidizing intracellular low molecular weight thiols to their disulfide form. Thus, disulfide stress, can be considered to be a subcategory of oxidative stress that leads to the accumulation of non-native disulfide bonds in the cytoplasm.

Diamide has been used to define the thiol-disulfide stress response of multiple bacteria including *M. tuberculosis*. Disulfide stress response of *Streptomyces coelicolor* A3 was shown to involve the σ^R regulon [5]. In *M. tuberculosis*, microarray analysis and reverse transcription revealed the role of sigma factor σ^H [6]. Global analysis of *Bacillus subtilis* mRNA expression pattern and 2D differential proteomics upon exposure to diamide revealed that 350 genes were induced [7].

In this paper, we identify the mycobacterial cellular processes that are affected by thiol specific oxidative stress. By using proteomics, we describe the differential protein expression profile of *Mycobacterium bovis* BCG associated with the thiols-damaging agent diamide. We complement these studies by transcription analysis of *M. bovis* BCG genes encoding proteins identified in the proteomics section. Using these methods we have observed at least 60 proteins that are differentially expressed in response to diamide treatment and some of these proteins have been shown before to be functionally related to oxidative stress response.

2. Materials and methods

2.1. Bacterial cultures

M. bovis BCG (ATCC-35734) was grown in 200 ml Middlebrook 7H9 broth (Difco, Sparks, MO, USA) with 0.05% (v/v) Tween 80 (Sigma, St. Louis, MO, USA) by shaking at 37 °C. Middlebrook medium was supplemented with either OADC (oleic acid, albumin, catalase, dextrose) or 1% glucose.

2.2. Liquid culture for diamide induction

M. bovis BCG cultures were grown in Middlebrook 7H9 broth supplemented with OADC at 37 °C to an OD_{600} of 0.4–0.6. Bacteria were harvested by centrifugation, washed and resuspended in Middlebrook 7H9 broth containing 1% glucose (OADC was removed to eliminate the protective effect of the catalase present in this supplement). After incubating the cultures for 24 h at 37 °C, one aliquot of the cultures was treated with 5 mM diamide (Sigma, St. Louis, MO, USA) for 2 and 4 h at 37 °C. The untreated aliquot served as a control. Bacteria were pelleted by centrifugation and the cell pellets were washed three times with phosphate buffered saline (PBS, pH 7.4) containing 1% Tween 80 and then stored at −70 °C until further processing. For RNA extraction, 0.5 ml 6 M guanidinium chloride solution was added to the pellet immediately after centrifugation and the samples were stored at −70 °C.

2.3. Sample preparation for two-dimensional gel electrophoresis

Frozen cell pellets were resuspended in lysis buffer [8] containing complete protease inhibitor cocktail (Roche Applied Science). The 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).

2.4. Isoelectric focusing and two-dimensional gel electrophoresis

Isoelectric focusing (IEF) was carried out using the sample-loading buffer (8 M urea, 4% (w/v) CHAPS, 40 mM Tris base, 65 mM dithiothreitol (DTT)) in strip holders of the IPGphor system (Amersham Pharmacia Biotech, Uppsala, Sweden). IEF was performed with Immobline Drystrips (Amersham Pharmacia Biotech Uppsala, Sweden) of either pH 4–7 or pH 3–10. Depending upon the length of strips (13, 18 or 24 cm), 50–150 μg of total protein was applied to the strips and the strips were rehydrated for 12 h. The 13, 18 and 24 cm strips were focused for 32,000, 52,000 and 62,000 Volt hours (Vh), respectively.

Second dimensional gel electrophoresis was carried out with a 12.5% SDS–polyacrylamide or Duracryl (Genomic Solutions Inc., Ann Arbor, MI, USA) gel. IPG strips were equilibrated 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 2× SDS running buffer containing trace amounts of bromophenol blue. The gels were silver stained for protein spot comparisons according to previously published protocols [9]. At least three different gels were run for each sample. For subsequent mass spectrometric analysis gels were rerun and Coomassie or silver staining was performed. The gel pictures were scanned and then analyzed using the Image Master software analysis program (Amersham Biosciences).

2.5. Matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS)

Protein spots of interest were excised from the gels, and digested in situ with trypsin (Sigma, St. Louis, MO, USA). After an overnight trypsin digestion at

37 °C, the samples were centrifuged and stored at −20 °C. MALDI-MS was performed on a Voyager DE STR TOF (time of flight) Mass Spectrometer (Applied Biosciences, Foster City, CA, USA) equipped with a delayed extraction ion source. Samples were prepared using a saturated solution of either 2,5-dihydroxybenzoic acid (DHB) or alpha-cyano-4 hydroxycinnamic acid. The peptides were extracted, concentrated and desalted using ZipTips (Millipore Corporation, Billerica, MA, USA). The peptide mass mapping was performed on 0.5 μl tryptic digest mixture using DHB or cinnamic acid matrix. The samples were air dried at room temperature on the MALDI plate. A standard sample mixture was also prepared and plated. The instrument was operated in the delayed extraction mode. Each mass spectrum was an average of 250 laser shots and each spectra was calibrated using the external standards to assign monoisotopic masses. 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) and Protein Prospector Software (UCSF Mass Spectrometry Facility) were used to interpret spectra and identify proteins.

2.6. RNA extraction and real time quantitative PCR

M. bovis BCG RNA was extracted by using the FastRNA Pro Blue kit (Qbiogene, Carlsbad, CA, USA). Cells were lysed in the presence of glass beads in a FastPrep instrument (Qbiogene) following the instructions provided with the FastRNA Pro kit. After extraction with chloroform and precipitation with ethanol, RNA was dissolved in DEPC treated water. Contaminating DNA was removed by digestion with RNase-free Turbo-DNase (Ambion, Texas, USA) according to the supplier's instructions and the RNeasy minikit (Qiagen) was used for the subsequent cleanup procedures. PCR was performed on an aliquot of DNase-treated RNA as a template to confirm the absence of DNA. Reverse transcription (RT) reactions on 0.5 μg RNA were performed using the RevertAid H Minus First strand cDNA synthesis kit (MBI Fermentas, Burlington, Ontario, Canada) following the supplied protocol. Reactions without reverse transcriptase served as the controls. Real time PCR was carried out on the DNA Engine Opticon instrument (MJ Research, Waltham, MA, USA) using the PCR master mix (Finnzyme, Espao, Finland) containing SYBR green dye. The 20 μl reactions consisted of PCR master mix (Finnzyme), 2 μl template and 300 nM of each primer (Table 1). In each case, the test gene and the normalizing gene (*sigA*) were assayed along with a set of standard samples (genomic DNA). Results were normalized to the amount of *sigA* mRNA [6,10].

3. Results

3.1. Differential expression of M. bovis BCG proteins in response to diamide treatment

Treatment of exponentially growing *M. bovis* BCG with 5 mM diamide for 2 h had no effect on the bacterial cell viability with limited decrease of the growth rate from 0.03 to 0.018 h^{-1}. Even the 4 h diamide treatment resulted only in small decrease in viability (Fig. 1), indicating that further experiments could be performed with the 5 mM diamide concentration.

A two dimensional electrophoretic map of *M. bovis* BCG total proteins resolved on SDS–PAGE displayed

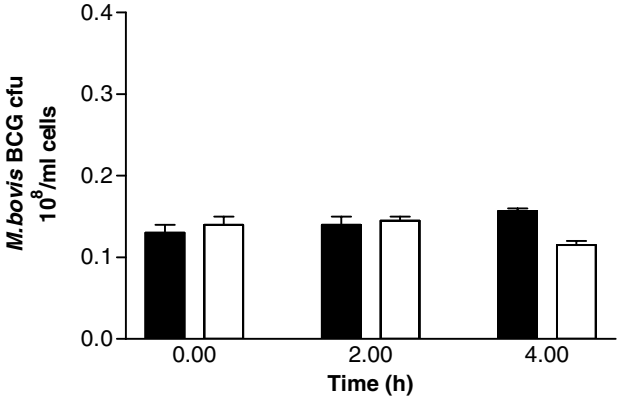

Fig. 1. Effect of diamide on *M. bovis* BCG viability. Viable counts determined as colony forming units (cfu) were measured for untreated (■) and 5 mM diamide treated (□) samples for 4 h. Error bars indicate standard error.

Table 1
Primers sequences used for qRT-PCR

Gene	Location	Forward primer	Reverse primer
ahpC	2726193	ACCAGCTCACCGCTCTCATC	GCGGTCCTCGAACTCGTCAT
asnB	2464997	GTACGTCGGCTGGAATCAGG	AGCACTGCCGTGATCTCGTC
nrdE	3412085	GAACGCCGACGAGAAGATCC	GATGTCAGCGAACGGCACAC
tpx	2183372	ACGAGCGTGCGGCGGCAAGTGG	GTTGCCGTCCGCGCCGATCACC
sigA	3017835	CTCGGTTCGCGCCTACCTCA	GCGCTCGCTAAGCTCGGTCA
Rv2971	3326101	AAGTGCTGCTGCGGTGGAAC	CCGCCAACTCGAAGTCGAAG

M. tuberculosis gene coordinate were used for the location determination.

968 protein spots for *M. bovis* BCG cell lysate which was not subjected to diamide stress (Fig. 2). 1020 protein spots were detected in the diamide treated *M. bovis* BCG cell lysate. The diamide treatment resulted in an increase of sixty proteins which represent approximately 15% of the genome (Fig. 2).

Details of the number of matched peptides and the percentage sequence coverage obtained for each protein identified are given in Table 2. Sixteen proteins are differentially expressed upon diamide treatment (Table 2). The pI and molecular weight of the identified proteins on the gel were close to that predicted by the database although most of the proteins spots identified were in the acidic range, between the pI of 4.0–5.6. Based on the gene annotation from the *M. tuberculosis* H37Rv genome sequence database (Tuberculist, Institute Pasteur, Paris), four proteins belong to the functional category of virulence, detoxification and adaptation (AhpC, Tpx, GroEL2, and GroEL1), eight proteins can be classified under the intermediary metabolism and respiration (LeuC, LeuD, Rv0224c, Rv3029c, AsnB, Rv2971, PheA and HisH), two proteins belong to lipid metabolism (EchA14 and EchA17) and one, NrdE belongs to the information pathway category.

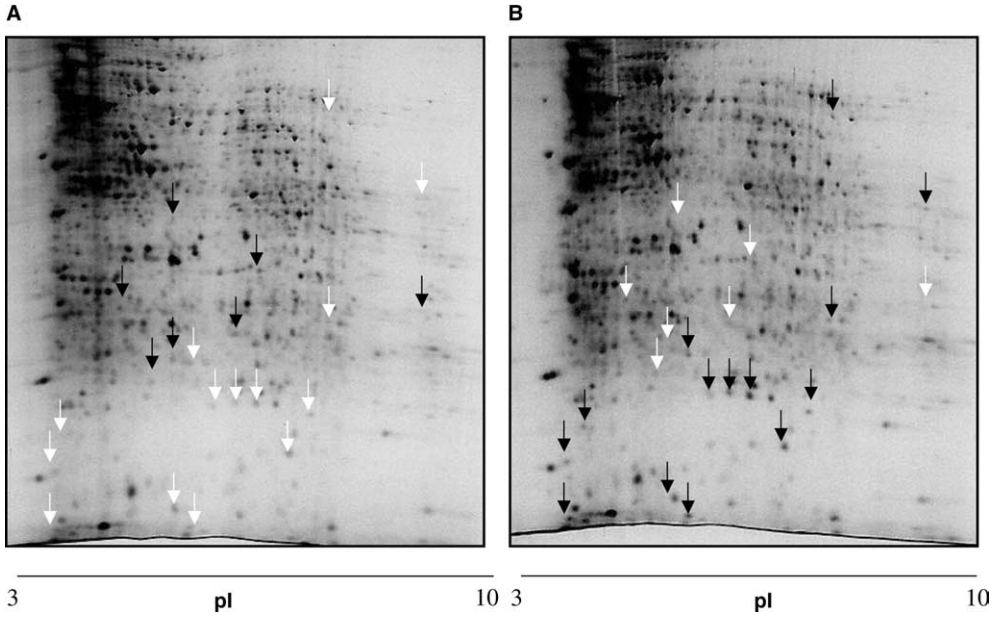

Fig. 2. Comparative representation of *M. bovis* BCG 2D gel protein expression profile. (A) Uninduced in vitro grown culture. (B) Diamide induced culture for 2 h at 37 °C. Black arrows represent upregulated proteins, white arrow the corresponding protein spot location.

Table 2
M. bovis BCG proteins identified by Maldi mass-spectrometery in response to diamide treatment

Name	Accession #*M.tb*	Accession #*M. bovis*	2dgel pI	Data base pI	2dgel MW (kD)	Data base MW (kD)	Peptides matched	Sequence coverage (%)	Mascot probability score[b]
AhpC	Rv2428	Mb2454	4.4	4.28	20–26	21	11	54	165
Tpx	Rv1932	Mb1967	4.2	4.12	12–19	17	5	47	52
HP[a]	Rv2204c	Mb2227c	4.2	4.18	10–17	12	5	54	45
GroEL 2	Rv0440	Mb0448	4.8	4.56	32–36	56	11	20	79
LeuC	Rv2988c	Mb3012c	5.2	5.29	50–55	50	8	18	71
GroEL1	Rv3417c	Mb3451c	6.0	4.74	10–15	15	7	14	55
Methylase	Rv0224c	Mb0229c	7.0	9.29	25–30	27	3	12	51
FixA	Rv3029c	Mb3055c	4.0	4.37	27–30	28	8	33	114
AsnB	Rv2201	Mb2224	6.8	6.78	70–76	72	4	30	41
EchA14	Rv2486	Mb2511	4.5	6.24	19–26	26	4	23	44
Reductase	Rv2971	Mb2996	4.8	4.51	20–25	30	5	20	67
LeuD	Rv2987c	Mb3011c	4.8	4.89	20–25	21	3	18	45
NrdE	Rv3051C	Mb3077c	5.6	6.57	65–85	82	6	12	42
PheA	Rv3838c	Mb3868c	4.5	5.03	10–25	12	3	33	45
HisH	Rv1602	Mb1628	5.6	5.0	25–33	23.5	4	15	58
EchA17	Rv3039c	Mb3065c	6.8	6.5	27–30	30	5	10	52

[a] HP, hypothetical protein.
[b] Values greater than 49 are significant ($p < 0.05$).

Out of the 16 proteins identified by mass spectrometry, 14 were upregulated and two proteins, HisH and EchA17, were downregulated to undetectable levels upon diamide treatment. One of the most abundant proteins identified by mass spectrometry AhpC, showed about 5-fold increase in its expression level upon diamide treatment (Fig. 3A and Fig. 4). The expression levels of the other identified proteins varied from 1.25- to 5-fold increase (Fig. 4).

3.2. Transcription analysis of diamide induced proteins by qRT-PCR

In order to verify that the observed upregulation in protein expression is due to adaptive gene transcription in response to diamide treatment, transcriptional analysis was performed using reverse transcription combined with real time PCR. An expression of a normalizing gene (*sig* A) was not affected by diamide treatment as the mean value for diamide treated and untreated samples were 0.476 RFU and 0.439 RFU respectively. The following transcriptional levels of selected genes were thus normalized with the *sigA* values. An increase of more than 2-fold induction in the expression level for selected genes was observed (Fig. 5). Among the genes studied, the highest induction was observed for *tpx*, a 3.6-fold induction as compared to that of the control gene *sigA*. For *ahpC*, there was a 2.82-fold increase in the level of transcription, a value similar to the increase in abundance of the protein level. Among other genes analysed, *nrdE*, *asnB*, and the probable oxidoreductase encoded by ORF *Rv2971* exhibited 2.29-, 2.75- and 1.44-fold induction in their respective transcription levels. Our studies confirm that the increased expression at the mycobacterial protein level was reflective of the increased transcription in response to disulfide stress.

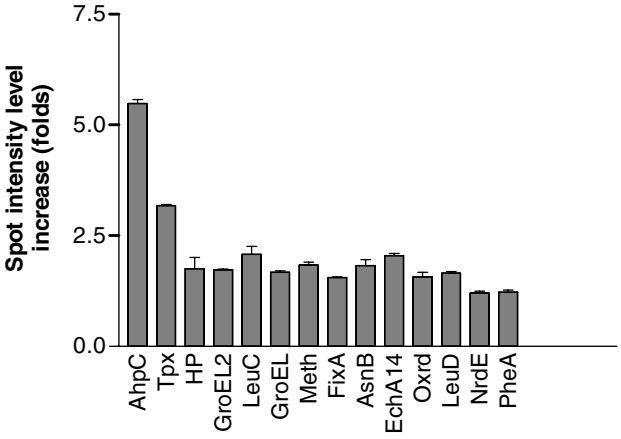

Fig. 4. Comparative protein spot intensity level. Each protein spot was compared to its corresponding protein spot in uninduced control gel and average values with standard errors provided by the Image Master II software values are plotted.

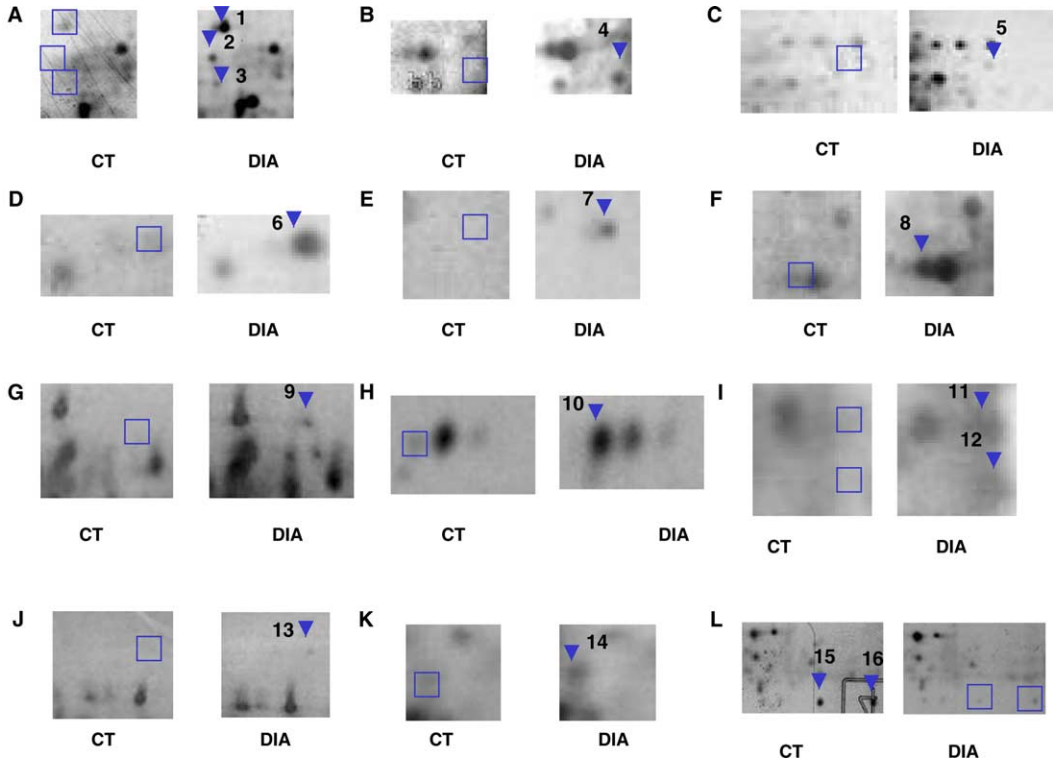

Fig. 3. Expression of MALDI-TOF identified *M. bovis* BCG proteins in response to diamide induced stress. (A) 1-AhpC, 2-Tpx, 3-Rv2204C. (B) 4-GroEL2. (C) 5-LeuC. (D) 6-GroEL. (E) 7-Rv0224C. (F) 8-FixA. (G) 9-AsnB. (H) 10-EchA14. (I) 11-Rv2971, 12-LeuD. (J) 13-NrdE. (K) 14-PheA. (L) 15-HisH, 16-EchA17. Arrows show protein expressed. CT, Control (uninduced), DIA- 5 mM diamide treatment for 2 h at 37 °C.

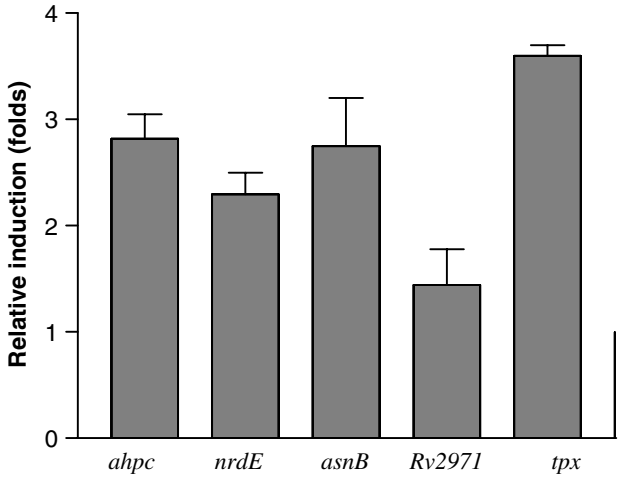

Fig. 5. Relative induction levels of thiol specific genes expression by Real Time quantitative PCR. The data are an average of three independent experiments. Error bars indicate standard error.

4. Discussion

Following a description of proteins present in cell lysates and culture filtrates proteins [11–13], several comparative studies of the proteome of *M. tuberculosis* have been reported. These studies include the changes in the mycobacterial proteome upon application of stressors such as hypoxia [14,15], reactive oxygen and nitrogen species [16], nutrition starvation [17], shaking and stationary culture conditions [18] and analyses of mycobacterial proteins following phagocytosis by macrophages [19,20]. Comparative studies of mycobacterial strains [8,21–24], were also performed using proteomics. A database for the mycobacterial proteins has been established [25]. Our interest is in mycobacterial thiol-mediated protection against xenobiotics [26,27]. Therefore, we took the proteomic approach to observe the effect of disulfide stress induced by the thiol specific oxidant diamide.

In our study, among the proteins induced by diamide stress, the chaperone GroEL2 is the only protein reported to be highly expressed under many other stresses and growth conditions. For example, the transcription of GroEL1 and GroEL2 was shown to be induced in *M. bovis* BCG following phagocytosis by THP-1 cells [20] possibly in response to the intracellular hostile environment within the macrophages. Under disulfide stress, GroEL would be needed for proper folding of different proteins that have been damaged by oxidation.

As described in Table 2, six of the proposed identifications fall below the mascot threshold of $p < 0.05$. Two of them, nrdE and AsnB, are confirmed in the present study by transcriptional analysis. Rv2204c, EchA14, LeuD and PheA match the experimental molecular weight and pI information, which increase the likelihood that the proposed identifications are correct.

One of the most abundant proteins in the lysate of the diamide treated *M. bovis* BCG culture is alkyl hydroperoxide reductase (AhpC), a member of the peroxiredoxin family of non-heme peroxidases which has been implicated in the detoxification of organic peroxides and hydrogen peroxide. In addition, expression of AhpC compensates for the loss of KatG activity in *katG*$^{-}$ mutants [28]. The AhpC system requires the thioredoxin-like oxidoreductase, AhpD, for activity [29]. Another peroxiredoxin, Tpx, is also significantly upregulated in response to disulfide stress. Like AhpC, Tpx is able to reduce organic peroxides and requires thioredoxin as a reductant [30]. Since diamide preferentially oxidizes low molecular thiols like thioredoxin, the upregulation of both these proteins may be due to the lack of available reduced thioredoxin, an essential cofactor in the peroxidase activity of both enzymes. Interestingly, the upregulation of the *tpx* transcript was higher than the *ahp* C transcript but the reverse was true for the intensity of the Tpx and AhpC protein spots. Since Tpx is an extracellular protein, its upregulation may be underestimated in the 2D gel analysis. This holds true for other proteins known to be present in the culture media and induced by diamide treatment; GroEL, FixA, and Rv2204c. These proteins may either be needed to function in the outer layer of *M. tuberculosis* or may mediate biological functions related to pathogenesis by interacting with host cell macrophages.

The third most abundant protein to be induced on diamide stress is NrdE, a component of the class 1b type of ribonucleotide reductase (NrdE) that reduces ribonucleotides to deoxyribonucleotides. NrdE, as a free radical containing enzyme, is extremely sensitive to nitrosative and oxidative stress. Upregulation of NrdE would be advantageous under stress conditions since reactive oxygen species inflict damage on DNA. Moreover, two low molecular weight thiol containing enzymes, thioredoxin and glutaredoxin, serve as hydrogen donors for ribonucleotide reductase. As with AhpC and Tpx, the absence of reduced thiols could result in induction *nrdE* gene as the cell tries to compensate for the decrease in deoxyribonucleotides levels.

The role of LeuC and LeuD 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 [31]. Prephanate dehydratase (PheA) is an enzyme within the biosynthesis pathway of phenylalanine, an amino acid proposed to have a role in siderophore biosynthesis. There are iron dependent repressor (IdeR) boxes upstream of the *pheA* gene and IdeR has been found to negatively regulate siderophore biosynthesis. Moreover, an *ideR* mutant in

M. smegmatis cannot mount an effective oxidative stress response [32].

The information regarding the roles of other proteins identified, Rv0224c (Mb0229c), FixA, AsnB, is limited. Rv0224c is possibly a methyl transferase and possesses a weak similarity with sterol-c methyltransferase. FixA codes for the small subunit of an electron transfer flavoprotein, which serves as a specific electron acceptor for dehydrogenases. Although flavins are thought to contribute to oxidative stress through their ability to produce superoxide, flavins also are frequently involved in reduction of hydro peroxides, products of oxygen-derived radical reactions.

An intensive study of *S. coelicolor* [5,33] and *M. tuberculosis* [34,6] demonstrated that most diamide induced genes are targets of a specific sigma factor, named σ^{R} in Streptomycetes and σ^{H} in mycobacteria. Using RT PCR [34] and microarray analysis response to diamide [6], the upregulation in the transcription of many genes was identified. Surprisingly, not all spots identified by our proteome analyses were identified by these transcriptome studies with the exception of *Rv0224c*, *groEL2*, *groEL1 and nrdE* (identified as induced by diamide in the supplementary materials or other independent studies). Furthermore, we could not identify the protein products of the genes identified in the microarrays on our 2D gels. Attempts to better identify spots on the 2D gels using mass spectrophotometry methods may assist us in finding the missing proteins. Also, differences between our experimental procedures and the limited capability of the 2D gel electrophoresis technique in describing the whole genome response, may account for part of the missing spots. We suggest that proteome and microarray methods should be used to complement each other in defining the cellular response to a define stressor.

To conclude, we have demonstrated that a global response is activated in *M. bovis* BCG upon exposure to thiol specific oxidative stress. We have shown that a number of enzymes, AhpC, Tpx, NrdE, dependent on thiol mediated enzymes, are induced by diamide. Analysis of *tpx*, *ahp* C and *nrd* E knock-out mutants will help to identify the mechanism by which mycobacteria respond to thiol-specific oxidative stress.

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