

# **Toxicogenomic response of** *Mycobacterium bovis* **to hydrogen peroxide**



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### ABSTRACT

**Background:** *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.

**Results:** 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.

<u>Mycobacterium bovis BCG genes that showed statistically significant</u> <u>mRNA level changes upon either 10 or 60 min exposure to hydrogen</u> <u>peroxide</u>

10min		60min							
Gene Annotation	P-value	Fold	P-value	Fold	Description	Functional group			
Group I: Upregulation (10min) - 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			

<u>Transcript level comparison of *Mycobacterium bovis* BCG genes</u> <u>between real-time PCR and microarray analyses</u>

Gene	mRNA level change with microarray Fold change		mRNA level chan PC		Sense primer sequence	Antisense primer sequence
Conto			Fold ch	nange		
	10min	60min	10min	60min		
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'

**Conclusions:** 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.

# MATERIAL AND METHOD

•*M. bovis* exposed to hydrogen peroxide(0.5mM) for 10 and 60min

•Affymetrix *M. bovis* BCG custom genechip analysis

Statistical analysis of microarray data *p*-value for the t-test ≤ 0.05
Fold change in transcript level ≥ 2.0
Presence or marginal calls ≥ 50% replicates on both the experimental and control sets

•Quantitative real-time PCR used for the validation of the microarray data

I	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	
			0.00500		putative 3-isopropylmalate		
BCG_3009c	0.00589	3.4 1.9	0.00589	1.5 1.6	dehydratase large subunit leuC putative transcriptional regulatory protein	Amino acid transport and metabolism Inorganic ion transport and metabolism	Transcription
BCG_1712c BCG 0020	0.0134	1.9	0.0134	1.5	Thioredoxin trxC (TRX) (MPT46)	Posttranslational modification,	Energy production and conversion
Group II: Upreg						protein turnover, chaperones	Energy production and conversion
						Secondary metabolites biosynthesis,	
BCG_2395c	0.00811	3.2			Polyketide synthetase mbtD	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 mbtl	Amino acid transport and metabolism	Coenzyme transport and metabolism
BCG_2394c	0.0132	2.0			Peptide synthetase mbtE	Secondary metabolites biosynthesis, transport and catabolism	
200_20040	0.0102	2.0					
BCG_1580c	0.0235	1.9			putative polyketide synthase associated protein papA4		
BCG_2140	0.0156	1.9			PPE family protein		
BCC 0226	0.0040	4 7			PDE family protoin		
BCG_0326	0.0246	1.7			PPE family protein putative drugs-transport		
BCG_1411	0.0272	1.7			transmembrane ATP-binding protein ABC transporter	Defence mechanism	
BCG_1410	0.00425	1.7			putative drugs-transport transmembrane ATP-binding protein ABC transporter	Defence mechanism	Inorganic ion transport and metabolism
BCG_0802c	0.00622	1.5			putative 3-hydroxyisobutyrate dehydrogenase mmsB	Lipid transport and metabolism	
							Secondary metabolites biosynthes
BCG_1406 Group III: No ch	0.0207 nange (10 mi	1.5 i <b>n) - Upre</b>	gulation (60n	nin) 3 ge	putative acyl carrier protein nes	Lipid transport and metabolism	transport and catabolism
BCG_3227c			0.0205	1.8	putative ATP-dependent DNA helicase	DNA replication, recombination and repair	
BCG_1377c			0.00117	1.6	putative methylated-DNAprotein- 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 ch	nange (10 m	in) - Dow	nregulation (	60min) 1	4 genes		
•		,					
BCG_0023c			0.011	-1.5	putative chromosome partitioning protein parB	Transcription	
					protein parB putative chromosome partitioning	Cell cycle control, mitosis and	
BCG_0024c			0.0156	-1.8	protein parB putative chromosome partitioning protein parA putative glucose-inhibited division	Cell cycle control, mitosis and meiosis	
					protein parB putative chromosome partitioning protein parA	Cell cycle control, mitosis and	
BCG_0024c			0.0156	-1.8	protein parB putative chromosome partitioning protein parA putative glucose-inhibited division	Cell cycle control, mitosis and meiosis	Inorganic ion transport and metabolism
BCG_0024c BCG_0025c			0.0156	-1.8	protein parB putative chromosome partitioning protein parA putative glucose-inhibited division protein B GID	Cell cycle control, mitosis and meiosis Cell wall/membrane biogenesis	
BCG_0024c BCG_0025c BCG_0119 BCG_0557			0.0156 0.015 0.0254 0.0169	-1.8 -1.5 -1.5	protein parB putative chromosome partitioning protein parA putative glucose-inhibited division protein B GID putative hydrogenase hycQ putative transmembrane protein	Cell cycle control, mitosis and meiosis Cell wall/membrane biogenesis	
BCG_0024c BCG_0025c BCG_0119 BCG_0557 BCG_1457c			0.0156 0.015 0.0254 0.0169 0.0222	-1.8 -1.5 -1.5 -1.7 -1.6	protein parB putative chromosome partitioning protein parA putative glucose-inhibited division protein B GID putative hydrogenase hycQ putative transmembrane protein PE-PGRS family protein	Cell cycle control, mitosis and meiosis Cell wall/membrane biogenesis Energy production and conversion	
BCG_0024c BCG_0025c BCG_0119 BCG_0557			0.0156 0.015 0.0254 0.0169	-1.8 -1.5 -1.5	protein parB putative chromosome partitioning protein parA putative glucose-inhibited division protein B GID putative hydrogenase hycQ putative transmembrane protein	Cell cycle control, mitosis and meiosis Cell wall/membrane biogenesis Energy production and conversion	
BCG_0024c BCG_0025c BCG_0119 BCG_0557 BCG_1457c			0.0156 0.015 0.0254 0.0169 0.0222	-1.8 -1.5 -1.5 -1.7 -1.6	protein parB putative chromosome partitioning protein parA putative glucose-inhibited division protein B GID putative hydrogenase hycQ putative transmembrane protein PE-PGRS family protein	Cell cycle control, mitosis and meiosis Cell wall/membrane biogenesis Energy production and conversion	
BCG_0024c BCG_0025c BCG_0119 BCG_0557 BCG_1457c BCG_1747			0.0156 0.015 0.0254 0.0169 0.0222 0.0334	-1.8 -1.5 -1.5 -1.7 -1.6	protein parB putative chromosome partitioning protein parA putative glucose-inhibited division protein B GID putative hydrogenase hycQ putative transmembrane protein PE-PGRS family protein putative initiation inhibitor protein	Cell cycle control, mitosis and meiosis Cell wall/membrane biogenesis Energy production and conversion Cell cycle control, mitosis and meiosis	
BCG_0024c BCG_0025c BCG_0119 BCG_0557 BCG_1457c BCG_1747 BCG_1751			0.0156 0.015 0.0254 0.0169 0.0222 0.0334 0.00439	-1.8 -1.5 -1.5 -1.7 -1.6 -1.6	protein parB putative chromosome partitioning protein parA putative glucose-inhibited division protein B GID putative hydrogenase hycQ putative transmembrane protein PE-PGRS family protein putative initiation inhibitor protein putative cytidylate kinase cmk putative 3-hydroxybutyryl-CoA	Cell cycle control, mitosis and meiosis Cell wall/membrane biogenesis Energy production and conversion Cell cycle control, mitosis and meiosis Nucleotide transport and metabolism	metabolism
BCG_0024c BCG_0025c BCG_0119 BCG_0557 BCG_1457c BCG_1747 BCG_1751 BCG_1754			0.0156 0.015 0.0254 0.0169 0.0222 0.0334 0.00439 0.021	-1.8 -1.5 -1.5 -1.7 -1.6 -1.6 -1.6	protein parB         putative chromosome partitioning         protein parA         putative glucose-inhibited division         protein B GID         putative hydrogenase hycQ         putative transmembrane protein         PE-PGRS family protein         putative cytidylate kinase cmk         putative 3-hydroxybutyryI-CoA         dehydrogenase fadB3	Cell cycle control, mitosis and meiosis Cell wall/membrane biogenesis Energy production and conversion Cell cycle control, mitosis and meiosis Nucleotide transport and metabolism Lipid transport and metabolism	Secondary metabolites biosynthes
BCG_0024c BCG_0025c BCG_0119 BCG_0557 BCG_1457c BCG_1747 BCG_1751 BCG_1754 BCG_2969c			0.0156 0.015 0.0254 0.0169 0.0222 0.0334 0.00439 0.021 0.021	-1.8 -1.5 -1.5 -1.7 -1.6 -1.6 -1.6 -1.5	protein parB         putative chromosome partitioning         protein parA         putative glucose-inhibited division         protein B GID         putative hydrogenase hycQ         putative transmembrane protein         PE-PGRS family protein         putative cytidylate kinase cmk         putative 3-hydroxybutyryl-CoA         dehydrogenase fadB3         putative fatty-acid-CoA ligase         putative chromosome partitioning	Cell cycle control, mitosis and meiosis Cell wall/membrane biogenesis Energy production and conversion Cell cycle control, and conversion Cell cycle control, mitosis and meiosis Nucleotide transport and metabolism Lipid transport and metabolism	Secondary metabolites biosynthes
BCG_0024c BCG_0025c BCG_0119 BCG_0557 BCG_1457c BCG_1747 BCG_1751 BCG_1754 BCG_2969c BCG_2969c BCG_3975c			0.0156 0.015 0.0254 0.0169 0.0222 0.0334 0.00439 0.021 0.021 0.026	-1.8 -1.5 -1.5 -1.7 -1.6 -1.6 -1.6 -1.5 -1.6	protein parB         putative chromosome partitioning         protein parA         putative glucose-inhibited division         protein B GID         putative hydrogenase hycQ         putative transmembrane protein         PE-PGRS family protein         putative cytidylate kinase cmk         putative 3-hydroxybutyryl-CoA         dehydrogenase fadB3         putative fatty-acid-CoA ligase         fadD22         putative chromosome partitioning         putative chromosome partitioning	Cell cycle control, mitosis and Cell wall/membrane biogenesis Energy production and conversion Energy production and conversion Cell cycle control, mitosis and meiosis Nucleotide transport and metabolism Lipid transport and metabolism Lipid transport and metabolism Cell cycle control, mitosis and	Secondary metabolites biosynthes

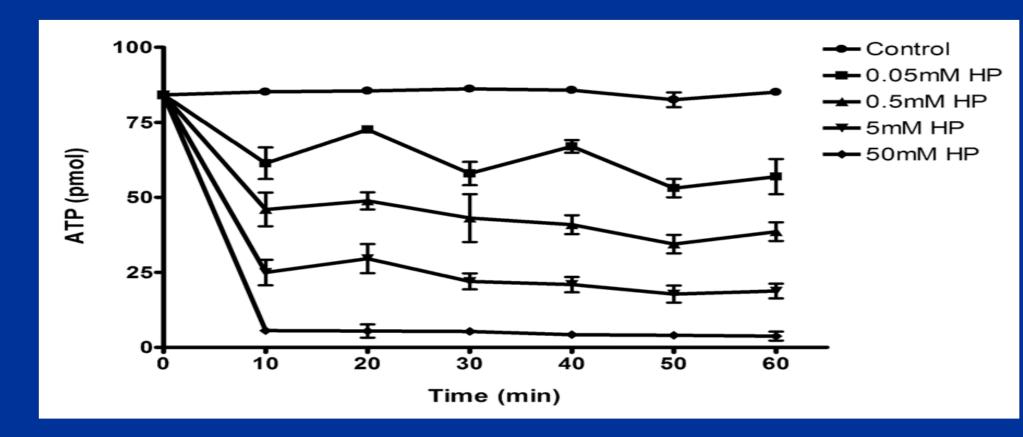
\* The real time PCR results are the mean of three biological replicates with three technical replicates for each gene.

#### CONCLUSION

In summary, we revealed that iron response genes were selectively upregulated during growth inhibition. This study also revealed how oxidative stress-induced genes were related and regulated in *M. bovis* BCG. Our results suggest that DNA repair proteins and catalases are among the most vital antioxidant defense systems of *M. bovis* BCG for preventing the lethal effects of reactive oxygen intermediates. A slowdown of membrane function-related genes was observed, implying that sublethal oxidative damage reduced transport through the cell membrane. We also found that growth inhibition was accompanied by the repression of cell wall/ membrane biogenensis genes. It was confirmed that oxidative stress could affect iron metabolism in that many of Fur-regulated genes were repressed upon exposure to hydrogen peroxide. Further, we showed the induction of iron uptake 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 during 10 min exposure with 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, we propose that repression of the Act-associated hemolytic activity in *M. bovis* may be affected by iron after hydrogen peroxide exposure. Subsequently, a ferric uptake regulatory gene (*fur*) repressed *act* gene expression in the presence of high amounts of iron at 10 min exposure. The repression of the glucose-inhibited division gene (*gid*) of *M. bovis* at 60 min exposure may indicate a significant reduction in hemolysis and cytotoxicity which may be linked to metabolic recovery.

## **RESULTS AND DISCUSSION**

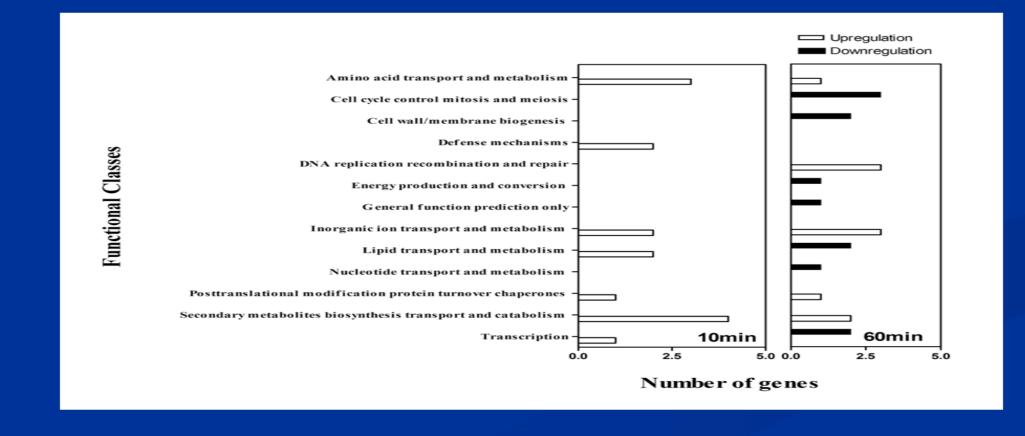
#### Growth Inhibition of *M. bovis* BCG treated with hydrogen peroxide



\* Changes in the amount of ATP produced by the growth culture of *M. bovis* BCG after treatment with hydrogen peroxide were measured in 10 minute intervals for one hour. 0 mM, control (*filled circle*), 0.05 mM (*filled square*), 0.5 mM (*filled triangle*), 5 mM (*inverted filled triangles*) and 50 mM (*filled diamond*).

\* The genes were grouped based on their regulation directions upon 10 and 60 min exposures.

Functional classification of genes showing statistically significant upregulation and downregulation in transcription levels after 10 and 60 minutes exposure to 0.5mM hydrogen peroxide



\* Upregulation (mRNA level changes of 1.5 fold or more, empty square) and downregulation (filled square).