Original Article

Proteomic Analysis of Hydroxyl Radical-Induced Resuscitation of Hypoxia-Induced Dormant Mycobacterial Cells

Abhishek Mishra

Department of Biotechnology, Goa University, Taleigao Plateau, Goa, India

Abstract

Background: The genus *Mycobacterium* has an ability to persist in hostile environments for years before its reactivation in favorable conditions. The major bottleneck in decades of mycobacterial research is a poor understanding of molecular mechanism which assists bacteria to attain dormancy and reactivation later. **Methods:** In this study, hydroxyl radical was quantified in aerobically growing mycobacterial cells using 2-deoxy-D-ribose assay. Furthermore, extraneous addition of hydroxyl radical in Wayne's dormancy model induced reactivation of dormant cells. The whole proteome of all three samples, namely, aerobic, Wayne dormancy, and hydroxyl radical reactivated cells was isolated, trypsin digested, and peptides are quantitatively characterized by liquid chromatography-elevated energy mass spectrometry. **Results:** This study reports the generation of highly reactive hydroxyl radical by *Mycobacterium smegmatis* during aerobic respiration. The hydroxyl radical levels can be managed by modulation of iron ions in the cellular pool. Proteomic characterization of resuscitation process highlights the increase in the level of ATPases, iron acquisition, redox response, changes in cell membrane dynamics, and cell wall hydrophobicity which is coupled with restoration of protein synthesis, carbohydrate, and lipid metabolism. In addition, two uncharacterized universal stress proteins MSMEG5245 and MSMEG3950 were uniquely identified in reactivated cells. **Conclusion:** Overall, the 1-hydroxypyridine-2-thione-induced reactivation process is a controlled and stepwise exit from dormancy.

Keywords: Dormancy, hydroxyl radical, *Mycobacterium smegmatis*, resuscitation

Introduction

The genus *Mycobacterium* of actinomycetes is classically described as an obligate aerobe. However, the genome of the genus challenges this idea by exhibiting machinery to survive in an anaerobic environment. *Mycobacterium smegmatis*, a nonpathogenic soil-dwelling bacterium, metabolically adapts from an aerobic-to-anaerobic state for its survival in a complex habitat. Similarly, its close relative *Mycobacterium tuberculosis* has an ability to switch from actively dividing to dormant states in response to hypoxia, nutrient starvation, iron deficiency, NO, CO, ethanol, and H_2O_2 exposure.[1-4] This confers a formidable challenge in the tuberculosis therapy as dormant bacilli are resistant to the drugs.[5] Unlike the other members of genus such as *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* cells of *M. smegmatis* and *M. tuberculosis* respond to the anaerobiosis in a completely different manner. Both of the later species adapt to the slow depletion of oxygen and shares characteristic features of dormancy at both molecular as well as phenotypic levels. This gives us an opportunity to study *M. smegmatis* as a model for *M. tuberculosis* dormancy and reactivation.

Reactive oxygen species (ROS) plays a dual role as a signaling and detrimental molecule in both prokaryotes and eukaryotes. ROS signaling is evident in the various cellular and physiological functions such as calcium signaling, quorum sensing, multicellularity, pattern formation, redox regulation, apoptosis, necrosis, and cancer signaling.[6-12] Furthermore, recent reports have identified ROS as an important antibiotic-induced cellular stress. These studies demonstrate that bactericidal antibiotics even with a diverse mechanism of action increase ROS production within cells through the Fenton's reaction.[13-15] Moreover, several recent

Address for correspondence: Dr. Abhishek Mishra, Department of Biotechnology, Goa University, Taleigao Plateau, Goa - 403 206, India.
E-mail: ab.mish@gmail.com

Access this article online

Quick Response Code:

Website: www.ijmyco.org

DOI: 10.4103/ijmy.ijmy_211_17

How to cite this article: Mishra A. Proteomic analysis of hydroxyl radical-induced resuscitation of hypoxia-induced dormant mycobacterial cells. Int J Mycobacteriol 2018;7:7-15.

studies suggest that antibiotic resistance could possibly depend on the pathogen's ability to survive ROS stress.[16-18] Most of the previous *in vivo* signaling studies were focused on two major ROS, superoxide and H_2O_2, due to their stability in the cellular environment.[19,20] The signaling role of biologically transient and highly reactive hydroxyl radical has limited evidence. However, growing pool of evidence suggests that the hydroxyl radical signaling operates through the interaction with specific atoms which leads to the covalent modification of the targeted proteins.[8] Recent report suggests that the antibiotic-induced hydroxyl radical has a differential susceptibility toward the dormant subpopulation residing in a larger antibiotic-susceptible population. Furthermore, the stimulation of hydroxyl radical production was found effective for the eradication of the dormant cells.[21]

This study shows the successful reactivation of Wayne hypoxia dormant cells by extraneous addition of the hydroxyl radical. Using liquid chromatography-elevated energy mass spectrometry (LC-MS^E) global changes in the expression of *M. smegmatis* proteins was observed after interaction with hydroxyl radicals. Furthermore, the key pathways during resuscitation were highlighted in this report to improve the understanding of overall process.

Methods

Chemicals, strains, and media

All the chemicals were purchased from Sigma-Aldrich, USA. Dubos medium was purchased from DIFCO, USA. *M. smegmatis* strain mc^2 155 was grown in a defined minimal medium (pH 6.6) containing 0.5 g potassium dihydrogen phosphate, 0.2 g sodium citrate, 60 mg magnesium sulfate, 0.5 g asparagine, and 2 ml glycerol in 100 ml of distilled water.

Wayne hypoxia-induced dormant bacilli

Wayne's 0.5 headspace ratio model was followed to induce dormancy. Wherein, the culture tube 20 mm × 125 mm containing 25.5 ml of the medium and 8 mm magnetic spin bar was inoculated with 1% v/v of O. D_{580} ~1 culture.[22] The culture tubes were incubated at 37°C for 8 days with an airtight seal using rubber septa and gently stirred at 112 RPM on a magnetic stirrer. The oxygen depletion was observed by decolorization of methylene blue (1.5 μg/ml) in the medium. The viable cells were counted by plating on Dubos agar plates.

Susceptibility testing

Susceptibility testing for *M. smegmatis* was done using the colony-forming unit per milliliter (CFU/ml). Initial stock solution (10 mM) and subsequent dilutions of 1-hydroxypyridine-2-thione (HOPT) was prepared in dimethyl sulfoxide (DMSO). At NRP-II stage, after 6 days when *M. smegmatis* culture OD_{600} ~0.35 and methylene blue is decolorized, HOPT was added and accordingly plated on Dubos agar plates. The inhibitory activity of HOPT on aerobically growing *M. smegmatis* was tested by following the method. Briefly, 1% mid-log phase of a culture at a cell density of 1 OD_{620nm} was inoculated in 20 ml of defined minimal medium and was incubated for 3 days under aerobic conditions at 37°C with shaking at 150 rpm (Thermo Electron Corp., Marietta, OH, USA). HOPT was added into the defined medium at the time of inoculation, with the negative control being an equal volume of only DMSO. Growth of *M. smegmatis* was estimated after a 3 days incubation period by determining the CFU/ml on Dubos-agar plates.

Assay for hydroxyl radical production

Hydroxyl radical production was estimated as described earlier with some modification by incubating *M. smegmatis* in 20 ml of 20 mM phosphate buffer containing 20 mM 2-deoxy-D-ribose.[23] The formation of the breakdown product malondialdehyde was determined by mixing 750 μL of centrifuged incubation medium with 250 μL of 2-thiobarbituric acid and 250 μL of trichloroacetic acid. After heating in boiling water for exactly 10 min, cooling in tap water, and clarifying by centrifugation, the reaction product was measured spectroscopically ($\lambda = 560$ nm) against reagent blanks.

Preparation of *Mycobacterium smegmatis* whole-cell proteome

A previously described method was used for the whole-cell proteome preparation.[24] Briefly, bacterial cells were harvested by centrifugation at 10,000 RCF for 30 min, and the pellet was resuspended in extraction/solubilization buffer (8M urea, 2M thiourea, 4% w/v dithiothreitol, and 2% w/v CHAPS 2%). After that eight cycle of sonication was done at 75 Hz with 30 s pulse with a break of 45 s. The cell extract was centrifuged at 11,000 RCF and supernatant was extracted by tricarboxylic acid (TCA) and acetone in the ratio 1:8. This consortium was kept at −20°C for 2 h and then centrifugation was done at 15,600 RCF for 45 min.

Trypsin digestion of *Mycobacterium smegmatis* whole proteome

A previously described method was used for trypsin digestion.[24] Briefly, 10 μg of the protein was treated with 50 μl of 0.1% RapigestSF™ (Waters, Milford, MA, USA) as per manufacturer instruction. After treatment, 1 μg/μl trypsin was added to the solution and incubated overnight at 37°C. Next day, acid precipitation was done by adding concentrated HCl and the solution was incubated at 37°C for 20 min followed by centrifugation at 30,000 RCF for 30 min. The supernatant was collected and transferred to a clean LC-MS tube and subjected to a LC-MS.

Liquid chromatography-elevated energy mass spectrometry analysis

For LC-MS^E, an earlier method was used.[24] Briefly, before LC-MS^E analysis, each digested protein sample was spiked with a predigested alcohol dehydrogenase from *Saccharomyces cerevisiae* internal standard (Waters, Milford, MA, USA) at a level of 50 fmol per 10 μl injection. For sample analysis, 10 μL aliquots of proteome tryptic digests were analyzed in triplicates

by LC-MS^E using a nano ACQUITY ultra performance liquid chromatography and Premier Q-Tof mass spectrometer equipped with a NanoLock Spray ion source (Waters, Milford, MA). Samples were injected onto a C18 cartridge (300 µm × 1 cm length) at a flow rate of 10 µl/min. Next, peptides were separated by BEH C18 stationary phase (1.7 µm particle size and flow rate of 300 nl/min) using a linear gradient from 2% to 40% B over 120 min (A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile). The LC-MS^E was operated in the alternating 2 s scans of low (4 V) or high (10 − 32 V) collision energies are used. For external mass calibration, Glu-fibrino peptide at a concentration of 200 fmol/µl was infused in the spray ion source. Before first sample injection, tryptic digest of bovine serum albumin was analyzed as standard.

Global protein expression of active, dormant, and resuscitated bacilli

For the analysis of global expression of proteins in *M. smegmatis* under different conditions, a recently developed method LC-MS^E (Waters, USA) was used, in which the whole-protein mixture is analyzed in a single injection. In this study, 210 differentially expressed proteins were identified using a label-free quantification known as LC/MS^E.[24-27] Noteworthy, the average signal intensity of the three most intense tryptic peptides remain constant at a given concentration irrespective of its protein size. This enables the stoichiometric comparison and absolute quantification of proteins in different samples.

Data processing and database searching

A previously described method was used for data processing (Mishra and Sarkar, 2015). Briefly, each raw data file was processed using ProteinLynx Global Server (PLGS) Version 2.2.5 software (Waters) to generate mass lists for protein identification. After processing, file was then searched against the UniProt protein database (http://www.uniprot.org/) using PLGS version 2.2.5. The absolute protein quantification was done using the alcohol dehydrogenase from *S. cerevisiae*.

Results

Hydroxyl radical generation by aerobically growing culture of *Mycobacterium smegmatis*

To avoid the nonspecific interaction with the macromolecules present in complex media a defined minimal media was used for the experiments. After careful standardization, 2-deoxy-D-ribose assay was used for the estimation of hydroxyl radical in *M. smegmatis*.[23] Briefly, 2-deoxy-D-ribose could be cleaved by hydroxyl radical to form malondialdehyde which further reacts with thiobarbutaric acid to give a colored adduct that could be measured spectrophotometrically at 560 nm. There was a constant increase in the oxidation of 2-deoxy-D-ribose for 8 h in aerobic culture of *M. smegmatis* [Figure 1]. Moreover, the production of hydroxyl radical increased significantly in the presence of ferrous ion but decreased in the presence of ferric ion [Figure 1]. Whereas, DMSO a well-known electron spin trap showed dose-dependent decrease in hydroxyl radical production.

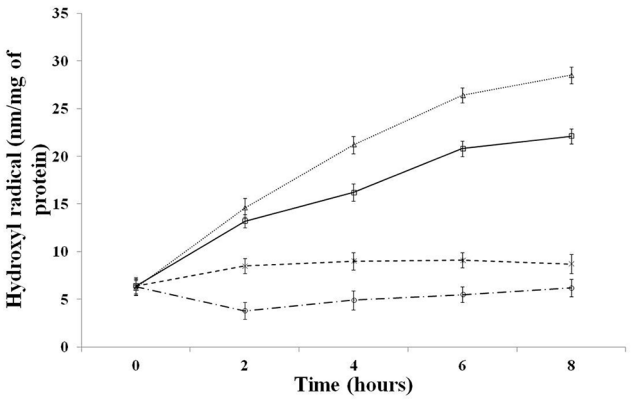

Figure 1: Effect of iron salts on oxidation of 2-deoxy-ribose by aerobically growing culture of *Mycobacterium smegmatis Mycobacterium smegmatis* cells were incubated with 20 mM of 2-deoxy-D-ribose in phosphate at 37°C at 130 RPM in the presence of (□) control, (Δ) $FeSO_4$ (0.1 mM), (x) 2% dimethyl sulfoxide, and (○) $FeCl_3$ (0.1 mM). Oxidation of 2-deoxy-D-ribose by aerobically growing culture of *Mycobacterium smegmatis* shows generation of hydroxy radical by organism. Result is an average of three identical experiments

Effect of hydroxyl radical generator 1-hydroxypyridine-2-thione on hypoxia-induced dormant bacilli

Hydroxyl radical generation by HOPT in the defined minimal media was standardized using 2-deoxy-D-ribose assay before test its effect on aerobic and Wayne hypoxia model. In addition, two separate set of tubes were kept as blank, one set with HOPT without ultraviolet (UV) exposure and the other set was exposed to UV light without HOPT treatment. The result indicated that HOPT and UV exposure alone had no effect on the growth or survival of dormant bacilli. Whereas, HOPT exposed to UV for 10 min was able to reactivate hypoxia-induced dormant cells at the same concentration with cell numbers increased by 0.4–0.6 log CFU/ml count in 24 h. Importantly, methylene blue recoloration was not observed throughout the experiment on addition of HOPT or/and UV exposure. This indicates that there was no significant oxygen was produced on addition of HOPT and growth stimulation was exclusively due to hydroxyl radical generation. However, the similar concentrations of HOPT in aerobic culture had detrimental effect due to the presence of high oxygen concentration which may have escalated the overall ROS production [Figure 2]. Interestingly, previous reports suggest that *M. smegmatis* can grow and survive in the absence of oxygen by metabolically shift to hydrogen fermentation. This ability of organism can be attributed to the diverse set of hydrogenases present in the cell.[28]

Global expression change in dormant, 1-hydroxypyridine-2-thione-treated dormant and active bacilli

Overall, 210 differentially expressed proteins were identified in aerobic, Wayne model, and HOPT-treated Wayne model cultures of *M. smegmatis*, using LC-MS^E. Analysis of the three-independent experiments using PLGS V 2.2.5 software revealed 42 proteins with the most significant fold change in protein expression. The mass list of peptides was converted

to a readable format using MASSLYNX and PLGS 2.2.5 software (Waters Corporation, USA). Furthermore, each translated peptide component is annotated as an exact-mass, retention time pair. A statistical and relative quantitative analysis was performed using the triplicate of the same set of experiments [Figure 3]. It illustrates the binary comparison of replicate injections for the average intensity measurements between the different samples. For each protein, the redundant quantitative measurement obtained from the multiple tryptic peptides were used to determine an average relative folds change. For each average folds – change a 95% confidence interval was determined from the standard deviation of total number of tryptic peptides.

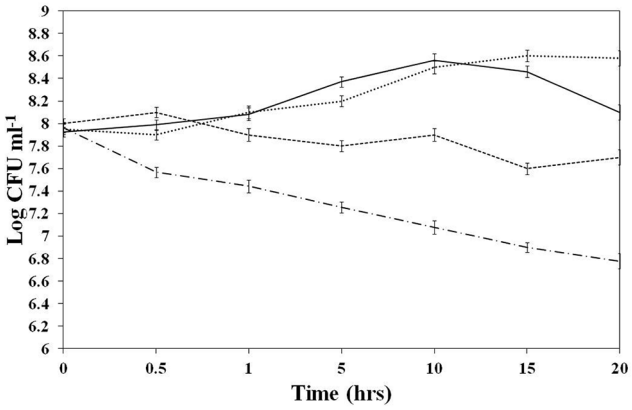

Figure 2: Effect of 1-hydroxypyridine-2-thione on dormant *Mycobacterium smegmatis* bacilli effect of 1-hydroxypyridine-2-thione on aerobic and dormant bacilli was monitored by measuring the colony-forming unit at different time intervals. In Wayne's dormancy model on the 6th day (culture OD_{600} 0.35) (---), 10 μl dimethyl sulfoxide (vehicle control) and (…) 10 μM 1-hydroxypyridine-2-thione was added. Similarly, in aerobic culture, OD_{600} ~ 0.6 (–) 10 μl dimethyl sulfoxide (vehicle control) and (● — ●) 10 μM 1-hydroxypyridine-2-thione was added. With respective addition, culture was plated on Dubos agrose at mentioned time. The rest of the details of the experiment were provided in "Materials and Methods." The plot was made using average values ± standard deviation obtained from three identical experiments

Metabolic enzymes

Nitrogen regulatory protein MSMEG2426 was detected in dormant cells as well as in HOPT-treated reactivated cell, approximately in the same concentration [Table 1]. The glutamate-binding protein MSMEG2727 was downregulated during the dormancy by 4 folds and upregulated by 2 folds in the HOPT-treated reactivated cells [Table 2]. This indicated that the shifting of dormant bacilli toward active form due to the addition of HOPT. Similarly, pyruvate kinase, an essential enzyme in carbohydrate metabolism, was upregulated by 10 folds in dormant cells and 5 folds in HOPT-treated reactivated cells [Table 3]. Glycerol kinase-3, a key enzyme in the regulation of glycerol uptake remained comparable in dormant and aerobic bacilli; however, it was found to be upregulated 3 folds in HOPT-treated dormant *M. smegmatis* cells [Table 1]. In HOPT-treated cells, 3-oxoacyl acyl carrier protein synthase (MSMEG4327) of beta-ketoacyl-ACP synthases family was upregulated by 5 folds and 2.5 folds in comparison to in dormant and aerobic cells, respectively [Table 1]. Another enzyme similar to 3-oxoacyl acyl carrier protein synthase, MSMEG1204, was uniquely present in HOPT-treated reactivated cells. This indicates its possible requirement for the lipid biosynthesis during resuscitation process [Table 1]. A strong upregulation of isocitrate dehydrogenase, a key TCA

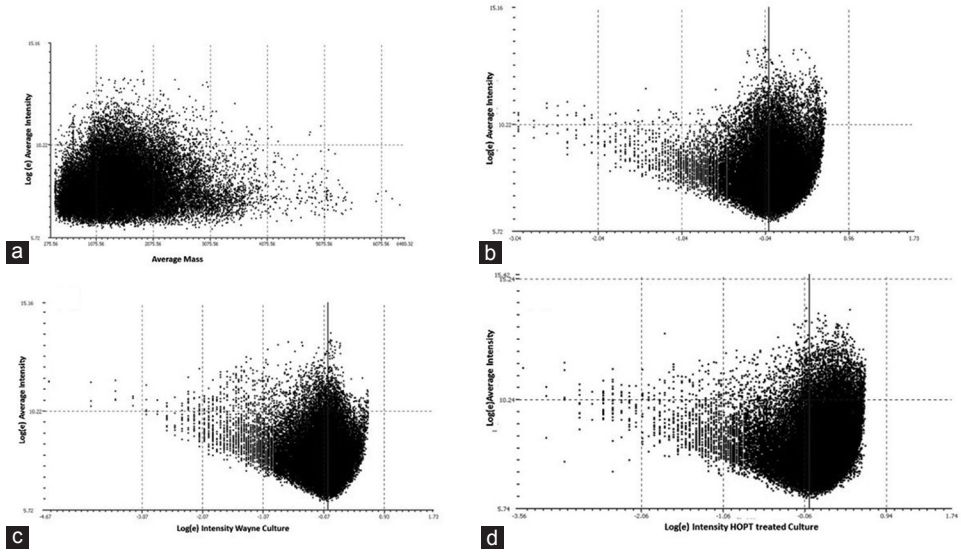

Figure 3: Relative quantitative analysis of peptides identified in all three samples each peptide component detected by liquid chromatography-elevated energy mass spectrometry is annotated as an exact-mass, retention time pair (a) The natural log of the average intensities (all clusters) plotted against average Mass. Binary comparisons of the log intensity measurements obtained from the exact-mass, retention time pairs for all clusters versus (b) Aerobic (c) Wayne hypoxia (d) 1-hydroxypyridine-2-thione-treated cells. For each protein, the redundant quantitative measurement obtained from the multiple tryptic peptides were used to determine an average relative folds change. For each average folds – change a 95% confidence interval was determined from the standard deviation of total number of tryptic peptides

Table 1: Proteins upregulated in 1-hydroxypyridine-2-thione-treated dormant cells

Proteins	Protein ID	Coverage	Number of peptide identified	PLGS score	Aerobic/ dormant folds change	HOPT/ dormant folds change	HOPT/ aerobic folds change
3 oxoacyl acyl carrier protein synthase 1	MSMEG4327	33.4	28	3152.4	2	5	2.5
3 oxoacyl acyl carrier protein synthase 2	MSMEG1204	37.2	17	2245.6	Unique to HOPT	Unique to HOPT	Unique to HOPT
Alcohol dehydrogenase	MSMEG2079	26.2	10	870.2	ND in aerobic	4	ND in aerobic
Alcohol dehydrogenase iron containing	MSMEG6242	27.3	14	860.7	0.25	1	4
ATP synthase subunit beta	AtpD/MSMEG4936	42.3	21	2002.3	28	58	2
ATP synthase epsilon chain	AtpC/MSMEG4935	39.8	18	2272.9	0.5	2	4
Branched chain amino acid aminotransferase	ilvE/MSMEG4276	29.2	11	1457.3	Unique to HOPT	Unique to HOPT	Unique to HOPT
Cell division protein	FtsZ/MSMEG4222	41.6	24	1136.5	ND in dormant	ND in dormant	3
Glycerol kinase 3	glpK/MSMEG6759	31.4	16	1433.2	1	4	4
Nitrogen regulatory protein PII	MSMEG2426	20.5	6	1098.1	ND in aerobic	1	ND in aerobic
Oxidoreductase short-chain dehydrogenase reductase family	MSMEG0372	27.1	9	1162.1	0.3	4	13
Quinone oxidoreductase	MSMEG3106	42.1	13	2129.6	1	3	3
Serine hydroxymethyltransferase	glyA	23.4	7	1113.5	1	3	3
Superoxide dismutase Cu Zn	SodC/MSMEG0835	18.9	6	778.6	1.5	3	2
Superoxide dismutase Mn	SodA/MSMEG6427	78.1	18	1431.1	0.5	1.5	3
Universal stress protein	MSMEG5245	56.8	9	889.7	Unique to HOPT	Unique to HOPT	Unique to HOPT
Universal stress protein	MSMEG3950	28.7	7	432.1	Unique to HOPT	Unique to HOPT	Unique to HOPT

PLGS: Protein Lynx Global Server, HOPT: 1-hydroxypyridine-2-thione, ND: Not detected, ATP: Adenosine triphosphate, SodA: Antioxidant enzyme such as iron-dependent superoxide dismutase, SodC: Membrane-bound copper-dependent superoxide dismutase

cycle enzyme, by 10 folds was observed in dormant cells and 5 folds in HOPT-treated reactivated cells. Succinyl CoA ligases, MSMEG5524, and MSMEG5525 were upregulated by 3 folds during dormancy as well as 1.5 folds in cells treated with HOPT in comparison to aerobic cells [Table 3]. Along similar lines, a strong upregulation of adenosine triphosphate (ATP) subunits, 30 folds and 45 folds upregulation of *atpA*, and 28 folds and 58 folds upregulation *atpD*, respectively, in aerobic and HOPT-treated dormant cells in comparison with the dormant cells was observed [Table 2]. This suggests an increased requirement of ATP during resuscitation and growth of bacilli and the potential involvement of *atpD* in the process [Table 1].

Cell division and cell wall proteins

β-ketoacyl-ACP thiolase involved in the synthesis of mycolic acids was downregulated in dormant bacilli by 4 folds and 6 folds in comparison to aerobic and HOPT-treated dormant cells [Table 2]. Noteworthy, the expression of enzyme in HOPT-treated dormant cell were 1.5 folds more than in aerobic cells. The potential of alcohol dehydrogenases for maintaining the hydrophobic character of the cell envelope is described in previous reports. In this study, two different alcohol dehydrogenase MSMEG2079 (iron containing) and MSMEG6242 (Zinc containing) were detected during the mass spectrometric analysis. Iron-containing alcohol dehydrogenase was absent in aerobic cells and 4 folds more in HOPT-treated dormant cells than dormant cells [Table 1]. Although zinc containing alcohol dehydrogenase was comparable in dormant and HOPT-treated dormant cells, it was upregulated by 4 folds in comparison to aerobic cells [Table 1]. Together, these results indicated that the cell wall of aerobic bacilli is relatively more hydrophobic than the dormant bacilli. Inositol-3-phosphate synthase enzyme that catalyzes the conversion of glucose-6-phosphate to myoinositol-3-phosphate was upregulated by more than 3 folds in dormant and 10 folds in HOPT-treated dormant cells in comparison to aerobic cells [Table 3]. Similarly, cell division protein FtsZ was not detected in dormant cells but upregulated by 3 folds in HOPT-treated dormant bacilli in comparison to aerobic cells [Table 1].

Transcription regulators, chaperons, universal stress proteins, and antioxidant enzymes

A stress protein and chaperone, *dnaK* was downregulated by 2 folds in dormant condition, but upregulated by 1.5 folds in HOPT-treated dormant cells [Table 2]. Similarly, the upregulation of Elongation Factor Tu by 5 folds in dormant as well as in HOPT-treated dormant cells was observed. Universal stress protein (USP) and MSMEG3811 (cAMP binding protein) was also downregulated in hypoxia and transiently

Table 2: Proteins downregulated in dormant condition

Proteins	Protein ID	Coverage	Number of peptide identified	PLGS score	Aerobic/ dormant folds change	HOPT/ dormant folds change	HOPT/ aerobic folds change
Alkyl hydroperoxide reductase subunit C	AhpC/MSMEG4891	74.8	24	6819.5	4	4	1
ATP synthase subunit alpha	AtpA/MSMEG4938	40.1	12	2455.7	35	40	1
Beta-ketoadipyl CoA thiolase	MSMEG5273	29.6	8	2178.1	4	6	1.5
Branched chain amino acid ABC transporter substrate binding protein	MSMEG3247	41.6	14	1454.2	3	3	1
Chaperone protein	dnaK/MSMEG0709	22.6	16	1127.8	2	5	1.5
D3 phosphoglycerate dehydrogenase	serA/MSMEG2378	34.4	13	1265.7	3	5	1.7
Electron transfer flavoprotein alpha subunit	etfA/MSMEG2352	38.2	13	589.3	3	4.5	1.5
Electron transfer flavoprotein beta subunit		23.5	12	1522.4	3	4	1.4
Extracellular solute binding protein family protein 3	MSMEG0114	26.5	18	600.2	2	4	2
Extracellular solute binding protein family protein 5	MSMEG0643	27.8	23	1381.8	2	2	1
Glyceraldehyde 3 phosphate dehydrogenase	gapA/MSMEG3084	27.4	23	1839.7	3	5	1.6
Glutamate binding protein	MSMEG2727	43.7	25	454.3	4	2	0.5
Meromycolate extension acyl carrier protein	acpM/MSMEG4326	54.4	7	6617.2	5	3	0.6
Polyribonucleotide nucleotidyltransferase	pnp/MSMEG2656	34.5	14	1185.4	3	3	1
Sulfonate binding protein	MSMEG0550	27.3	15	352.4	5	3	0.6
Iron-dependent repressor	IdeR	32.6	16	1133.6	Unique to aerobic	Unique to aerobic	Unique to aerobic
Universal stress protein	MSMEG3811	72.3	22	3363.7	3	1.5	0.5

Ahp: Alkyhydroxy peroxidase, PLGS: Protein Lynx Global Server, HOPT: 1-hydroxypyridine-2-thione, IdeR: Iron-dependent regulator, ATP: Adenosine triphosphate, CoA: Coenzyme A, ABC: ATP-binding cassette transporter

Table 3: Proteins upregulated in Wayne hypoxia (dormant) cells

Proteins	Protein ID	Coverage	Number of peptides identified	PLGS score	Aerobic/ dormant folds change	HOPT/ dormant folds change	HOPT/ aerobic folds change
Adenosylhomocysteinase/ S-adenosyl-L-homocysteine hydrolase	ahcY/MSMEG1843	19.2	11	2167.8	0.1	0.5	5
Elongation factor Tu	tuf/MSMEG1401	45.4	27	2962.3	0.2	0.2	1
GMP synthase glutamine hydrolyzing	guaA/MSMEG1610	28.6	7	1188.1	Unique to dormant	Unique to dormant	Unique to dormant
Helix turn helix motif	MSMEG5136	74.5	7	837.3	Unique to dormant	Unique to dormant	Unique to dormant
Inositol 3 phosphate synthase	ino1/MSMEG6904	24.2	23	914.7	0.3	3	10
Isocitrate dehydrogenase NADP	MSMEG1654	39.9	18	4786.1	0.1	0.5	5
MarR family protein regulatory protein	MSMEG3415	41.5	24	1025.8	Unique to dormant	Unique to dormant	Unique to dormant
Pyruvate kinase	Pyk	26.9	10	2041.9	0.1	0.5	5
Succinyl CoA ligase ADP forming subunit alpha	sucD/MSMEG5524	31.7	21	2310.4	0.3	1.5	0.5
Succinyl CoA ligase ADP forming subunit beta	sucC/MSMEG5525	62.4	18	687.3	0.3	1.5	0.5

Tu: Thermo unstable, GMP: Guanosine monophosphate, PLGS: Protein Lynx Global Server, HOPT: 1-hydroxypyridine-2-thione, ADP: Adenosine diphosphate, CoA: Coenzyme A, NADP: Nicotinamide adenine dinucleotide phosphate

increase in HOPT-treated hypoxic cells, which suggest the possible role during resuscitation of the bacilli [Table 1]. These proteins may have role in ROS-induced DNA damage repair as they downregulate during hypoxia. Two uncharacterized USPs MSMEG5245 and MSMEG3950 were uniquely found in HOPT-treated cells probably because of their important function in resuscitation of dormant bacilli [Table 1]. However, the expression of these unique USPs in resuscitated cells needs further characterization to understand their role in reactivation.

Discussion

Mycobacteria are obligate aerobes; however, they have an extraordinary ability to survive under hypoxia. The shift to nitrogen metabolism during dormancy is a hallmark event of metabolic plasticity in *Mycobacterium*.[22,29,30] The Wayne's dormancy model is obtained by slow depletion of oxygen in the medium and comprehensively mimics the dormant phenotype.[22] In this study, reactivation of Wayne dormancy model provide an unprecedented opportunity to demarcate the metabolic profile of the process.

The glycolytic pathway enzyme catalyzes the transfer of a phosphate group from phosphoenolpyruvate to adenosine diphosphate (ADP) which yields one molecule each of pyruvate and ATP. The upregulation of pyruvate kinase indicates the importance of glycolysis or gluconeogenesis pathway for the carbohydrate metabolism during dormancy.[29,30] Similarly, fatty acids are primarily catabolized by sequential rounds of β-oxidation.[30,31] Glycerol-3-kinase may be an essential enzyme during resuscitation of dormant bacilli as its production is crucial for the membrane and fatty acid metabolism. Along with glycerol kinase, it involves fatty-acyl glycerol phosphates that leads to the interconversion of CDP-diacyl-glycerol and phosphatidyl-glycerol phosphates.[29,30] These results were consistent with the previous finding and emphasize the involvement of membrane metabolism in biomass production.[32]

Acetyl-coA derived from the catabolism of fatty acids or sugars is utilized by the TCA cycle, which provides biosynthetic precursors and reducing equivalents for the cell.[30] In dormant cells, a shift in carbohydrate metabolism was observed where TCA cycle reroutes toward the glyoxalate cycle as described in the earlier report.[30] However, in HOPT-treated cells their expression was found in between the dormant and aerobic cells which indicates the occurrence of mixed population in resuscitation. As described in the results, a transition phase between dormancy and resuscitation could be defined in HOPT-treated hypoxia cells. The expression of unique 3-oxoacyl carrier protein in resuscitated cells suggests the requirement of fatty acid metabolism in the resuscitation. This justified the different variants of TCA cycle in *Mycobacterium* sp. as well as the importance of glycolytic and TCA cycle pathway during dormant condition of the bacilli.

ATP synthase utilizes the proton-motive force across the bacterial membrane for the synthesis of ATP. Interestingly, in oxygen-limited condition, this reaction could be reversed to maintain a proton-motive force.[33] A strong downregulation of ATP synthase is characteristic of dormant bacilli in different models.[34] The differences in the expression of different subunits as well not detection of some may be attributed to the limitation of isolation of subunits during proteome preparation. The rapid decrease in ATP level during dormancy led to cessation of ribosomal assembly and protein synthesis. A strong induction of ATP synthase during resuscitation suggests that ATP synthesis is the primary event in resuscitation.

Mycobacteria genus synthesizes two forms of siderophores, carboxymycobactin, and mycobactin, to capture iron. They differ in the length of an alkyl substitution, thereby leading to a difference in polarity and solubility.[35] The carboxymycobactin or mycobactin deficient *M. tuberculosis* is unable to replicate in low-iron media or macrophages.[36] Many reports consider iron acquisition as an important step in pathogen survival and attaining successful infection.[37,38] Similarly, soil-dwelling *M. smegmatis* requires enhanced siderophore synthesis for biofilm formation and maturation.[39] Iron-dependent regulator (*IdeR*) plays a dual role as both transcription regulator and iron acquisition protein.[40] In this study, *IdeR* proteins were expressed only in actively dividing cells but completely absent in dormancy. Similarly, the cellular production of hydroxyl radical increased significantly in the presence of ferrous ion but decreased in the presence of ferric ion. This indicates that iron acquisition is an important step to maintain the concentration of hydroxyl radical by *in vivo* Fenton's reaction. However, *IdeR* was not upregulated in HOPT-treated cells which could possibly be due to extraneous addition of hydroxyl radical.

Mycobacteria cell wall is composed of long-chain fatty acid, mycolic acids, plays a critical role in structure and function.[41] This structure confers the bacteria to resist any chemical injury, low permeability to antibiotics, damage from dehydration, and an ability to survive within the phagolysosomes of the macrophages.[42] Inositol phosphate capping of the nonreducing termini of lipoarabinomannan is an essential step in achieving dormancy type cell wall in fast-growing *Mycobacterium* sp.[43] FtsZ concentration and its controlled interaction with other cell division proteins is a key regulator of bacterial cell division.[44] The HOPT-induced resuscitation highlights the upregulation of different cell membrane and cell division proteins that affect the cell membrane dynamics in terms of its content, polarity, and hydrophobicity.

MarR family regulatory proteins generally show a low concentration under *in vitro* and nonstressed conditions, but strongly upregulated under *in vivo* and stressed conditions.[45,46] Similarly, MarR proteins were uniquely detected in dormant (Wayne model) and not in either aerobic or HOPT-treated dormant cultures [Table 3]. MarR family members are strong autoregulators; this explains the absence of MarR regulatory proteins in HOPT-treated dormant cells [Table 3].

Elongation factor plays an important role in oxidative stress and in bacterial association with macrophages.[47,48] Upregulation of elongation factor and USPs during dormancy and HOPT treatment suggests their role in transition and regulation of genes during different stages. Although the functional roles of USPs are still needed to be determined, their homologues are known for DNA damage repair.[49] On the other hand, antioxidant enzyme such as iron-dependent superoxide dismutase (*SodA*), membrane-bound copper-dependent superoxide dismutase (*SodC*), and alkyhydroxy peroxidase (*AhpC*) were earlier reported to downregulate in dormant stage.[49-51] Although all

three enzymes mentioned were found to be essential at the time of infection to counter oxidative burst by host macrophages and subsequently downregulate after 15 days postinfection.[30] *AhpC* together with *AhpD* constitutes an NADH-dependent peroxidase which act against diverse reactive nitrogen intermediates and oxidative stress generated by the host as a primary defense.[51] In case of HOPT-treated reactivated cells expression of the antioxidant proteins was comparable to aerobic stage. This was 4 folds higher than untreated Wayne hypoxic cells [Table 2]. Antisense RNA of *SodA* was found to significantly decrease the survival of tubercular bacilli under aerobic condition.[49] As reported earlier, *SodA* was induced in dormancy by 2 folds as well as 3 folds in HOPT-treated Wayne culture[30,50] [Table 1]. *SodC*, which was considered as insignificant in infection as its mutants survived in guinea pigs, was downregulated by 0.75 folds during hypoxia-induced dormancy and upregulated by 3 folds in those cells when treated with HOPT [Table 1]. Thioredoxin reductase, a putative enzyme that was earlier found to inhibit oxidative stress-dependent killing of *M. smegmatis* was not detected in both hypoxic and HOPT-treated Wayne hypoxic cultures. In tuberculosis, this enzyme was reported to be downregulated in Wayne hypoxia and mouse infection model.[30] The restoration of stress proteins and antioxidants enzymes profile in HOPT-induced resuscitated cells as actively dividing cells confirms the complete activation of dormant cells into active state.

In this study, a simple hydroxyl radical-induced reactivation system using *in vitro* Wayne dormancy model is developed. Using this model, we could highlight the metabolic pathways translationally relevant during dormancy and resuscitation program. In accordance to previous reports, this study found similar shift in metabolism from the available set of proteins when compared between active and dormant state.[29,30,52-54] The biological processes, such as, carbohydrate and lipid metabolism, cell wall biosynthesis, transcription, and damage repair that are minimal during dormancy were transiently reactivated during resuscitation program. The quantitative proteome analysis could justify that the resuscitation process is a controlled reestablishment of metabolic process and step-wise exit from dormancy. Finally, this study suggests a novel Wayne dormancy–HOPT reactivation model which will help in identifying checkpoints and understanding the reactivation process.

Acknowledgment

The author would like to thank Director, CSIR-National Chemical Laboratory, Pune, India, for providing financial support and laboratory facility and also thank Council of Scientific and Industrial Research, New Delhi, India, for research fellowship.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, *et al.* Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. J Exp Med 2003;198:705-13.
2. Kumar A, Toledo JC, Patel RP, Lancaster JR Jr., Steyn AJ. *Mycobacterium tuberculosis* DosS is a redox sensor and DosT is a hypoxia sensor. Proc Natl Acad Sci U S A 2007;104:11568-73.
3. Fontán P, Aris V, Ghanny S, Soteropoulos P, Smith I. Global transcriptional profile of *Mycobacterium tuberculosis* during THP-1 human macrophage infection. Infect Immun 2008;76:717-25.
4. Shiloh MU, Manzanillo P, Cox JS. *Mycobacterium tuberculosis* senses host-derived carbon monoxide during macrophage infection. Cell Host Microbe 2008;3:323-30.
5. Zhang Y. Persistent and dormant tubercle bacilli and latent tuberculosis. Front Biosci 2004;9:1136-56.
6. Frey RL, He L, Cui Y, Decho AW, Kawaguchi T, Ferguson PL, *et al.* Reaction of N-acylhomoserine lactones with hydroxyl radicals: Rates, products, and effects on signaling activity. Environ Sci Technol 2010;44:7465-9.
7. Bechtel W, Bauer G. Modulation of intercellular ROS signaling of human tumor cells. Anticancer Res 2009;29:4559-70.
8. Hidalgo E, Ding H, Demple B. Redox signal transduction via iron-sulfur clusters in the soxR transcription activator. Trends Biochem Sci 1997;22:207-10.
9. Bloomfield G, Pears C. Superoxide signalling required for multicellular development of *Dictyostelium*. J Cell Sci 2003;116:3387-97.
10. Lee JH, Yeo WS, Roe JH. Induction of the sufA operon encoding Fe-S assembly proteins by superoxide generators and hydrogen peroxide: Involvement of oxyR, IHF and an unidentified oxidant-responsive factor. Mol Microbiol 2004;51:1745-55.
11. McInnis J, Wang C, Anastasio N, Hultman M, Ye Y, Salvemini D, *et al.* The role of superoxide and nuclear factor-kappaB signaling in N-methyl-D-aspartate-induced necrosis and apoptosis. J Pharmacol Exp Ther 2002;301:478-87.
12. Oliveira-Sales EB, Colombari DS, Davisson RL, Kasparov S, Hirata AE, Campos RR, *et al.* Kidney-induced hypertension depends on superoxide signaling in the rostral ventrolateral medulla. Hypertension 2010;56:290-6.
13. Dwyer DJ, Kohanski MA, Hayete B, Collins JJ. Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. Mol Syst Biol 2007;3:91.
14. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A common mechanism of cellular death induced by bactericidal antibiotics. Cell 2007;130:797-810.
15. Kolodkin-Gal I, Engelberg-Kulka H. The extracellular death factor: Physiological and genetic factors influencing its production and response in *Escherichia coli*. J Bacteriol 2008;190:3169-75.
16. Gusarov I, Shatalin K, Starodubtseva M, Nudler E. Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. Science 2009;325:1380-4.
17. Nguyen D, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K, *et al.* Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 2011;334:982-6.
18. Shatalin K, Shatalina E, Mironov A, Nudler E. H2S: A universal defense against antibiotics in bacteria. Science 2011;334:986-90.
19. Rhee SG. Redox signaling: Hydrogen peroxide as intracellular messenger. Exp Mol Med 1999;31:53-9.
20. Stone JR, Yang S. Hydrogen peroxide: A signaling messenger. Antioxid Redox Signal 2006;8:243-70.
21. Grant SS, Kaufmann BB, Chand NS, Haseley N, Hung DT. Eradication of bacterial persisters with antibiotic-generated hydroxyl radicals. Proc Natl Acad Sci U S A 2012;109:12147-52.
22. Wayne LG, Sohaskey CD. Nonreplicating persistence of *Mycobacterium tuberculosis*. Annu Rev Microbiol 2001;55:139-63.
23. Biaglow JE, Manevich Y, Uckun F, Held KD. Quantitation of hydroxyl radicals produced by radiation and copper-linked oxidation of ascorbate by 2-deoxy-D-ribose method. Free Radic Biol Med 1997;22:1129-38.
24. Mishra A, Sarkar D. Qualitative and quantitative proteomic analysis

of Vitamin C induced changes in *Mycobacterium smegmatis*. Front Microbiol 2015;6:451.
25. Silva JC, Denny R, Dorschel CA, Gorenstein M, Kass IJ, Li GZ, *et al.* Quantitative proteomic analysis by accurate mass retention time pairs. Anal Chem 2005;77:2187-200.
26. Silva JC, Denny R, Dorschel C, Gorenstein MV, Li GZ, Richardson K, *et al.* Simultaneous qualitative and quantitative analysis of the *Escherichia coli* proteome: A sweet tale. Mol Cell Proteomics 2006;5:589-607.
27. Chakraborty AB, Berger SJ, Gebler JC. Use of an integrated MS – multiplexed MS/MS data acquisition strategy for high-coverage peptide mapping studies. Rapid Commun Mass Spectrom 2007;21:730-44.
28. Greening C, Berney M, Hards K, Cook GM, Conrad R. A soil actinobacterium scavenges atmospheric H2 using two membrane-associated, oxygen-dependent [NiFe] hydrogenases. Proc Natl Acad Sci U S A 2014;111:4257-61.
29. Shi L, Sohaskey CD, Kana BD, Dawes S, North RJ, Mizrahi V, *et al.* Changes in energy metabolism of *Mycobacterium tuberculosis* in mouse lung and under *in vitro* conditions affecting aerobic respiration. Proc Natl Acad Sci U S A 2005;102:15629-34.
30. Shi L, Sohaskey CD, North RJ, Gennaro ML. Transcriptional characterization of the antioxidant response of *Mycobacterium tuberculosis in vivo* and during adaptation to hypoxia *in vitro*. Tuberculosis (Edinb) 2008;88:1-6.
31. Stewart GR, Robertson BD, Young DB. Tuberculosis: A problem with persistence. Nat Rev Microbiol 2003;1:97-105.
32. Jamshidi N, Palsson BØ. Investigating the metabolic capabilities of *Mycobacterium tuberculosis* H37Rv using the *in silico* strain iNJ661 and proposing alternative drug targets. BMC Syst Biol 2007;1:26.
33. Boyer PD. A research journey with ATP synthase. J Biol Chem 2002;277:39045-61.
34. Sassetti CM, Rubin EJ. The open book of infectious diseases. Nat Med 2007;13:279-80.
35. De Voss JJ, Rutter K, Schroeder BG, Su H, Zhu Y, Barry CE 3rd, *et al.* The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. Proc Natl Acad Sci U S A 2000;97:1252-7.
36. Rodriguez GM, Smith I. Identification of an ABC transporter required for iron acquisition and virulence in *Mycobacterium tuberculosis*. J Bacteriol 2006;188:424-30.
37. Gold B, Rodriguez GM, Marras SA, Pentecost M, Smith I. The *Mycobacterium tuberculosis* IdeR is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages. Mol Microbiol 2001;42:851-65.
38. Monfeli RR, Beeson C. Targeting iron acquisition by *Mycobacterium tuberculosis*. Infect Disord Drug Targets 2007;7:213-20.
39. Ojha A, Hatfull GF. The role of iron in *Mycobacterium smegmatis* biofilm formation: The exochelin siderophore is essential in limiting iron conditions for biofilm formation but not for planktonic growth. Mol Microbiol 2007;66:468-83.
40. Timm J, Post FA, Bekker LG, Walther GB, Wainwright HC, Manganelli R, *et al.* Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. Proc Natl Acad Sci U S A 2003;100:14321-6.
41. Brennan PJ. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 2003;83:91-7.
42. Barry CE 3rd, Lee RE, Mdluli K, Sampson AE, Schroeder BG, Slayden RA, *et al.* Mycolic acids: Structure, biosynthesis and physiological functions. Prog Lipid Res 1998;37:143-79.
43. Khoo KH, Dell A, Morris HR, Brennan PJ, Chatterjee D. Inositol phosphate capping of the nonreducing termini of lipoarabinomannan from rapidly growing strains of *Mycobacterium*. J Biol Chem 1995;270:12380-9.
44. Dziadek J, Rutherford SA, Madiraju MV, Atkinson MA, Rajagopalan M. Conditional expression of *Mycobacterium smegmatis* ftsZ, an essential cell division gene. Microbiology 2003;149:1593-603.
45. Ellison DW, Miller VL. Regulation of virulence by members of the MarR/SlyA family. Curr Opin Microbiol 2006;9:153-9.
46. Grkovic S, Brown MH, Skurray RA. Regulation of bacterial drug export systems. Microbiol Mol Biol Rev 2002;66:671-701.
47. Raman S, Song T, Puyang X, Bardarov S, Jacobs WR Jr., Husson RN, *et al.* The alternative sigma factor SigH regulates major components of oxidative and heat stress responses in *Mycobacterium tuberculosis*. J Bacteriol 2001;183:6119-25.
48. Hickey TB, Thorson LM, Speert DP, Daffé M, Stokes RW. *Mycobacterium tuberculosis* cpn60.2 and DnaK are located on the bacterial surface, where cpn60.2 facilitates efficient bacterial association with macrophages. Infect Immun 2009;77:3389-401.
49. Edwards KM, Cynamon MH, Voladri RK, Hager CC, DeStefano MS, Tham KT, *et al.* Iron-cofactored superoxide dismutase inhibits host responses to *Mycobacterium tuberculosis*. Am J Respir Crit Care Med 2001;164:2213-9.
50. Piddington DL, Fang FC, Laessig T, Cooper AM, Orme IM, Buchmeier NA, *et al.* Cu, Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. Infect Immun 2001;69:4980-7.
51. Ng VH, Cox JS, Sousa AO, MacMicking JD, McKinney JD. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: Countering the phagocyte oxidative burst. Mol Microbiol 2004;52:1291-302.
52. Blokpoel MC, Smeulders MJ, Hubbard JA, Keer J, Williams HD. Global analysis of proteins synthesized by *Mycobacterium smegmatis* provides direct evidence for physiological heterogeneity in stationary-phase cultures. J Bacteriol 2005;187:6691-700.
53. Kim SY, Lee BS, Shin SJ, Kim HJ, Park JK. Differentially expressed genes in *Mycobacterium tuberculosis* H37Rv under mild acidic and hypoxic conditions. J Med Microbiol 2008;57:1473-80.
54. Starck J, Källenius G, Marklund BI, Andersson DI, Akerlund T. Comparative proteome analysis of *Mycobacterium tuberculosis* grown under aerobic and anaerobic conditions. Microbiology 2004;150:3821-9.