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ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · NOVEMBER 2009

Impact Factor: 5.33 · DOI: 10.1021/es902255q · Source: PubMed

Microarray Analysis of *Mycobacterium bovis* BCG Revealed Induction of Iron Acquisition Related Genes in Response to Hydrogen Peroxide.

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Received July 27, 2009. Revised manuscript received October 30, 2009. Accepted November 07, 2009.

Mycobacterium bovis BCG strain Pasteur 1173P2 responds with adaptive and protective strategies against oxidative stress. Despite advances in our understanding of the responses to oxidative stress in many specific cases, the connectivity between targeted protective genes and the rest of cell metabolism remains obscure. This study was therefore carried out to investigate the genome-wide response of *M. bovis* BCG to hydrogen peroxide after 10 and 60 min of treatment. ATP measurements were carried out in order to monitor the changes in *M. bovis* BCG growth over a 1 h period.

The *fur*A gene in *Mycobacterium bovis*, a pleiotropic regulator that couples iron metabolism to the oxidative stress response was involved in the response to hydrogen peroxide stress. There were also increased levels of catalase/ peroxidase (KatG) and the biosynthesis operon of mycobactin.

This study revealed significant upregulation of the oxidative response group of *M. bovis*, amino acid transport and metabolism, defense mechanisms, DNA replication, recombination and repair, and downregulation of cell cycle control, mitosis, and meiosis, lipid transport and metabolism, and cell wall/membrane biogenesis.

This study shows that the treatment of *M. bovis* BCG with hydrogen peroxide induces iron acquisition related genes and oxidative stress response genes within one hour of treatment.

Introduction

Although many antituberculosis drugs have been developed over the past 30 years, tuberculosis (TB) remains a potential threat to primary public health. The efficacy of TB drugs is compromised by the long treatment times required (*1*) and the growing threat of multidrug resistant strains (*2*). Many countries use the *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccine as part of their TB control programs, especially for infants. BCG vaccines are live attenuated strains of *M. bovis*.

Both *Mycobacterium. tuberculosis* and *M. bovis* belong to the same complex known as the *M. tuberculosis* complex (*3*) and their genome sequences are almost identical (greater than 99.95% similarity). *M. bovis* has a reduced genome size due to the deletion of genetic information (*4*). Both *M. bovis* and *M. tuberculosis* are intracellular pathogens which are able to survive inside macrophages, where there is respiratory burst during infection (*5, 6*).

Iron is an essential cofactor for most bacteria, although iron acquisition requires specialized mechanisms (*7, 8*). Mycobacteria acquire iron by synthesizing and secreting siderophores (*8, 9*) such as extracellular exochelin and mycobactin (*7*). *M. tuberculosis*, synthesizes mycobactins and these are critical for growth in iron-limiting conditions in macrophages and in mice (*10, 11*). The expression of iron acquisition genes is typically tightly regulated and is induced under iron-limiting conditions. In *M. tuberculosis*, the expression of over 150 open reading frames (ORFs) is responsive to iron depletion, including the mycobactin biosynthetic operon under control of the IdeR regulator (*12, 13*). Mycobacteria also contain one or more FurA-family genes that are involved in the uptake of metals and in oxidative stress response (*14, 15*).

The ability of pathogenic microorganisms to acquire iron from host cells has been implicated as a factor in their virulence (*16, 17*). Many microorganisms secrete iron binding siderophores under low-iron growth conditions making it possible for them to compete for the host's iron held in specific molecules. The roles of the various types of microbial iron acquisition systems in host-pathogen interactions depend on the nature of the infection and the location of the pathogen within the host. Microbes that infect extracellular spaces of host cells utilize different strategies for iron acquisition compared to those that invade and multiply within host cells (*18, 19*).

Hydrogen peroxide (HP) is routinely used in hospitals and health care facilities for surface sterilization. Hydrogen peroxide is also used as a fresh produce disinfectant (*20*); as a household disinfectant (*21*); and has been reported to be suitable for use in broiler production for disinfection during the incubation of eggs (*22*). By reacting with intracellular iron, HP can form hydroxyl radicals by way of the Fenton reaction, which damages several cellular molecules including lipids, proteins, and DNA (*23, 24*). Superoxide is also capable of promoting oxidative damage by increasing the concentration of intracellular iron (*25−27*). In mycobacteria, iron deficiency can lead to oxidative stress by decreasing the activity of heme-containing enzymes that are involved in protection against toxic reactive oxygen intermediates (*46*). As such, the regulation of iron levels in the cells and the response to oxidative stress are closely linked. In particular, FurA, which is a ferric uptake regulation protein is suggested to be an iron-dependent regulator of the *kat*G gene, which codes for a catalase-peroxidase involved in the response of *M. tuberculosis* to oxidative stress (*28*).

Our previous data revealed the transcriptome analysis of the cellular response of *Staphylococcus aureus* and *Pseudomonas aeruginosa* to hydrogen peroxide-induced oxidative stress. This data indicated that the response to oxidative stress includes the induction of genes involved in virulence, DNA repair, anaerobic metabolism (*29*), pyocins, and iron regulation (*30*). However, the mechanisms of action by which this

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TABLE 1. Transcript Level Comparison of *Mycobacterium bovis* BCG Genes between Real-Time PCR and Microarray Analyses[a]

gene	mRNA level change with microarray		mRNA level change with real-time PCR		sense primer sequence	antisense primer sequence
	fold change		fold change			
	10 min	60 min	10 min	60 min		
BCG_1947c	5.5	2.7	18.0 (±0.1)	5.5 (±0.3)	5′- AAC ATC AAA GTG TCC TTC GCC GAC −3′	5′- GCA AAG GAT TCC ACG TCG GTT TGT −3′
BCG_1948c	4.9	2.3	10.3 (±0.1)	2.9 (±0.2)	5′- TCG GAC CAT AAC GGC TTC CTG TT −3′	5′- GAT GTG ATC GCG AAG TGT CGG ATA −3′
BCG_2396c	4.5	1.9	7.1 (±0.1)	5.3 (±0.2)	5′- CTT TCA CAC CGC GGT TCA AGC TAT −3′	5′- TGC TGC TTG GAG AAC TCG ACG AAA −3′
BCG_2397c	3.6	2.0	7.5 (±0.1)	4.8 (±0.3)	5′- ATC TCG CGA CTT TCC CAT CAG TGT −3′	5′- GTC AAC GCA AGT TCG AAT ACC GCA −3′
BCG_3009c	3.4	1.5	7.1 (±0.1)	2.5 (±0.4)	5′- AAA GCA TTG GCG TAC ATG GCC TTC −3′	5′- TTC AAT GCG ACC GTT GGT ACA CGA −3′
BCG_0020	1.6	1.5	4.5 (±0.1)	3.3 (±0.1)	5′- AAA GTT ACC GAC GCA TCC TTT GCC −3′	5′- TAC CAT CTT GCA AGG TCC ACA CCA −3′
BCG_2395c	3.2		11.7 (±0.1)		5′- AGG ACT ACG ACC TGG TAG GAA ACA −3′	5′- TGC GCA GTA CCG GAG TAA AGA ACT −3′
BCG_3008c	2.4		3.0 (±0.6)		5′- AAG CCT TTC ACA CCC ACT CTG GTA −3′	5′- TCA GAA AGA CCG CGG GAA TGA TCT −3′
BCG_2400c	2.1		5.9 (±0.4)		5′- TGG CTC GTG ATG ACC TGG AAT CAA −3′	5′- TAA TCT CCT CAA GCG AAG AGC GCA −3′
BCG_2394c	2.0		2.6 (±0.2)		5′- TAT TGG AGG GAC GCA TGT CGC ATA −3′	5′- TTC GGA ATC CGC GAA TCT TGA CCT −3′
BCG_1410	1.7		5.0 (±0.2)		5′- GGA TCC GGT GAA TTT GTT GCC GTT −3′	5′- AGT TCG GTT TCG TCG AGT GTG ACT −3′
16S rRNA[a]	1.0	1.0	1.0	1.0	5′- TGC AAG TCG AAC GGA AAG GTC TCT −3′	5′- AAG ACA TGC ATC CCG TGG TCC TAT −3′

[a] The real time PCR results are the mean of three biological replicates with three technical replicates for each gene.

disinfectant kills *M. bovis* BCG and the extent to which *M. bovis* BCG is resistant to HP have not been elucidated.

To advance our understanding in this unknown territory, we investigated HP-driven changes in global genome expression in *M. bovis* by using whole-genome microarrays and validated the genes by a second method, quantitative polymerase chain reaction (PCR).

Materials and Methods

Bacterial Strains and Growth Conditions. *Mycobacterium bovis* BCG strain Pasteur 1173P2 was obtained from the American Type Culture Collection (ATCC 35748). The *M. bovis* BCG stock culture was inoculated into 200 mL Middlebrook 7H9 broth (Difco, Sparks, MO) supplemented with 0.1% (v/v) tween 80 (Sigma-Aldrich Co., St. Louis, MO) and 10% (v/v) OADC (oleic acid, albumin, dextrose, catalase) (BBL Co., Franklin Lakes, NJ) (*31*),. The growth culture was incubated at 37 °C with shaking at 200 rpm, to reach an OD_{600nm} of 0.3−0.4 after 5 days. One milliliter aliquots of this prepared stock culture were maintained in 10% (v/v) glycerol at −80 °C for subsequent use.

One aliquot of the prepared of *M. bovis* stock culture was inoculated into the growth medium (prepared as described above) and incubated at 37 °C with shaking at 200 rpm to reach an OD_{600nm} of 0.3−0.4 after 5 days. Cells were harvested and resuspended in 200 mL of Luria−Bertani (LB) medium as described in our previous papers (*32*−*34*) containing 0.1% Tween 80 and incubated for 24 h at 37 °C. In meantime, 10 mL of cells was transferred into 200 mL of new M7H9 medium for continuous culture.

Quantification of ATP from bacterial culture. All steps were performed at room temperature (22−25 °C) unless indicated. LB growth cultures were dispensed into designated 50 mL tubes prewarmed to 37 °C and hydrogen peroxide was added to the cultures to reach a range of several final concentrations: 0, 0.05, 0.5, 5, and 50 mM. To obtain a value for background luminescence, 1 mL of the untreated culture was spun down. The pellet was washed in 1 mL 1 × phosphate-buffered saline (PBS) buffer (Invitrogen, Carlsbad, CA) and centrifuged. The pellet was resuspended in 200 μL PBS solution by gentle vortexing or pipetting.

The BacTiter-Glo buffer (Promega Co., San Luis Obispo, CA) and lyophilized substrate were thawed and equilibrated to room temperature for approximately 30 min before use. The buffer and substrate were mixed by inverting the bottle to obtain a homogeneous solution. One hundred microliters of the reagent mix was added to designated control wells in an opaque-walled 96 well-plate. Sterile PBS buffer was added to control wells and luminescence was measured using the luminometer, Glomax (Promega Co., San Luis Obispo, CA), to obtain a value for background luminescence. Thereafter, PBS containing the cells was added to test wells containing 100 μL of the reagent mix. Well contents were briefly mixed and luminescence measured over the selected test times

RNA Isolation. Total RNA was isolated from *M. bovis* not treated with hydrogen peroxide (control) and from *M. bovis* treated with 0.5 mM hydrogen peroxide after 10 and 60 min using the RiboPure bacteria kit (Ambion, Inc., Austin, TX) (*35*). The mycobacterial cells treated with hydrogen peroxide and untreated cells were harvested and resuspended in PBS buffer. The mini-bead beater-16 (Bio spec products Inc., Bartlesville, OK) was used for breaking down the *Mycobacterium* cells for RNA extraction. Beating was performed for 1 min (five times), with intermittent storage on ice after each beating period for 2 min. The quantity of eluted RNA was determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The RNA 6000 Nano LabChip with an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) was used to check the quality and purity of extracted RNA.

cDNA Synthesis, Labeling, Hybridization, Staining, And Scanning. cDNA synthesis, cDNA fragmentation, labeling, hybridization, staining, and washing steps were performed according to the manufacturer's protocol for the Affymetrix *M. bovis* BCG custom GeneChip arrays (Affymetrix, Inc., Santa Clara, CA) as described in our previous papers (*29, 30, 35, 36*).

Affymetrix *M. bovis* BCG Custom Genechip Analysis. The custom array was constructed by Affymetrix using the sequence of *Mycobacterium bovis* BCG strain Pasteur 1173P2. The arrays were scanned with the Affymetrix GeneChip Scanner 3000. To analyze the array data, GeneChip Operating Software (GCOS) v. 1.2 (Affymetrix, Inc., Santa Clara, CA) and GeneSpring GX v. 7.3 (Agilent Technologies, Inc., Santa Clara, CA) were utilized with the following parameters: *alpha* 1, 0.04; *alpha* 2, 0.06; *tau*, 0.015; target signal, 500. Fold changes were calculated as the ratio between the signal averages of three biological controls (untreated) and three biological experimental (hydrogen peroxide -treated) for 10 and 60 min exposures.

Real Time Reverse Transcriptase PCR Analysis. To determine the validity of the array data, transcript level changes obtained with the microarray analysis were compared with those from quantitative real time reverse transcriptase PCR (real time RT PCR). Genes and primer sequences employed for the real-time PCR analysis are listed in Table 1. We examined the relative levels of 12 genes with a range of fold changes (2.5- to 18-fold) by real time RT PCR analysis, which were specifically involved in the biosynthesis of mycobactin or metabolism of *M. bovis*. Table 1 shows that our microarray results were corroborated with the real time

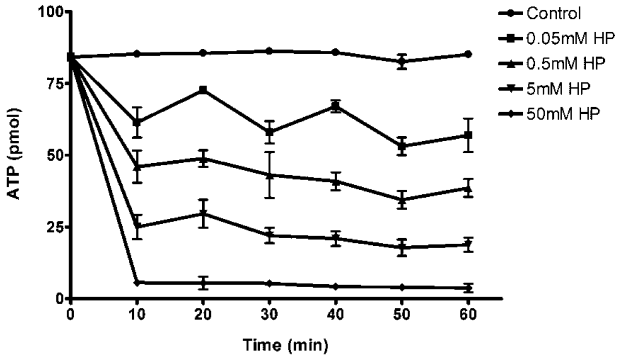

FIGURE 1. Growth Inhibition of *M. bovis* BCG treated with hydrogen peroxide. Changes in the amount of ATP produced by the growth culture of *M. bovis* BCG after treatment with hydrogen peroxide were measured in 10 min intervals for 1 hour. 0 mM, control (●), 0.05 mM (■), 0.5 mM (▲), 5 mM (▼),and 50 mM (◆). The error bars represent the positive and negative standard deviations from the mean of three replicates.

RT PCR analysis, which provides independent verification of transcript level changes of the genes that we discuss in this report. The housekeeping gene, 16s rRNA, was used as an endogenous control.

The real time RT PCR was performed by employing iCycler iQ Real-Time PCR detection system with iScript cDNA synthesis kit and IQ SYBR Green Supermix (BioRad Laboratories, Inc., Hercules, CA). For each gene, three biological replicates with three technical replicates each were employed. Reaction mixtures were initially incubated for 3 min at 95.0 °C, followed by 40 cycles of 10 s at 95.0 °C, 30 s at 55.0 °C, and 20 s at 72.0 °C. PCR efficiencies were derived from standard curve slopes in the iCycler software v. 3.1 (BioRad Laboratories, Inc., Hercules, CA). Melt-curve analysis was also performed to evaluate PCR specificity and resulted in single primer-specific melting temperatures. In this report, relative quantification based on the relative expression of a target gene versus 16s rRNA gene was utilized to determine transcript level changes.

Results and Discussion

Growth Inhibition by Hydrogen Peroxide. To investigate response of *M. bovis* BCG to oxidative stress, we performed a transcriptome analysis with microarrays. We chose a test concentration of 0.5 mM hydrogen peroxide since it caused a strong sublethal growth inhibition for the first 60 min post-treatment (Figure 1).

Transcriptional Profiles in Response to Hydrogen Peroxide. To investigate the effect of time on the transcriptional changes in response to hydrogen peroxide exposure, we isolated total RNA after 10 and 60 min exposure to 0.5 mM hydrogen peroxide and conducted three independent microarray experiments in the absence (control) and the presence (experimental) of 0.5 mM hydrogen peroxide (see Figure 1). To further identify genes with statistically marked changes in expression levels, we applied the following criteria to each of the 10 min, 60 min, and control-experimental microarray data sets: (i) a *p*-value for a 1-way ANOVA should be equal to or less than 0.05, (ii) an absolute fold change in transcript level should be equal to or greater than 1.5, and (iii) a gene should have a presence or marginal call (*37*) from 50% or more replicates on both the experimental and control replicate sets. In this experiment, we utilized Affymetrix custom microarray expression chips for *M. bovis* BCG. Of the 5366 genes represented on the *M. bovis* GeneChip, 230 genes showed statistical significance based on a 1-way ANOVA. We found that mRNA levels of 35 genes of *M. bovis* were significantly altered in response to hydrogen peroxide by 1.5 fold or more upregulation or downregulation. The raw data of 5366 genes control (0 min) and experimental genes after 10 and 60 min exposure to 0.5 mM of hydrogen peroxide) has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE 14272 (Additional file 1).

Analysis of Gene Expression Changes after 10 and 60 min. To examine how genes with transcript level changes were distributed with regard to their functions, we further classified these 35 differentially expressed genes (genes that were upregulated or downregulated by a fold change of 1.5 fold or more) according to the Gene Classification based on COG functional categories in the genome of National Center for Biotechnology information (NCBI) (Table 2).

In Figure 2, the differences between the numbers of up and downregulated genes in each functional class after 10 and 60 min exposure to 0.5 mM of hydrogen peroxide are illustrated. Figure 2 illustrates that the functional classes contained only upregulated genes at 10 min. This result may be linked to the significant initial growth inhibition after 10 min observed in *M. bovis* BCG after treatment with 0.5 mM hydrogen peroxide.

Functional Classifications Analysis. To identify genes with similar transcription patterns during the time course, we classified the 35 differentially regulated genes into four groups on the basis of their transcription directions. Table 2 shows the genes in each group and their *n*-fold changes and *p*-values in response to 10- and 60-min exposures.

Group I: Genes Upregulated upon 10 and 60 min Exposures. Group I consisted of seven genes whose expression levels increased in response to 10 and 60 min of exposure (Table 2). The most dominant class was "inorganic ion transport and metabolism," which possessed three of the genes in group I. Moreover, two of the genes in this class encode proteins that convey cations and iron-carrying compounds. In particular, the gene encoding the ferric uptake regulation protein (*fur*A) and the catalase-peroxidase-peroxynitritase T gene (*kat*G), both belonging to the class of inorganic ion transport and metabolism were upregulated after both treatment times. In both Gram-negative bacteria and some Gram-positive bacteria, the expression of the genes involved in iron uptake is regulated by Fur, a repressor protein that uses ferrous iron as a corepressor (*46*). In low iron conditions, the Fur protein, using Fe^{2+} as a corepressor, binds to a 19-bp operator sequence located upstream of the Fur-regulated genes (*38*−*41*). The presence of an oxidative stress-inducible promoter, immediately upstream of the *furA* gene, has been reported previously for *M. tuberculosis*, *Mycobacterium smegmatis*, and *Mycobacterium bovis* BCG (*14, 42, 43*). The *kat*G gene showed the highest fold increases of 5.5 after 10 min and 2.7 after 60 min and encodes a catalase-peroxidase-peroxynitritase T in *M.* bovis (*14*).

The furA-katG region of *Mycobacterium tuberculosis* is highly conserved among mycobacteria and both genes are induced upon oxidative stress. The promoter of furA is an oxidative stress-responsive promoter controlled by the FurA protein (*44*). FurA is reported to be a specialized iron-dependent regulator that controls the *kat*G gene, which encodes a catalase-peroxidase enzyme involved in the response of *M. tuberculosis* to oxidative stress (*28*). It is known that iron metabolism is coordinately regulated with oxidative stress defenses because iron promotes the formation of hydroxyl radicals, which indiscriminately damage all cellular components (*45*). The induction of *kat*G, therefore, is in line with the need to scavenge hydrogen peroxide to prevent the oxidation of ferrous iron and the production of oxygen radicals through the Fenton reaction (*8, 46*−*48*). The up-regulation of both *fur*A and *kat*G in this study after both treatment times is therefore in line with the theory that the regulation of iron levels and oxidative stress response are interconnected.

TABLE 2. *Mycobacterium bovis* BCG Genes That Showed Statistically Significant mRNA Level Changes upon Either 10 or 60 min Exposure to Hydrogen Peroxide[a]

	10 min		60 min				
gene annotation	*p*-value	fold	*p*-value	fold	description	functional group	
Group I: Upregulation (10 min)−Upregulation (60 min) 7 genes							
BCG_1947c	**0.00124**	**5.5**	**0.00124**	**2.7**	**catalase-peroxidase-peroxynitritase T katG**	**inorganic ion transport and metabolism**	
BCG_1948c	**0.00111**	**4.9**	**0.00111**	**2.3**	**ferric uptake regulation protein furA**	**inorganic ion transport and metabolism**	
BCG_2396c	0.00381	4.5	0.00381	1.9	polyketide synthetase mbtc	secondary metabolites biosynthesis, transport and catabolism	
BCG_2397c	0.00139	3.6	0.00139	2.0	phenyloxazoline synthase mbtb	secondary metabolites biosynthesis, transport and catabolism	
BCG_3009c	0.00589	3.4	0.00589	1.5	putative 3-isopropylmalate dehydratase large subunit leuc	amino acid transport and metabolism	
BCG_1712c	0.0134	1.9	0.0134	1.6	putative transcriptional regulatory protein	inorganic ion transport and metabolism	transcription
BCG_0020	**0.028**	**1.6**	**0.028**	**1.5**	**thioredoxin trxC (TRX) (MPT46)**	**posttranslational modification, protein turnover, chaperones**	**energy production and conversion**
Group II: Upregulation (10 min)−No change (60 min) 11 genes							
BCG_2395c	0.00811	3.2			polyketide synthetase mbtD	secondary metabolites biosynthesis, transport and catabolism	
BCG_3008c	0.0278	2.4			putative 3-isopropylmalate dehydratase smallsubunit leuD	amino acid transport and metabolism	
BCG_2400c	0.00508	2.1			putative isochorismate synthase mbtI	amino acid transport and metabolism	coenzyme transport and metabolism
BCG_2394c	0.0132	2.0			peptide synthetase mbtE	secondary metabolites biosynthesis, transport and catabolism	
BCG_1580c	0.0235	1.9			putative polyketide synthase associated protein papA4		
BCG_2140	0.0156	1.9			PPE family protein		
BCG_0326	0.0246	1.7			PPE family protein		
BCG_1411	0.0272	1.7			putative drugs-transport transmembrane ATP-binding protein ABC transporter	defense mechanism	
BCG_1410	0.00425	1.7			putative drugs-transport transmembrane ATP-binding protein ABC transporter	defense mechanism	inorganic ion transport and metabolism
BCG_0802c	0.00622	1.5			putative 3-hydroxyisobutyrate dehydrogenase mmsB	lipid transport and metabolism	
BCG_1406	0.0207	1.5			putative acyl carrier protein	lipid transport and metabolism	secondary metabolites biosynthesis, transport and catabolism
Group III: No change (10 min)−Upregulation (60 min) 3 genes							
BCG_3227c			0.0205	1.8	putative ATP-dependent DNA helicase	DNA replication, recombination and repair	
BCG_1377c			0.00117	1.6	putative methylated-DNA--protein-cysteine Methyltransferase ogt	DNA replication, recombination and repair	
BCG_3226c			0.00657	1.6	putative ATP-dependent DNA helicase	DNA replication, recombination and repair	
Group IV: No Change (10 min)−Downregulation (60 min) 14 genes							
BCG_0023c			0.011	−1.5	putative chromosome partitioning protein parB	transcription	
BCG_0024c			0.0156	−1.8	putative chromosome partitioning protein parA	cell cycle control, mitosis and meiosis	
BCG_0025c			0.015	−1.5	putative glucose-inhibited division protein B GID	cell wall/membrane biogenesis	
BCG_0119			0.0254	−1.5	putative hydrogenase hycQ	energy production and conversion	inorganic ion transport and metabolism
BCG_0557			0.0169	−1.7	putative transmembrane protein		
BCG_1457c			0.0222	−1.6	PE-PGRS family protein		
BCG_1747			0.0334	−1.6	putative initiation inhibitor protein	cell cycle control, mitosis and meiosis	
BCG_1751			0.00439	−1.6	putative cytidylate kinase cmk	nucleotide transport and metabolism	
BCG_1754			0.021	−1.6	putative 3-hydroxybutyryl-CoA dehydrogenase fadB3	lipid transport and metabolism	
BCG_2969c			0.0419	−1.5	putative fatty-acid-CoA ligase fadD22	lipid transport and metabolism	secondary metabolites biosynthesis, transport and catabolism
BCG_3975c			0.026	−1.6	putative chromosome partitioning protein parB	transcription	
BCG_3976c			0.0283	−1.7	putative chromosome partitioning protein parA	cell cycle control, mitosis and meiosis	
BCG_3977c			0.0134	−1.5	putative glucose-inhibited division protein B gid	cell wall/membrane biogenesis	
BCG_3978c			0.00572	−1.5	hypothetical protein similar to jag protein	general function prediction only	

[a] The genes were grouped based on their regulation directions upon 10 and 60 min exposures. The genes related to oxidative stress are highlighted in gray.

Although the control of the response to oxidative-stress in mycobacteria has not been clearly elucidated, KatG is one of the protective enzymes that has been described (*47*). KatG also plays a role in mycobacterial pathogenesis and is an important virulence factor in *M. tuberculosis* (*42*, *49*), since its expression is required for growth and persistence in the

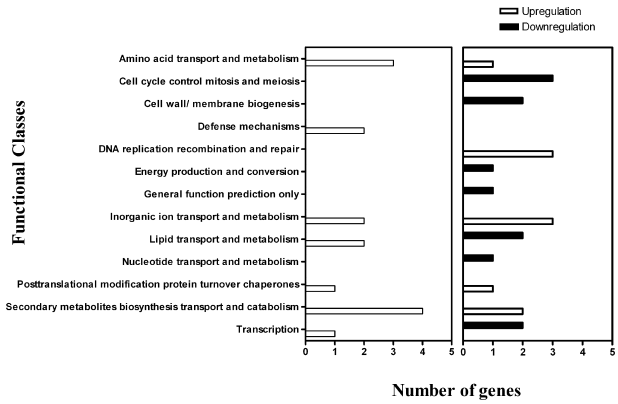

FIGURE 2. Functional classification of genes showing statistically significant upregulation and downregulation in transcription levels after 10 and 60 min exposure to 0.5 mM hydrogen peroxide. Upregulation (mRNA level changes of 1.5 fold or more, empty bars) and downregulation (filled bars).

mouse and guinea pig model systems (*50*). The importance of a better understanding of the elements involved in *kat*G regulation is increased by its implication in the innate susceptibility and acquired resistance to the front-line antituberculosis drug isoniazid (isonicotinic acid hydrazide) (*51, 52*). Indeed, inactivation of *kat*G is frequently found among isoniazid-resistant *M. tuberculosis* strains

Further supporting the theory that iron uptake was enhanced was the upregulation of BCG_2396c (*mbt*C) - BCG_2397c (*mbt*B) after 10 and 60 min. BCG_2396c (*mbt*C) - BCG_2397c (*mbt*B) code for siderophore (mycobactin, iron-chelating compound) biosynthesis proteins (*10*). Siderophore mediated iron uptake is one of the most important mechanisms that bacteria use to acquire iron from the environment (*53*). Further, superoxide, generated during the process of oxygen reduction, releases free iron from iron−sulfur proteins, thus increasing the levels of intracellular free iron (*26*).

Group I also contained one gene associated with post-translational modification, protein turnover, and chaperones in *M. bovis*. Interestingly, this gene encodes the thioredoxin, *trx*C. Thioredoxins are proteins that act as antioxidants by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. Thioredoxins are found in nearly all known organisms and are essential for life in mammals (*54, 55*).

Another notable finding was the presence of BCG_3009c (*leu*C) in group I and BCG_3008c (*leu*D) in group II, which implies that both *leuD* and *leuC* were induced at 10 min. The *leuD* and *leuC* genes encode individual protein subunits that dimerize to form a functional isopropylmalate isomerase (IPMI) heterodimer, involved in leucine biosynthesis (*56*). These two genes have been identified as members of common operons in numerous prokaryotic organisms (*57*) often with additional members of the leucine biosynthesis pathway. Sequence analysis of the *leuC* and *leuD* genes suggested that cysteine residues for iron−sulfur binding (*34*) and other amino acid residues are involved in isomerase activity. Hence, the bacterial and mitochondrial aconitases, IPMI and the iron-responsive-element-binding protein form a family of structurally related proteins (*58, 59*). These relationships raise the possibility that the iron-responsive-element-binding protein may be an IPMI and that the *M. bovis* IPMI may have an iron-responsive regulatory function.

Another possibility could be that iron uptake could be enhanced to ensure proper functioning of enzymes involved in protection against oxidative stress. This result suggests that the induction of these genes may help protect against hydrogen peroxide induced oxidative stress by controlling intracellular iron levels.

Group II: Genes Upregulated upon 10 min Exposure. Group II of Table 2 also shows that several genes related to iron responsive proteins of *M.* bovis were present. For example, BCG_2394c (*mbt*E) - BCG_2395c (*mbt*D) - BCG_2400c (*mbt*I) encodes a polyketide synthetase for siderophore (mycobactin, iron-chelating compound) biosynthesis proteins. Further, BCG_1580c (*pap*A4) encodes a putative polyketide synthase associated protein. The metabolite synthesized by papA4 and pks5 has not yet been identified (*60*).

Group II of Table 2 indicates that the class of "lipid transport and metabolism" was upregulated after 10 min. The BCG_0802c (*mms*B) encodes a putative 3-hydroxy isobutyrate dehydrogenase which catalyzes the oxidation of 3-hydroxyisobutyrate to methylmalonate semialdehyde into biosynthesis of polyketide backbone.

The last and perhaps most striking result is that BCG_1410 and BCG_1411 of group II encode a putative drug-transport transmembrane ATP-binding protein ABC transporter involved classified under the "defense mechanism" functional class. Moreover, the BCG_1410 gene encodes a two region protein; a siderophore-interacting protein (inorganic ion transport and metabolism) and an ABC-type multidrug transport system (defense mechanism). The siderophore-interacting protein, the ViuB protein, is a mycobactin utilization protein which shares the domain structure of ferredoxin reductase like family (*61−64*). This result strengthens confidence in the prior inferences made about the role of iron (which may be sequestered by mycobactins) in the oxidative protection processes.

In this study, hydrogen peroxide also upregulated the gene expression of PE/PPE families of proteins upon 10 min exposure. The PE and PPE families of proteins are acidic glycine-rich proteins encoded by approximately 10% of the genome of *M. tuberculosis* (*65*). Comparative genome sequencing of several mycobacterial species indicates that, PE/PPE gene families are unique to *M. tuberculosis* but there are a few homologues in *M. bovis*, *M. leprae*, *M. marinum*, and other species (*66*). It is widely speculated that PE/PPE families of proteins may be responsible for generating antigenic variation (*67−70*). Serological studies have shown the presence of antibodies specific to some of the proteins from these two classes of proteins (*67, 71*). In addition, another study has reported a role for PE/PPE proteins in the transportation of antimicrobials across the outer membrane of *M. tuberculosis* (*72*). This suggests that PE/PPE proteins may play several unrelated roles in the physiology in different mycobacterial species.

Group III: Genes Upregulated upon 60 min Exposure. Table 2 also shows that several genes related to DNA replication, recombination and repair of *M.* bovis were present in group III. For example, BCG_1377c (*ogt*) encodes a putative methylated DNA protein-cysteine methyltransferase that is a DNA repair gene. BCG_3226c - BCG_3227c encode putative ATP-dependent DNA helicase. In our previous report, DNA repair-related genes of *Pseudomonas aeruginosa* were highly induced in the presence of hydrogen peroxide (*30*). Indeed, it is known that hydrogen peroxide causes oxidative DNA damage by generating hydroxyl or ferryl radicals (*23, 25, 73*). Palma et al. demonstrated that genes of the *P. aeruginosa* SOS regulon exhibit increases in mRNA level upon exposure to 1 mM hydrogen peroxide (*74*). This result suggests that DNA repair mechanisms are selectively induced to maintain DNA integrity for the synthesis of proteins vital for cell survival.

Group IV: Genes Downregulated upon 60 min Exposures. In group IV in Table 2, we noted that genes belonging to the functional class of "cell cycle control, mitosis, and meiosis" were downregulated upon 60 min exposure. Intriguingly, we observed the downregulation of BCG_0023c (*par*B) and BCG_0024c (*par*A). The *par*AB genes encoding partitioning

proteins, i.e., ParA (ATPase) and ParB (DNA-binding proteins) that are components of the segregation machinery (*75*).

The Gid protein encoded by BCG_0025c is a putative glucose-inhibited division protein B which is functionally classified in the 'cell wall/ membrane biogenesis' class in *M. bovis* BCG. Another study showed that there was significant reduction in the hemolytic and cytotoxic activity associated with the cytotoxic enterotoxin (Act) in *Aeromonas hydrophila* mutants containing transposon insertions in the *gid*A gene regions (*76*). Sha et al. demonstrated that the hemolytic activity associated with Act in *Aeromonas hydrophila* was also affected by a number of environment stimuli, such as iron, calcium, pH, temperature, and glucose (*77*). In addition, a ferric uptake regulatory gene (*fur*) has been shown to repress the expression of the *act* gene in the presence of high amounts of iron (*77*).

BCG_1457c which codes for PE_PGRS family protein was downregulated at 60 min (Table 2). The PE_PGRS family protein belong to a subgroup of the PE protein family that have the distinct feature of containing a highly repetitive domain rich in the amino acids glycine and alanine (*65, 78*).

The product of the alkyl hydroperoxide reductase gene (*ahp*C) of *M. tuberculosis* is thought to be involved in protecting the organism against oxidative stress. In a study by Springer et al., contrary to expectations, *ahpC* expression in virulent strains of *M. tuberculosis* and *Mycobacterium bovis* grown in vitro was repressed, often below the level of detection, whereas expression in the avirulent vaccine strain *M. bovis* BCG was constitutively high (*79*). However, the expression of *ahp*C did not change in this study.

In summary, we revealed that iron response genes were selectively upregulated during growth inhibition. This study also revealed how oxidative stress-induced genes were regulated in *M. bovis* BCG. Our results suggest that DNA repair proteins and catalases are among antioxidant defense systems of *M. bovis* BCG for preventing the lethal effects of reactive oxygen intermediates. The downregulation of membrane function-related genes was observed, implying that sublethal oxidative damage may reduce transport through the cell membrane. Our results also show that oxidative stress may affect iron metabolism in that many of Fur-regulated genes were repressed upon exposure to hydrogen peroxide. Further, we showed iron uptake may be induced through the regulation of the *fur* and *kat*G genes, induction of mycobactin biosynthesis and upregulation of biosynthesis of the polyketide backbone through putative drug-transport transmembrane ATP-binding protein ABC transporter after 10 min exposure to hydrogen peroxide. These results suggest that *M. bovis* might undergo an iron acquisition state for protection against oxidative damage upon exposure to hydrogen peroxide. In this study, hydrogen peroxide also upregulated the gene expression of PE/PPE family protein upon 10 min exposure. Further, our results show that the *gid*A gene was downregulated at 60 min, which may affect the Act-associated hemolytic activity in *M. bovis* BCG after hydrogen peroxide exposure.

Acknowledgments

This research is supported by the United States Environmental Protection Agency Grant No. T-83284001-2. Although the research described in this paper has been funded wholly by the United States Environmental Protection Agency, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the EPA; nor does the mention of trade names or commercial products constitute endorsement of recommendation of use.

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ES902255Q