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GENOMICS AND PROTEOMICS

Global transcriptome analysis of the *Mycobacterium bovis* BCG response to sodium hypochlorite

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Abstract Tuberculosis is a common and often deadly infectious disease caused by mycobacteria, mainly *Mycobacterium tuberculosis* and infrequently by other subspecies of the *M. tuberculosis* complex, such as *M. bovis*. Sodium hypochlorite (bleach) is routinely used in hospitals and health care facilities for surface sterilization; however, the modes of action of bleach on *M. bovis* BCG and how this organism develops resistance to sodium hypochlorite have not been elucidated. In this study, we performed a global toxicogenomic analysis of the *M. bovis* response to 2.5 mM sodium hypochlorite after 10 and 20 min. *M. bovis* BCG growth was monitored by measuring the quantity of ATP in picomoles produced over a short exposure time (10–60 min) to sodium hypochlorite. This study revealed significant regulation of oxidative stress response genes of *M. bovis* BCG, such as oxidoreductase, peroxidase, heat shock proteins and lipid transport, and metabolism genes. We interpreted this response as a potentially more lethal interplay between fatty acid metabolism, sulfur metabolism, and oxidative stress. Our results also suggest that sodium hypochlorite repressed transcription of genes involved in cell wall synthesis of *M. bovis*. This study shows that the treatment of *M. bovis* BCG with bleach inhibits the biosynthesis of outer cell wall mycolic acids and also induces oxidative damage.

Keywords Toxicogenomics · Microarray · *Mycobacterium bovis* BCG · Sodium hypochlorite · Bleach

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Introduction

Tuberculosis (TB) is a common and often deadly infectious disease caused by mycobacteria, mainly *Mycobacterium tuberculosis* and infrequently by other subspecies of the *M. tuberculosis* complex, such as *M. bovis*. The 2004 World Health Organization mortality and morbidity statistics show that 14.6 million chronic active TB cases, 8.9 million new cases, and 1.6 million deaths occurred that year, the majority of which happened in developing countries. According to a report from the Centers for Disease Control and Prevention, from 1993 to 2006, the number of TB cases in the USA decreased by 45%, from 25,107 to 13,779. This decline occurred unequally in the USA: among the US-born population, the numbers of cases fell by 66% while the number of cases among foreign-born people in the USA increased by 5% (Cain et al. 2008). Recently, the Bill and Melinda Gates foundation contributed to support the fight against global health threats from infectious diseases such as AIDS, malaria, and TB. Bill Gates has also appealed to the world's industrialized nations to show an increased commitment to the fight against the global threat of infectious diseases (Pasterkamp 2001).

TB remains a major potential threat to public health, although many antituberculosis drugs have been developed over the past 30 years. Many countries use the *M. 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*.

Although drugs that target actively growing *M. tuberculosis* are available, their efficacy is compromised by the lengthy treatment times (Pym and Cole 1999) and the increasing problem of multidrug resistance (Espinal 2003). An additional problem is the ability of *M. tuberculosis* to enter a nonreplicating, latent state after engulfment by activated macrophages in the lung (Gomez and McKinney 2004). It is estimated that one third of the world's population harbors a latent infection of this kind and is at risk of reactivation of disease (Cahn et al. 2003).

The majority of the disinfectants that have been approved to be registered by the US Environmental Protection Agency (EPA) exploit chlorination for their antimicrobial action (Chang et al. 2007; Small et al. 2007a, b). Sodium hypochlorite (bleach) is routinely used in hospitals and health care facilities for surface sterilization; however, the mechanism of action by which this disinfectant kills and the extent to which *M. bovis* is resistant to sodium hypochlorite have not been elucidated.

In addition to confirming upregulation of oxidative stress response genes, this study revealed significant and coincident regulation of oxidative response genes of *M. bovis* BCG, such as oxidoreductase, *ahp*CD, *kat*G, *hsp*, and lipid transport and metabolism genes. We interpreted this response as a potentially more lethal interplay between fatty acid metabolism, sulfur metabolism, and oxidative stress.

Materials and methods

Bacterial strains and growth conditions

M. bovis BCG was obtained from the American Type Culture Collection (ATCC 35748). Middlebrook 7H9 Broth dehydrated base (which may be supplemented with either glycerol or polysorbate 80) in combination with Middlebrook ADC Enrichment and Middlebrook 7H9 Broth prepared tubes (containing ADC Enrichment) when supplemented with glycerol (2 ml/l) support the growth of mycobacteria including *M. tuberculosis* (http://www.bd.com/ds/technicalCenter/inserts/difcoBblManual.asp). Two hundred milliliters of growth medium in a 1-L Erlenmeyer flask was prepared as follows: 40 ml 5 M Middlebrook 7H9 broth (Difco, Sparks, MO, USA) supplemented with 20 ml OADC (oleic acid, albumin, dextrose, catalase; BBL Co., Franklin Lakes, NJ) and 140 ml distilled water (Dosanjh et al. 2005;Graham and Clark-Curtiss 1999). Two hundred microliters of Tween 80 (Sigma-Aldrich Co., St. Louis, MO) was added to the growth medium to prevent clumping of the bacteria. The *M. bovis* BCG stock culture (ATCC 35748) was inoculated into the Middlebrook 7H9 broth and 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* BCG stock culture was inoculated into the M7H9 growth medium 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 (Chang et al. 2007; Jang et al. 2008; Small et al. 2007b) containing 0.1% Tween 80 and incubated for 24 h at 37°C. In the meantime, 10 ml of cells was transferred into 200 ml of new M7H9 medium for continuous culture.

Disinfectant treatment and ATP measurements

We used the BactTiter-Glo™ assay (Promega Co.) to rapidly assess cell viability of *M. bovis* BCG. This assay is based on quantifying the adenosine triphosphate (ATP) present in bacterial cultures to determine the number of live cells. In principle, the assay generates a glow-type luminescent signal produced by the luciferase reaction. In this study, luminescent signals generated by the bacterial culture were detected and quantified by a luminometer (Glomax Promega Co.). This assay was easy to perform, highly sensitive, and is a rapid and convenient technique for monitoring the growth of mycobacterium. The time needed to obtain a disinfectant susceptibility pattern was reduced to less than 1 week as compared with 4 weeks with conventional methods. The BacTiter-Glo buffer (Promega Co., San Luis Obispo, CA) and lyophilized substrate were thawed and equilibrated to room temperature and dispensed into designated control wells. Sterile phosphate-buffered saline (PBS) containing untreated cells was added to control wells, and luminescence was measured using the Glomax™ luminometer (Promega Co., San Luis Obispo, CA).

One milliliter of the prepared *M. bovis* stock culture was added to the M7H9 medium and incubated at 37°C with shaking at 200 rpm to reach an OD600 of 0.3–0.4 after 5 days. Due to the slow growing nature of *M. bovis* BCG, changes in the amounts of ATP produced by peracetic acid-treated cells were utilized to monitor the changes in the number of viable cells. However, catalase present in OADC interferes with ATP measurements. As such, cells grown in M7H9 were harvested and resuspended in 200 ml of LB broth containing 0.1% Tween 80 and incubated for 24 h at 37°C to reach an OD600 of 0.3–0.6. By using LB broth, the

amount of ATP produced by cells (control) and cells exposed to sodium hypochlorite could be reliably quantified and used to monitor the changes in the growth of sodium hypochlorite-treated *M. bovis* BCG over a short period of time (10-min intervals for 1 h). The amount of ATP produced by cells exposed to sodium hypochlorite was measured using the BacTiter Glo™ microbial cell viability assay and the Glomax™ luminometer (Promega Co., San Luis Obispo, CA). The BacTiter Glo™ assay measures the amount of luminescence generated by the cells being tested in relative light units (RLU). In order to correlate the amount of luminescence with the quantity of ATP produced in picomoles (pmol), serial dilutions of 100 pmol ATP (Promega Co., San Luis Obispo, CA) were performed and the corresponding RLU were determined using the BacTiter Glo™ microbial cell viability assay. From these two sets of ATP concentrations, a standard curve relating the amount of luminescence (RLU) and the corresponding amount of ATP in picomoles was obtained (Supplementary Fig. 1).

All steps were performed at room temperature. The BacTiter-Glo buffer (Promega Co., San Luis Obispo, CA) and lyophilized substrate were thawed and equilibrated at room temperature for 30 min before use. The buffer and substrate were mixed to obtain a homogenous solution. A 100-μL aliquot of buffer–substrate solution was added to designated control wells of a 96-well plate. To obtain a value for background luminescence before disinfectant treatments, 1 ml of reaction mixture in the 50-ml reaction tube in which sodium hypochlorite was replaced by water (in LB broth as described previously) was spun down. The pellet was resuspended in 1 ml 1x PBS (Invitrogen, Carlsbad, CA) and centrifuged. The pellet was resuspended in 200 μl PBS. A 100-μl aliquot of the resuspended pellet of untreated cells was added to control wells containing the buffer–substrate mixture, and the luminescence was measured. LB growth cultures (40 ml) were dispensed into designated 50-ml tubes, and 11 M sodium hypochlorite stock was added to the cultures to reach several final concentrations: 0, 2.5, 2.75, and 3.0 mM. At 10-min intervals for up to 1 h, ATP measurements were performed for the different sodium hypochlorite concentrations as described for the untreated cells.

RNA isolation

Total RNA was isolated from *M. bovis* not treated with sodium hypochlorite (control) and from *M. bovis* treated with 2.5 mM sodium hypochlorite for 10 and 20 min using the RiboPure bacteria kit (Ambion, Inc., Austin, TX; Jang et al. 2008). The mycobacterial cells treated with sodium hypochlorite 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 for 2 min after each beating period. 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 (Chang et al. 2005, 2006a, b; Jang et al. 2008).

Affymetrix *M. bovis* BCG custom genechip analysis

The custom array was constructed by Affymetrix using the sequence of *Mycobacterium bovis* BCG strain Pasteur 1173P2: No. of probe pair sets, 3,977 (cds); 1389 (ig). Sixteen probes per gene (desired number) with a minimum of eight probes per gene were used for GeneChip construction. 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 (sodium hypochlorite-treated) for 10- and 20-min exposures.

Real-time PCR analysis

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

To determine the validity of the array data, transcript-level changes obtained with the microarray analysis were compared with those from quantitative real-time PCR. Genes and primer sequences employed for the real-time PCR analysis are listed in Table 1. The housekeeping gene, 16 s rRNA, was used as an endogenous control. The real-time 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

Table 1 Transcript level comparison of *Mycobacterium bovis* BCG genes between real-time PCR and microarray analyses. The real time PCR results are the mean of three biological replicates with three technical replicates for each gene

Gene	mRNA level change with microarray[a]		mRNA level change with real-time PCR[b]		Sense primer sequence	Antisense primer sequence
	Fold change		Fold change			
	10min	20min	10min	20min		
BCG_1108	33.2	25.1	20.1(± 0.1)	20.5(± 0.0)	5′- GTT TGG TCG CAT TGA CGT CGT GTT -3′	5′- TTG CTG CTT CAT GAT CGG CAA GAC -3′
BCG_1533	3.7	4.8	1.4(± 0.4)	2.6(± 0.2)	5′- TCA CGT GCG TTC GAG ATC ATG TTG -3′	5′- TCC ACA GCG TAC GTT TGG TCA ACT -3′
BCG_0020	4.8	7.1	2.2(± 0.1)	5.7(± 0.2)	5′- AAA GTT ACC GAC GCA TCC TTT GCC -3′	5′- TAC CAT CTT GCA AGG TCC ACA CCA -3′
BCG_3043[c]	2.0	2.8	2.6(± 0.3)	4.2(± 0.0)	5′- ATA TTC CGC AGT TGA TCG CTT CGC -3′	5′- GCG CGA TAT CCA GCA AGG TAT TGA -3′
BCG_2447		2.3		2.4(± 0.2)	5′- CCA CTG CTA ACC ATT GGC GAT CAA -3′	5′- AGG GCA CAC GAA CGT GAA GTC TTT -3′
BCG_2448		2.5		2.0(± 0.1)	5′- ACG CCA AAG ACA TCA AGC TGA ACC -3′	5′- GCT TCA GCG CCA ATG TCA GCT AAT -3′
BCG_0280[c]	−2.3	−2.4	−5.9(± 0.3)	−4.3(± 0.1)	5′- AGG ACT ACG ACC TGG TAG GAA ACA -3′	5′- TGC GCA GTA CCG GAG TAA AGA ACT -3′
BCG_3162	−2.2	−2.1	−6.7(± 0.2)	−3.6(± 0.1)	5′- ACG AAG CCA GCG ACA ACG ACT ATT -3′	5′- CGA AAG CCT GGC GTT GTT TCA CAT -3′
BCG_0877[c]		−2.5		−1.9(± 0.1)	5′- TGC CGG TAC TGA AGA AAT GGC GTA -3′	5′- GTT GCT TGG ACA CCT CGA ACT TGT -3′
BCG_1154		−2.9		−2.6(± 0.1)	5′- ATG CGA GAT CCT GCT GAT CCT CAA -3′	5′- CCA CCA ATC CTC GAG TAT GAA GTG CT -3′
16S rRNA[c]	1.00	1.00	1.00	1.00	5′- TGC AAG TCG AAC GGA AAG GTC TCT -3′	5′- AAG ACA TGC ATC CCG TGG TCC TAT -3′

[a] The microarray results are the mean of three replicates of each gene

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

[c] Internal control: 16S rRNA

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

Growth inhibition by sodium hypochlorite

To study the effect of NaOCl-induced stress on *M. bovis*, we performed a transcriptome analysis upon exposure to sodium hypochlorite by using whole-genome microarrays. *M. bovis* was exposed to 2.5 mM sodium hypochlorite because we confirmed that this concentration caused sublethal inhibition on cell growth for the first 10 min posttreatment (see Fig. 1). This concentration was selected also based on the fact that US Food and Drug Administration (2003) regulations recommend a maximum concentration of 2.7 mM of sodium hypochlorite for disinfecting food processing equipment and food contact surfaces (Chang et al. 2007).

In this study, to better understand how *M. bovis* initially responds to 2.5 mM sodium hypochlorite, we chose to perform a toxicogenomic analysis after 10- and 20-min exposure times compared with control (without sodium hypochlorite; Fig. 1).

Transcriptional profiles in response to sodium hypochlorite

To investigate early transcriptional changes in response to sodium hypochlorite exposure, we isolated total RNA after

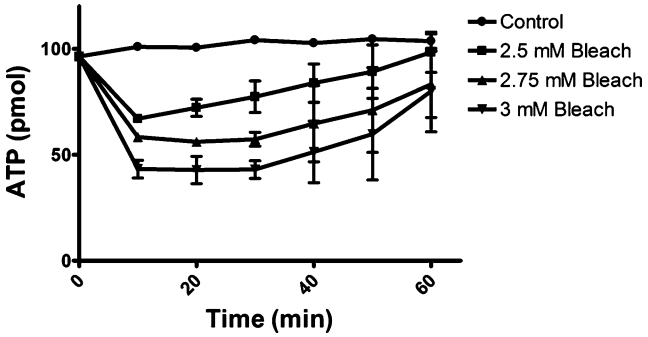

Fig. 1 Growth inhibition of *M. bovis* BCG by sodium hypochlorite over 60 min. ATP measurements in picomoles were monitored in 10-min intervals. The sodium hypochlorite concentrations were as follows: 0 mM, control (*filled circle*), 2.5 mM (*filled square*), 2.75 mM (*filled triangle*), and 3 mM (*inverted filled triangles*)

10 and 20 min exposure to 2.5 mM sodium hypochlorite and conducted three independent microarray experiments in the absence (control) and the presence (experimental) of 2.5 mM sodium hypochlorite (see Fig. 1). To further identify genes with statistically marked changes in expression levels, we applied the following criteria to each of the 10-min, 20-min, and control-experimental microarray data sets: (1) a p value for a 1-way analysis of variance (ANOVA) should be equal to or less than 0.05, (2) an absolute change, in multiples, in transcript level should be equal to or greater than 2, and (3) a gene should have a presence or marginal call (Affymetrix 2004) from 50% or more replicates on both the experimental and control replicate sets. In this experiment, we ordered custom microarray expression chips for *M. bovis* BCG to Affymetrix. Of the 5,366 genes represented on the *M. bovis* GeneChip, 1,399 genes showed statistical significance based on a 1-way ANOVA. We found that at existing mRNA levels, 261 genes of *M. bovis* BCG were significantly altered in response to sodium hypochlorite by a change in multiples of two or more upregulation or downregulation. The raw data of 5,366 control (0 min) and experimental genes (after 10- and 20-min exposures to 2.5 mM of sodium hypochlorite) have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus and are accessible through GEO Series accession number GSE 13423.

Analysis of gene expression changes in 10-min and 20-min exposures

To examine how genes with transcript level changes are distributed with regard to their functions, we further classified these 261 genes that were either upregulated or downregulated by a change in multiples of two or more according to the Gene Classification based on COG functional categories in the genome of NCBI.

In Fig. 2, the differences between the numbers of up- and downregulated genes in each functional class after 10- and 20-min exposures to 2.5 mM of sodium hypochlorite

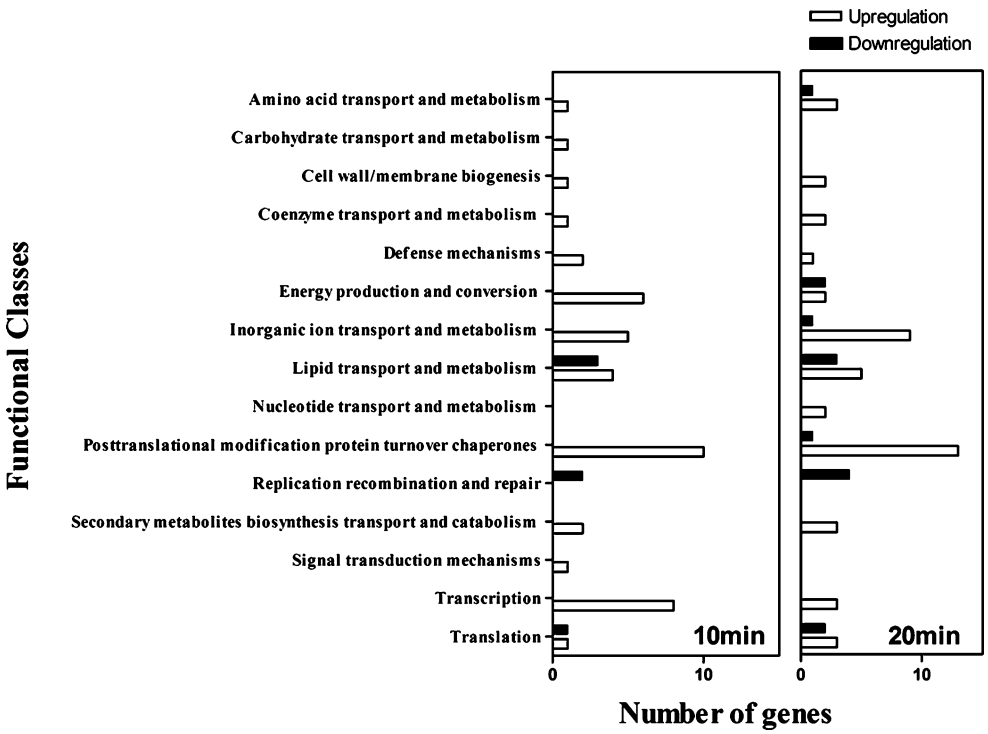

Fig. 2 Function classification of number of genes with statistically significant upregulation and downregulation in mRNA level. Functional classification of genes with upregulation (*empty bar*) and downregulation (*filled bar*) mRNA level changes of twofold or more after 10- and 20-min exposures to 2.5 mM sodium hypochlorite

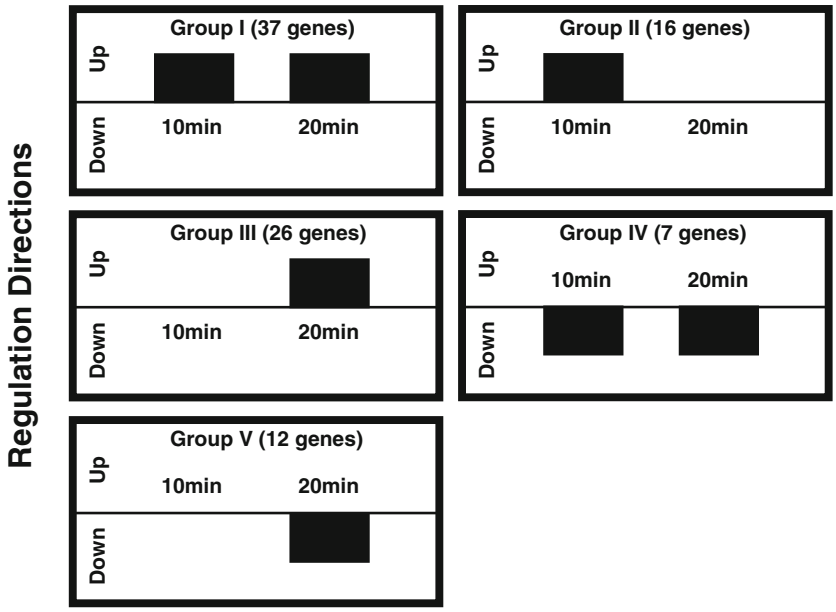

Fig. 3 Groups of differentially regulated 98 genes with known functional class, which are categorized by their transcription directions upon 10- and 20-min exposures. Group I contained 37 genes upregulated upon both exposure times while group II had 16 genes upregulated at 10 min and had no significant changes upon 20-min exposure. Further, group III possessed 26 genes that were upregulated in response to 20-min exposure. Group IV contained seven genes downregulated upon both exposure times. Finally, group V had 12 genes that exhibited downregulation after 20 min exposure

are illustrated. Note that Fig. 2 represents a total of 98 genes excluding the group of "function unknown, hypothetical protein and intergenic regions" (163 genes). Some interesting findings are as follows: (1) more genes in the class of "Energy production and conversion" were significantly downregulated after 20-min exposure compared with after 10-min exposure; (2) the number of genes involved in inorganic ion transport and metabolism and posttranslational modification, protein turnover, and chaperones were increased after 20-min exposure compared with after 10-min exposure. In general, Fig. 2 illustrates that the functional classes contained more downregulated genes at 20-min exposure compared with 10-min exposure. This result suggests that the functional class profiles were notably different at 20-min exposure compared with 10-min exposure, and this difference might explain why *M. bovis* BCG underwent initial growth inhibition followed by partial growth recovery upon exposure to sodium hypochlorite.

Functional classifications analysis

As expected, sodium hypochlorite treatment triggered the expression of genes involved in the antioxidant adaptation and protection process, which are conserved among Eubacteria. In bacteria, sodium hypochlorite has been shown to elicit responses similar to those produced by hydrogen peroxide. Bacterial treatment with these oxidative chemicals causes the generation of superoxide anions (oxygen singlets) and hydroxyl radicals, which are presumed to account for the major bactericidal activity (Albrich and Hurst 1982; Candeias et al. 1993; Dukan and Touati 1996; Imlay and Linn 1986; Khan and Kasha 1994a, b; hence, it has been speculated that sodium hypochlorite functions by similar mechanisms as other oxidizing agents (Miller and Britigan 1997).

To further identify genes with similar transcription patterns during the time course, we categorized the 98 genes with known functions into five groups on the basis of their transcription directions (Fig. 3). Figure 3 displays the number of genes (98) within groups I through V.

Discussion

We constructed Table 2 with the 98 *M. bovis* BCG genes that were most strongly upregulated or downregulated in response to sodium hypochlorite after 10- and 20-min exposures. These genes were also divided into five groups based on their transcription directions.

Group I: genes upregulated upon 10- and 20-min exposures

Group I of Table 2 contains eight genes associated with posttranslational modification, protein turnover, and chaperones in *M. bovis*. Interestingly, five of these genes encode the thioredoxin, *trx*B, or *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 (Holmgren 1989; Nordberg and Arner 2001). Very significantly, oxidoreductase showed the highest multiple in increases of 33.2 after 10 min and 25.1 after 20 min in this experiment.

Table 2 *Mycobacterium bovis* BCG genes that showed statistically significant mRNA level changes upon either 10- or 20-min exposure to sodium hypochlorite

Affymetrix Probe ID	ORF no.	10min		20min		Description	Gene symbol	Functional class	
		p value	Fold change	*p* value	Fold change				
Group I: Upregulation (10 min) -upregulation (20 min) 37 genes									
MBOV0704S00001096_at	BCG_1108	4.25E–05	33.2	4.25E–05	25.1	Putative oxidoreductase		Lipid transport and metabolism	Secondary metabolites biosynthesis, transport, and catabolism
MBOV0704S00000362_at	BCG_0370	1.93E–06	13.2	1.93E–06	19.5	Putative dehydrogenase/reductase		General function prediction only	
MBOV0704S00001518_at	BCG_1532	0.000287	10.9	0.000287	14.8	Putative thioredoxin trxB1	*trx*B1	Posttranslational modification, protein turnover, chaperones	Energy production and conversion
MBOV0704S00001095_at	BCG_1107	4.27E–07	18.0	4.27E–07	13.9	Putative transcriptional repressor protein		Transcription	
MBOV0704S00003175_at	BCG_3198	1.76E–05	8.3	1.76E–05	9.1	Putative short-chain dehydrogenase/reductase		Lipid transport and metabolism	Secondary metabolites biosynthesis, transport, and catabolism
MBOV0704S00000017_s_at	BCG_0020	3.04E–05	4.8	3.04E–05	7.1	Thioredoxin trxC (TRX) (MPT46)	*trx*C	Posttranslational modification, protein turnover, chaperones	Energy production and conversion
MBOV0704S00003942_s_at	BCG_3972	8.46E–06	4.7	8.46E–06	7.0	Thioredoxin trxC	*trx*C	Posttranslational modification, protein turnover, chaperones	Energy production and conversion
MBOV0704S00003941_s_at	BCG_3971	0.000146	5.0	0.000146	6.5	Putative thioredoxin reductase trxB2	*trx*B2	Posttranslational modification, protein turnover, chaperones	
MBOV0704S00000016_s_at	BCG_0019	0.000202	4.9	0.000202	6.4	Putative thioredoxin reductase trxB2 (TRXR)	*trx*B2	Posttranslational modification, protein turnover, chaperones	
MBOV0704S00000358_at	BCG_0366c	0.00104	2.6	0.00104	5.8	Putative cytochrome P450 135A1 cyp135A1	*cyp*135A1	Secondary metabolites biosynthesis, transport and catabolism	
MBOV0704S00002623_at	BCG_2644c	0.000214	4.7	0.000214	5.7	Putative transmembrane protein			
MBOV0704S00001519_at	BCG_1533	0.00166	3.7	0.00166	4.8	Putative enoyl-CoA hydratase echA12	*ech*A12	Lipid transport and metabolism	
MBOV0704S00003209_at	BCG_3232c	0.00299	3.4	0.00299	4.4	Putative molybdenum cofactor biosynthesis protein moeB1	*moe*B1	Coenzyme transport and metabolism	Inorganic ion transport and metabolism
MBOV0704S00001698_at	BCG_1712c	6.83E–05	2.8	6.83E–05	4.4	Putative transcriptional regulatory protein		Transcription	Inorganic ion transport and metabolism
MBOV0704S00000414_at	BCG_0422c	0.00101	2.1	0.00101	4.4	Putative endopeptidase ATP binding protein (chain b) clpB	*clp*B	Posttranslational modification, protein turnover, chaperones	
MBOV0704S00002122_at	BCG_2140	0.000115	2.6	0.000115	3.7	PPE family protein	*ppe*37		

Table 2 (continued)

Affymetrix Probe ID	ORF no.	10min		20min		Description	Gene symbol	Functional class	
		p value	Fold change	*p* value	Fold change				
MBOV0704S00002395_at	BCG_2413c	0.000213	3.2	0.000213	3.6	Putative sulfate-transport integral membrane protein ABC transporter cysW	*cys*W	Inorganic ion transport and metabolism	
MBOV0704S00001758_at	BCG_1772c	0.00492	3.6	0.00492	3.5	Putative transmembrane protein			
MBOV0704S00002394_at	BCG_2412c	0.000475	2.9	0.000475	3.4	Putative sulfate-transport ATP-binding protein ABC transporter cysA1	*cys*A1	Inorganic ion transport and metabolism	
MBOV0704S00003176_at	BCG_3199	0.0016	2.5	0.0016	3.0	Putative amidase		Translation	
MBOV0704S00000356_at	BCG_0364	2.99E–05	2.7	2.99E–05	3.0	putative transcriptional regulatory protein (possibly arsR-family)		Transcription	Inorganic ion transport and metabolism
MBOV0704S00003018_at	BCG_3041c			0.0252	3.0	PE family protein	PE27A		
MBOV0704S00001566_at	BCG_1580c	0.000499	2.1	0.000499	2.9	Putative polyketide synthase associated protein papA4	*pap*A4		
MBOV0704S00001998_at	BCG_2014	0.00255	3.4	0.00255	2.8	Putative metal cation transporter P-type atpase A ctpF	*ctp*F	Inorganic ion transport and metabolism	
MBOV0704S00003020_at	BCG_3043c	0.00312	2.0	0.00312	2.8	PE family protein			
MBOV0704S00002631_at	BCG_2652c	0.00185	3.5	0.00185	2.8	Putative transmembrane alanine and leucine rich protein			
MBOV0704S00002396_at	BCG_2414c	0.00144	2.4	0.00144	2.7	Putative sulfate-transport integral membrane protein ABC transporter cysT	*cys*T	Posttranslational modification, protein turnover, chaperones	
MBOV0704S00001264_at	BCG_1278c	0.00051	2.7	0.00051	2.4	Putative tetronasin-transport ATP-binding protein ABC transporter		Defense mechanisms	
MBOV0704S00002397_at	BCG_2415c	0.0193	2.1	0.0193	2.3	Putative sulfate-binding lipoprotein subI	*sub*I	Inorganic ion transport and metabolism	
MBOV0704S00001235_at	BCG_1249	0.00647	2.5	0.00647	2.3	Putative pyrroline-5-carboxylate dehydrogenase rocA	*roc*A	Energy production and conversion	
MBOV0704S00003761_at	BCG_3788	0.012	2.7	0.012	2.3	Putative two-domain membrane protein		Amino acid transport and metabolism	Inorganic ion transport and metabolism
MBOV0704S00002740_at	BCG_2761c	0.000941	2.1	0.000941	2.2	Putative transcriptional regulatory protein		Transcription	
MBOV0704S00002739_at	BCG_2760c	0.000147	2.1	0.000147	2.2	Conserved 35 kda alanine rich protein	35kd_ag	Transcription	Signal transduction mechanisms
MBOV0704S00002685_at	BCG_2706c	1.50E–05	2.3	1.50E–05	2.2	Putative integral membrane alanine and leucine rich protein			
MBOV0704S00003773_at	BCG_3801c	0.00104	2.6	0.00104	2.1	Putative oxidoreductase		Inorganic ion transport and metabolism	
MBOV0704S00002369_at	BCG_2387c	0.00208	2.1	0.00208	2.0	Putative chaperone protein dnaJ2	*dna*J2	Posttranslational modification, protein turnover, chaperones	
MBOV0704S00002056_at	BCG_2072c	0.000978	2.0	0.000978	2.0	Putative transmembrane protein	*fxs*A		

Group II: Upregulation (10 min) -no change (20 min) 16 genes								
MBOV0704S00003760_at	BCG_3787	0.00774	3.2		Putative oxidoreductase			
MBOV0704S00002032_at	BCG_2048c	0.0128	2.7		Putative phosphofructokinase pfkB	*pfk*B	Carbohydrate transport and metabolism	
MBOV0704S00000111_at	BCG_0114	0.00367	2.6		Putative transcriptional regulatory protein		Transcription	
MBOV0704S00001265_at	BCG_1279c	0.000851	2.5		Putative transcriptional regulatory protein		Transcription	
MBOV0704S00003523_at	BCG_3551c	0.0146	2.4		Putative esterase/lipase lipF	*lip*F	Lipid transport and metabolism	
MBOV0704S00003255_at	BCG_3278c	0.00339	2.4		Putative transcriptional regulatory protein (probably tetR-family)		Transcription	
MBOV0704S00000112_at	BCG_0115	0.0128	2.3		Putative oxidoreductase		Energy production and conversion	
MBOV0704S00001263_at	BCG_1277c	5.93E–05	2.3		Putative tetronasin-transport integral membrane protein ABC transporter		Cell wall/membrane biogenesis	
MBOV0704S00003257_at	BCG_3280c	0.00182	2.2		Putative rubredoxin rubA	*rub*A	Energy production and conversion	
MBOV0704S00003256_at	BCG_3279c	0.00262	2.2		Putative rubredoxin rubB	*rub*B	Energy production and conversion	
MBOV0704S00002008_at	BCG_2024c	0.0036	2.1		Putative ferredoxin fdxA	*fdx*A	Energy production and conversion	
MBOV0704S00000113_at	BCG_0116	0.0308	2.1		Putative oxidoreductase		Energy production and conversion	Inorganic ion transport and metabolism
MBOV0704S00001030_at	BCG_1041	0.032	2.1		Putative adhesion component transport ATP-binding protein ABC transporter		Defense mechanisms	
MBOV0704S00002034_at	BCG_2050c	0.000263	2.0		Heat shock protein hspX	*hsp*X	Posttranslational modification, protein turnover, chaperones	
MBOV0704S00003224_s_at	BCG_3247c	0.000183	2.0		Putative two component sensor kinase		Signal transduction mechanisms	
MBOV0704S00001262_at	BCG_1276c	0.00724	2.0		Putative integral membrane protein		Posttranslational modification, protein turnover, chaperones	
Group III: No change (10 min) -upregulation (20 min) 26 genes								
MBOV0704S00000281_at	BCG_0289c			0.00112	2.6	Heat shock protein hsp	*hsp*	Posttranslational modification, protein turnover, chaperones
MBOV0704S00000856_at	BCG_0867c			0.00891	2.6	Putative thiosulfate sulfurtransferase cysA2	*cys*A2	Inorganic ion transport and metabolism
MBOV0704S00002453_at	BCG_2473c			0.00484	2.5	Putative molybdopterin-guanine dinucleotide biosynthesis protein A mobA	*mob*A	Coenzyme transport and metabolism
MBOV0704S00002428_at	BCG_2448			0.00315	2.5	Alkyl hydroperoxide reductase D protein ahpD	*ahp*D	
MBOV0704S00003054_at	BCG_3077c			4.33E–06	2.4	Putative glutaredoxin electron transport component of nrdEF (glutaredoxin-like protein) nrdH	*nrd*H	Posttranslational modification, protein turnover, chaperones

Table 2 (continued)

Affymetrix Probe ID	ORF no.	10min		20min		Description	Gene symbol	Functional class	
		p value	Fold change	*p* value	Fold change				
MBOV0704S00003019_at	BCG_3042c			0.000268	2.4	Putative secreted ESAT-6 like protein 9 esxR	*esx*R		
MBOV0704S00003318_s_at	BCG_3342c			0.000415	2.4	Putative anti-sigma factor		Amino acid transport and metabolism	Inorganic ion transport and metabolism
MBOV0704S00002427_at	BCG_2447			0.00207	2.3	Alkyl hydroperoxide reductase C protein ahpC	*ahp*C	Posttranslational modification, protein turnover, chaperones	
MBOV0704S00003226_s_at	BCG_3249c			0.000268	2.3	Putative anti-sigma factor		Amino acid transport and metabolism	Inorganic ion transport and metabolism
MBOV0704S00003052_at	BCG_3075c			0.0323	2.3	Putative ribonucleoside-diphosphate reductase (alpha chain) nrdE	*nrd*E	Nucleotide transport and metabolism	
MBOV0704S00003053_at	BCG_3076c			0.00202	2.3	Putative nrdI protein	*nrdI*	Nucleotide transport and metabolism	
MBOV0704S00000018_s_at	BCG_0021			0.00101	2.3	Putative hydrolase		Cell wall/membrane biogenesis	
MBOV0704S00003017_at	BCG_3040c			0.0014	2.3	PPE family protein	PPE46		
MBOV0704S00003943_s_at	BCG_3973			0.000989	2.2	Putative hydrolase		Cell wall/membrane biogenesis	
MBOV0704S00001993_at	BCG_2009c			0.0192	2.2	Putative metal cation transporter P-type atpase G ctpG	*ctp*G	Inorganic ion transport and metabolism	
MBOV0704S00000381_at	BCG_0389			0.00254	2.2	Putative chaperone protein dnaK	*dna*K	Posttranslational modification, protein turnover, chaperones	
MBOV0704S00000297_at	BCG_0305			0.0149	2.1	Putative integral membrane nitrite extrusion protein narU	*nar*U	Inorganic ion transport and metabolism	
MBOV0704S00003317_s_at	BCG_3341c			0.000727	2.1	Biotinylated protein TB7.3		Lipid transport and metabolism	
MBOV0704S00002379_at	BCG_2397c			0.0136	2.1	Phenyloxazoline synthase mbtB	*mbt*B	Secondary metabolites biosynthesis, transport, and catabolism	
MBOV0704S00002524_at	BCG_2545c			0.00371	2.1	Putative fatty acid synthase fas	*fas*	Lipid transport and metabolism	Secondary metabolites biosynthesis, transport, and catabolism
MBOV0704S00003225_s_at	BCG_3248c			0.00102	2.1	Biotinylated protein TB7.3		Lipid transport and metabolism	
MBOV0704S00003016_at	BCG_3039c			0.00113	2.1	Putative ESAT-6 like protein 8 esxQ	*esx*Q		
MBOV0704S00003859_at	BCG_3887c			0.000466	2.0	Putative polyketide synthase associated protein papA1	*pap*A1	Secondary metabolites biosynthesis, transport, and catabolism	
MBOV0704S00001931_at	BCG_1947c			0.00483	2.0	Catalase-peroxidase-peroxynitritase T katG	*kat*G	Inorganic ion transport and metabolism	

MBOV0704S00002738_at	BCG_2759c			0.000279	2.0	Putative membrane alanine rich protein			
MBOV0704S00002454_at	BCG_2474c			0.0066	2.0	Putative oxidoreductase subunit beta		Energy production and conversion	
Group IV: Downregulation (10 min) -downregulation (20 min) seven genes									
MBOV0704S00000864_at	BCG_0876c	0.000324	−2.5	0.000324	−3.9	Putative transcriptional regulatory protein		Translation	
MBOV0704S00002892_at	BCG_2915c	0.000902	−2.5	0.000902	−2.7	Putative integrase/recombinase xerC	*xer*C	Replication, recombination and repair	
MBOV0704S00000272_at	BCG_0280c	2.27E-05	−2.3	2.27E-05	−2.4	Putative 3-oxoacyl-[acyl-carrier protein] reductase fabG4	*fab*G	Lipid transport and metabolism	Secondary metabolites biosynthesis, transport, and catabolism
MBOV0704S00002596_at	BCG_2617c	0.000485	−2.0	0.000485	−2.3	Putative crossover junction endodeoxyribonuclease ruvC	*ruv*C	Replication, recombination and repair	
MBOV0704S00001876_at	BCG_1892c	0.00217	−2.1	0.00217	−2.1	Putative oxidoreductase		Lipid transport and metabolism	Secondary metabolites biosynthesis, transport, and catabolism
MBOV0704S00003139_at	BCG_3162	0.00142	−2.2	0.00142	−2.1	Putative acyl-CoA dehydrogenase fadE24	*fad*E24	Lipid transport and metabolism	
MBOV0704S00000208_at	BCG_0216c	0.016	−2.1			Putative lipoprotein lprO	*lpr*O		
Group V: No change (10 min) -downregulation (20 min) 12 genes									
MBOV0704S00003095_at	BCG_3118c			0.00185	−3.2	Putative oxidoreductase		Energy production and conversion	
MBOV0704S00001142_at	BCG_1154			0.000351	−2.9	Putative acyl-[acyl-carrier protein] desaturase desA2	*des*A2		
MBOV0704S00000865_at	BCG_0877c			0.00163	−2.5	Putative acyl-[acyl-carrier protein] desaturase desA1	*des*A1		
MBOV0704S00001443_at	BCG_1457c			0.00278	−2.4	PE-PGRS family protein	PE-PGRS25		
MBOV0704S00002595_at	BCG_2616c			0.00181	−2.4	Putative holliday junction DNA helicase ruvA	*ruv*A	Replication, recombination and repair	
MBOV0704S00001227_at	BCG_1241			0.00196	−2.4	Putative aminotransferase		Amino acid transport and metabolism	
MBOV0704S00003889_at	BCG_3917c			0.0226	−2.3	Monooxygenase ethA	*eth*A	Inorganic ion transport and metabolism	
MBOV0704S00002594_at	BCG_2615c			0.00156	−2.3	Putative holliday junction DNA helicase ruvB	*ruv*B	Replication, recombination, and repair	
MBOV0704S00000460_at	BCG_0468c			0.000825	−2.2	Putative polypeptide deformylase def	*def*	Translation	
MBOV0704S00001226_at	BCG_1240			0.000669	−2.1	Putative ferredoxin fdxC	*fdx*C	Energy production and conversion	
MBOV0704S00001266_at	BCG_1280c			0.000461	−2.1	Putative methyltransferase			
MBOV0704S00000561_at	BCG_0569			0.00192	−2.0	Putative thioredoxin protein		Posttranslational modification, protein turnover, chaperones	Energy production and conversion

The genes were grouped based on their regulation directions upon 10 and 20 min exposures

The *ech*A12 gene potentially encodes the enoyl-CoA hydratase enzyme involved in lipid transport and metabolism. It catalyzes the reversible hydration of unsaturated fatty acyl-CoA to beta-hydroxyacyl-CoA. It is functional in fatty acid elongation, and enoyl-CoA reductase is also essential for the reaction (Shimakata et al. 1980).

In this study, sodium hypochlorite also upregulated the gene expression of proteins of the proline-glutamic acid/proline-proline-glutamic acid (PE/PPE) family upon both 10- and 20-min exposures. It is interesting to note that PE and PPE family proteins are encoded by approximately 10% of the genome of *M. tuberculosis* (Cole et al. 1998). 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 (Cole 2002). It is widely speculated that PE/PPE families of proteins may be responsible for generating antigenic variation (Banu et al. 2002; Brennan et al. 2001; Delogu and Brennan 2001; Singh et al. 2001). Serological studies have shown the presence of antibodies specific to some of the proteins from these two classes of proteins (Banu et al. 2002; Espitia et al. 1999). In addition, another study has reported a role for PE/PPE proteins in the transportation of antimicrobials across the outer membrane of *M. tuberculosis* (Danilchanka et al. 2008).

Group II: genes upregulated upon 10-min exposure

Group II of Table 2 indicates that the class of "energy production and conversion" which is responsible for electron transfer in biological systems was upregulated after 10 min. In group II of Table 2, for instance, three genes encode oxidoreductase, two genes code for rubredoxin, and one gene codes for ferredoxin. An oxidoreductase is an enzyme that catalyzes the transfer of electrons from electron donor to electron acceptor. Like cytochromes, ferredoxins, and Rieske proteins, rubredoxins participate in electron transfer in biological systems. Rubredoxins are a class of low-molecular-weight iron-containing proteins found in sulfur-metabolizing bacteria and Archaea. Sometimes, rubredoxins are classified as iron–sulfur proteins; however, in contrast to iron–sulfur proteins, rubredoxins do not contain inorganic sulfide. Ferredoxins are iron–sulfur proteins that mediate electron transfer in a range of metabolic reactions. In noncyclic photophosphorylation, ferredoxin is the last electron acceptor and reduces the enzyme $NADP^+$ reductase.

A recent study revealed a protective role for the redox-regulated heat shock protein Hsp33 in bacteria against hypochlorous acid stress. When exposed to bleach, *Escherichia coli* Hsp33 becomes an active chaperone holdase that protects essential bacterial proteins against the formation of bleach-induced aggregates, thus increasing bacterial resistance to bleach (Winter et al. 2008). Our previous data showed that genes encoding heat shock proteins were induced in *Pseudomonas aeruginosa* in response to hydrogen peroxide exposure (Chang et al. 2005). In group II of Table 2, there is heat shock protein, *hsp*X, belonging to the class of "posttranslational modification, protein turnover, chaperones". In addition, another heat shock protein was upreglated in group III. It is therefore possible that heat shock proteins also play a role in the stress response of *M. bovis* to bleach.

Group III: genes upregulated upon 20-min exposure

The alkyl hydroperoxide reductase C and D (*ahp*CD) and catalase–peroxidase–peroxynitritase T (*kat*G) genes belonging to the class of inorganic ion transport and metabolism were present in group III of Table 2. Oxidant defense system genes using catalase (*kat*), alkyl hydroperoxide reductase (*ahp*), and glutathione peroxidase/reductase (Dukan and Touati 1996) were all upregulated. A previous study in our laboratory revealed that the transcription levels of *ahp* and *kat* which are related to oxidative stress response were increased in *P. aeruginosa* in response to bleach exposure (Small et al. 2007b). The catalase gene *kat*G (Rv2710), which is the only known catalase in *M. tuberculosis*, is required for activation of isoniazid (Wilson et al. 1998). All the isoniazid-resistant, *ahp*C-overexpressing strains are deficient in the activity of the mycobacterial catalase–peroxidase KatG. The mycobacterial catalase–peroxidase KatG is a multifunctional enzyme exhibiting catalase, broad-spectrum peroxidase, and peroxynitritase activities. It may also play a role in the intracellular survival of mycobacteria within macrophages and protection against reactive oxygen and nitrogen intermediates produced by phagocytic cells.

Group IV: genes downregulated upon 10- and 20-min exposures

In group IV in Table 2, we noted that genes belonging to the functional class of "lipid transport and metabolism" were downregulated upon both exposure times. Intriguingly, we observed the downregulation of BCG_0280c and BCG_3162 (*fab*G4 and *fad*E24). Mycolic acids are a key component of the mycobacterial cell wall providing structure and forming a major permeability barrier. In *M. tuberculosis*, mycolic acids are synthesized by type I and type II fatty acid synthases. One of the enzymes of the type II system is encoded by fabG4. Acyl CoA dehydrogenase is also the enzyme used to catalyze the first step of β-oxidation in fatty acid metabolism.

Group V: genes downregulated upon 20-min exposures

BCG_0877c (*des*A1) and BCG_1154 (*des*A2) which are possibly parts of an extensively modified long-chain fatty acid complex were downregulated at 20 min (Table 2). The acyl-ACP desaturases are one of the major functional

classes of soluble diiron enzymes (Fox et al. 2004). Genome sequencing has suggested that *M. tuberculosis* and related organisms also contain acyl-ACP desaturase-like proteins (Cole et al. 1998). This finding has intriguing biochemical implications as all mycobacteria have an outer cell wall containing an extensively modified long-chain fatty acid complex called mycolic acid (Barry et al. 1998). This waxy coating provides a protective barrier against macrophage attack, desiccation, water-soluble antibiotics, and other antimicrobial agents. A widely attributed hypothesis is that the biosynthesis of mycolic acid requires desaturases to form precursors with position-specific double bonds (Dubnau et al. 2000; Yuan et al. 1995). Subsequent modifications of the double bonds allow position-specific cyclopropanation, epoxidation, hydroxylation, methoxylation, and ketonization. Therefore, desaturation is likely an essential step in the biosynthesis of structurally and chemically diverse mycolic acids. Therefore, this outcome in conjunction with the extensive downregulation of the genes encoding mycolic acid biosynthesis suggests that sodium hypochlorite may inhibit biosynthesis of the mycolic acid in outer cell wall of *M. bovis*.

In summary, this paper describes the first genome-wide transcriptional analysis of *M. bovis* BCG response to sodium hypochlorite. Briefly, our data, based on the toxicogenomic analysis, showed the following results. First, sodium hypochlorite is an oxidant which initiates a stress response and induces the expression of heat shock proteins; secondly, this outcome in conjunction with the extensive downregulation of the genes encoding mycolic acid biosynthesis suggests that sodium hypochlorite may inhibit biosynthesis of the mycolic acid in outer cell wall of *M. bovis*.

Consequently, we are currently exploring whether the upregulation and/or downregulation of these genes help protect against sodium hypochlorite in *M. bovis* and how this event is linked to the bacterial growth inhibition and metabolism in the early response after10 and 20 min.

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