Journal of Medical Microbiology (2008), **57**, 1473–1480 **DOI** 10.1099/jmm.0.2008/001545-0

Differentially expressed genes in *Mycobacterium tuberculosis* H37Rv under mild acidic and hypoxic conditions

Su-Young Kim,[1,2] Byung-Soo Lee,[1] Sung Jae Shin,[1,2] Hwa-Jung Kim[1] and Jeong-Kyu Park[1,3]

Correspondence
Jeong-Kyu Park
jekpark@cnu.ac.kr
or
Sung Jae Shin
sjshin@cnu.ac.kr

[1]Department of Microbiology, College of Medicine, Chungnam National University, Daejeon 301-747, Republic of Korea

[2]Infectious Signaling Network Research Center, College of Medicine, Chungnam National University, Daejeon 301-747, Republic of Korea

[3]Cancer Research Institute, College of Medicine, Chungnam National University, Daejeon 301-747, Republic of Korea

The survival mechanism of dormant tubercle bacilli is unknown; however, accumulating evidence indicates that *Mycobacterium tuberculosis* can survive and persist in hypoxic and mildly acidic microenvironments. Such conditions are found in the acidic vacuoles of macrophages, which *M. tuberculosis* is known to target. We used DECAL (differential expression using customized amplification library) to identify the genes expressed under acidic and hypoxic conditions, following the cultivation of *M. tuberculosis* H37Rv at an acidic pH and/or under hypoxic or anoxic conditions *in vitro*. Of 960 clones analysed, 144 genes, consisting of 71 induced and 8 repressed genes, were identified by sequencing and divided into functional categories to characterize their cellular roles. In general, the genes induced under acidic and hypoxic conditions were involved in the biosynthesis of secondary metabolites (e.g. *pks4*), lipid metabolism, energy production (e.g. *pckA*) and cell wall biogenesis (e.g. *Rv0696* and *plcB*). The combination of genes identified may explain the energy processing and energy storage of *M. tuberculosis* during latent infection. These findings not only enhance our understanding of the mechanism of dormancy, but they also may be useful in the design of therapeutic tools and vaccines for latent tuberculosis.

Received 27 February 2008
Accepted 21 August 2008

INTRODUCTION

Tuberculosis (TB) is a major cause of morbidity worldwide. A recent survey estimated that one third of the world's population is infected with tubercle bacilli, and that around 3000 deaths occur annually due to TB in Korea (Mustafa Abu & Al-Attiyah, 2003); however, most individuals infected with *Mycobacterium tuberculosis* do not progress to active disease. Nevertheless, approximately 10 % of infected individuals develop reactivated TB during their lifetime (Kochi, 1991). Current TB chemotherapy involves bactericidal agents, which target actively growing bacteria; however, they do not eliminate latent or dormant bacteria persisting inside the host.

A bacillus may remain in its host for decades in a dormant state, meaning that an infected individual carries the potential for revival or reactivation of the disease (Wayne, 1994). Dormancy is characterized by nonreplication persistence (NRP) resulting from the presence of metabolically inactive organisms. Important factors to consider in terms of the initiation of NRP include nutrient depletion, shifts in pH, the production of specific growth-limiting products and oxygen depletion (Wayne & Hayes, 1996). Dormant tubercle bacilli exist in granulomas in mildly acidic and hypoxic microenvironments (Koul *et al.*, 2004; Russell, 2001; Salkin & Wayne, 1956). However, the survival mechanism of dormant mycobacteria and the reactivation mechanism of TB are unknown.

Wayne and colleagues developed an *in vitro* culture system to study mycobacterial dormancy and confirmed that oxygen depletion is a critical signal that triggers the dormancy response in bacilli (Wayne, 1994; Wayne & Hayes, 1996). Mycobacteria inhibit phagosome maturation by blocking the fusion of phagosomes with early endosomes and lysosomes, and by causing alterations in membrane proteins that normally promote the formation of an acidic phagolysosome (Koul *et al.*, 2004).

Abbreviations: CAL, customized amplification library; DECAL, differential expression using customized amplification library; TB, tuberculosis.

Tables of primer sequences and expression data are available as supplementary material with the online version of this paper.

The DECAL (differential expression using customized amplification library) method extends the applicability of DNA arrays to situations in which limited amounts of initial RNA are available (Alland *et al.*, 1998). Moreover, once a customized amplification library (CAL) is prepared, it may be used repeatedly.

Here, we analysed the genes that are induced or repressed in *M. tuberculosis* strain H37Rv under mildly acidic and hypoxic conditions *in vitro* using DECAL and sequence analysis. In addition, the functional roles of the genes were examined with regard to energy processing in a dormant state.

METHODS

Construction of the plasmid library. The *M. tuberculosis* H37Rv library was constructed in pBluescript II SK$^-$ (Stratagene) using genomic DNA isolated from *M. tuberculosis* H37Rv as described by Belisle & Sonnenberg (1998) and Sambrook & Russel (2001). The DNA was partially restricted with *Bam*HI. pBluescript II SK$^-$ was prepared by digestion with *Bam*HI and then dephosphorylated with calf intestinal alkaline phosphatase. The plasmid DNA was then ligated with the genomic fragments. The resulting recombinant plasmids were used to transform *Escherichia coli* DH-5α competent cells and ampicillin resistance was selected for on solid Luria–Bertani plates containing X-Gal and IPTG.

CAL production. The *M. tuberculosis* H37Rv CAL was constructed as described by Alland *et al.* (1998), with several modifications. Identification of the clones containing rRNA (5S, 16S and 23S) genes was achieved by dot blot hybridization and amplified by PCR with Uniamp primers. The amplification included a 5 min hot start followed by ten cycles of 1 min segments at 95, 65 and 72 °C. After the end of the tenth cycle, 4 U fresh *Taq* DNA polymerase was added, and 27 additional cycles were performed at 95 °C for 1 min, 65 °C for 2 min and 72 °C for 3 min. The sequences of the primers used are listed in Supplementary Table S1, available with the online journal. Plasmid DNA from the library was extracted and transferred onto a positively charged nylon membrane (Schleicher & Schuell). The PCR-amplified rRNA genes were then labelled and hybridized using a DIG non-radioactive nucleic acid labelling and detection system (Boehringer Mannheim) and dot blots. DNA from the negative clones was pooled and digested with *Bam*HI. The inserts from the digested DNA were purified from agarose gels with a QIAEX II agarose gel extraction kit (Qiagen). The purified DNA was then digested with *Alu*I, and the section corresponding to 400–1500 bp was extracted. The purified DNA was then ligated with Uniamp *Xho*I adapters

Culture conditions and RNA extraction procedure. *M. tuberculosis* H37Rv was grown in minimal Sauton medium as surface pellicles. The medium was then removed by filtration and the bacterial cells were transferred to a 0.2 μm vent-cap flask (Corning) containing normal (pH 7.2) or mildly acidic 7H9 medium (pH 6.0). The bacilli were cultured static for 8 days at 37 °C in a CO_2 incubator set at 21 or 13 % O_2, or in an anaerobic jar (Oxoid) containing AnaeroGen (Oxoid) and an anaerobic indicator (Oxoid): normal conditions (pH 7.2 and 21 % O_2), normal pH with hypoxia (pH 7.2 and 13 % O_2), normal pH with anoxia (pH 7.2 and 0 % O_2), mildly acidic pH with hypoxia (pH 6.0 and 13 % O_2), and mildly acidic pH with anoxia (pH 6.0 and 0 % O_2). The media were buffered with 100 mM MOPS and adjusted to pH 6.0. The culture turbidity (OD_{540}) of *M. tuberculosis* under these five conditions was measured. Steady-state growth was identified by monitoring the cultures for constant turbidity. After 8 days, tubercle bacilli had reached steady state growth under each condition. Total RNA from *M. tuberculosis* H37Rv grown under the above conditions was isolated using a Catrimox-14 RNA isolation kit (Takara), according to the manufacturer's instructions and a published protocol (Payton & Pinter, 1999). Briefly, the bacterial cells were pelleted, resuspended in Catrimox-14 and sonicated on ice. The cell lysate was then vortexed for 1 min and allowed to form micelles. The lysate was then centrifuged, and the pellet was resuspended in guanidine solution (4 M guanidine isothiocyanate, 0.2 M sodium acetate, pH 4.0). The suspension was treated with RNase-free DNase at a final concentration of 2 μg ml^{-1}, incubated for 60 min at 37 °C and extracted with phenol/chloroform/isoamyl alcohol. The aqueous layer was precipitated in 2-propanol, washed with 70 % ethanol and air dried. The RNA pellet was then resuspended in diethylpyrocarbonate-treated water and examined by gel electrophoresis.

Probe construction. The RNA was reverse transcribed with biotin-labelled random hexamers and biotin-dATP using SuperScript II (Gibco-BRL) at 50 °C for 1 h; RNase H was subsequently added for 30 min at 37 °C. Next, 300 ng CAL, 20 μg salmon sperm DNA and 20 μg tRNA were added to the cDNA in a final volume of 150 μl. The sample was then extracted with phenol/chloroform, ethanol-precipitated overnight, resuspended in 6 μl 3 mM EDTA containing 30 mM HEPPS (pH 8.0; Sigma), overlain with oil, and heated to 99 °C for 5 min; thereafter, 1.5 μl 5 M NaCl preheated to 69 °C was added. The sample was then incubated at 69 °C for 3 to 4 days, then diluted with 150 μl incubation buffer (1 × TE, 1 M NaCl and 0.5 % Tween 20) preheated to 69 °C and 50 μl washed, preheated streptavidin-coated magnetic beads (Roche Diagnostics) were added. The sample was incubated at 55 °C with occasional mixing for 30 min, and washed three times at room temperature and three times for 30 min at 69 °C with 0.1 % SDS and 0.2 × SSC by placing the microcentrifuge tubes on a hybridization shaker. The sample was then washed with 2.5 mM EDTA and eluted by boiling in 80 μl water. PCR was performed as described for CAL preparation using 20 μl sample in each reaction.

Screening. The plasmid library arrays were prepared using a Bio-Dot apparatus (Bio-Rad) by double-spotting the plasmid clones onto a nylon membrane. The PCR probes were labelled with [α-^{32}P]dCTP using a DNA-labeller probe DNA labelling kit (Intron). Unincorporated radiolabelled nucleotides were removed by passing the solution through a probe DNA purifying system (Intron) and hybridized to dot blots for 16–18 h as described elsewhere (Sambrook & Russel, 2001). The blots were then washed twice at room temperature for 15 min each in 2 × SSC and 0.1 % SDS, and at 68 °C for 30 min each in 0.1 × SSC and 0.1 % SDS. The spot density of dot blot hybridization was analysed using ArrayGauge (Fujifilm) software. The background was subtracted from the value of each spot on the array. The calculated numeric data were transformed and revealed coloured spots according to each standard. Based on normal conditions (pH 7.2 and aerobic), the fold change was calculated. The clones that hybridized at different intensities with the two probes were selected for further analysis.

Sequencing of the differentially expressed clones. The clones were sequenced using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). For each clone, the start and end sequences of the inserts were generated using a T7 primer, which annealed to either side of the multiple cloning site in pBluescript II SK$^-$. A BLAST search was conducted to identify matches to the *M. tuberculosis* H37Rv sequences deposited in the National

Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) by allowing the size and content of each insert to be extrapolated.

Confirmation of the genes by RT-PCR. Representative induced and repressed genes were randomly chosen to confirm their fold changes by RT-PCR. Briefly, cDNA was obtained from the total RNA with reverse transcriptase and random primers at 42 °C for 60 min. The reactions were stopped by heat inactivation for 5 min at 95 °C and chilled on ice. The cDNA was then amplified by PCR. The sequences of the primers used for amplification are shown in Supplementary Table S1, available with the online journal. The amplification consisted of 30 cycles of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 °C. The *rpoB* was used as an internal control. The products were then subjected to 1 % agarose gel electrophoresis and the intensity of each band was calculated using Quantity One software (version 4.1.0; Bio-Rad). All samples were compared to normal culture (oxygen pressure of 21 %, pH 7.2; condition 1) and the gene expression level of this condition corresponds to 1. The RT-PCR results were expressed as the fold induction relative to the control value of normal culture.

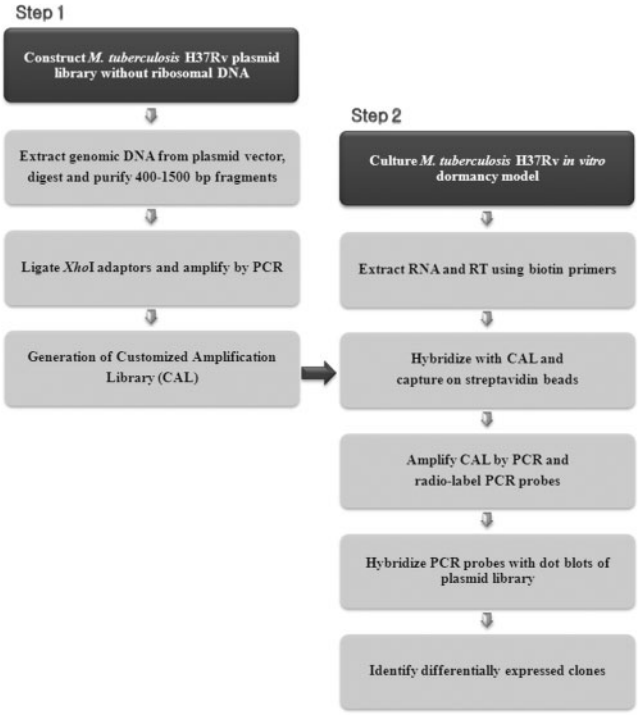

Fig. 1. Schematic of the steps in the use of DECAL for *M. tuberculosis* H37Rv. A plasmid library was screened for clones containing rDNA. Non-ribosomal plasmids were extracted, digested and purified to generate smaller and similar-sized fragments. These fragments were ligated to *Xho*I adaptors and PCR amplified. Total RNA was isolated from mycobacteria cultured under different conditions, reverse-transcribed using biotin-labelled random primers and hybridized to the CAL. The captured CAL was amplified to generate PCR probes, radiolabelled and hybridized with dot blots of the plasmid library. Differentially expressed spots were selected and identified.

RESULTS AND DISCUSSION

Generation of *M. tuberculosis* H37Rv CAL and probe

We applied DECAL to identify induced or repressed genes in *M. tuberculosis* under mild acidic and hypoxic conditions (Fig. 1). To generate a CAL for the *M. tuberculosis* H37Rv genome, genomic DNA from a plasmid library was extracted and digested. The fragments were then ligated to adapters and amplified by PCR (Fig. 1, step 1). The CAL was then used for optimal and efficient amplification of the mRNAs expressed under different conditions.

M. tuberculosis can successfully adapt to hypoxia *in vitro*, and the growth of mycobacteria under acidic conditions

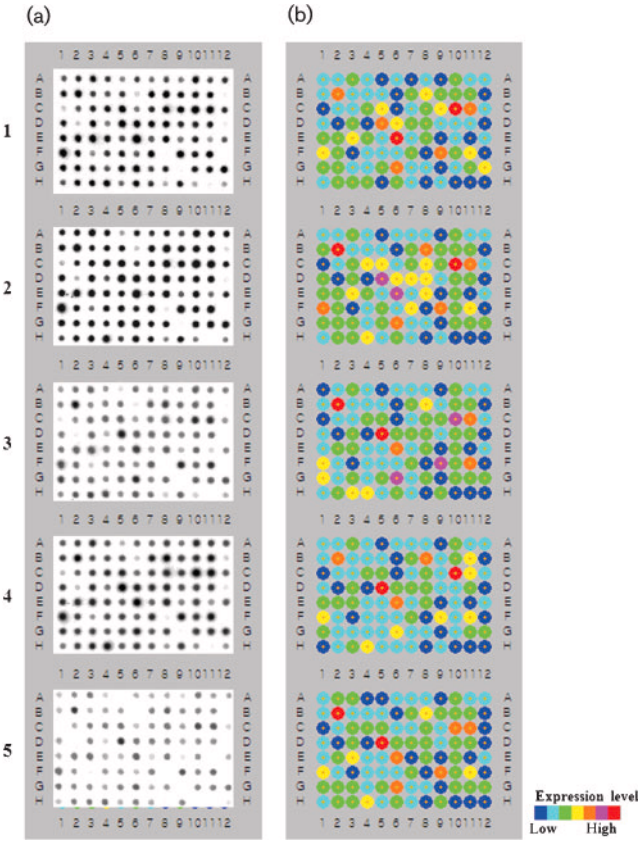

Fig. 2. Spot density of dot blot hybridization with PCR probes to the *M. tuberculosis* H37Rv plasmid library. Dot blots were hybridized with radiolabelled PCR probes for each of the following conditions: (1) normal conditions (pH 7.2 and 21 % O_2), (2) normal pH with hypoxia (pH 7.2 and 13 % O_2), (3) normal pH with anoxia (pH 7.2 and 0 % O_2), (4) mildly acidic pH with hypoxia (pH 6.0 and 13 % O_2), (5) mildly acidic pH with anoxia (pH 6.0 and 0 % O_2). The original results of dot blot hybridization are shown in (a). The results were then analysed using ArrayGauge software. Spot density is indicated by colour. The highest and lowest levels of expression are represented by red and blue, respectively (b).

Table 1. *M. tuberculosis* H37Rv genes with increased expression under mild acidic and hypoxic conditions

Locus	Annotated gene	Product	Fold change
Rv0017c	*rodA*	Probable cell division protein	1.8
Rv0107c	*ctpI*	Probable cation-transporter ATPase	2.3
Rv0108c		Hypothetical protein	2.3
Rv0109	PE_PGRS1	PE-PGRS family protein	2.0
Rv0211	*pckA*	Probable iron-regulated phosphoenolpyruvate carboxykinase	2.1
Rv0212c	*nadR*	Possible transcriptional regulatory protein	2.1
Rv0247c		Probable succinate dehydrogenase	1.6
Rv0248c		Probable succinate dehydrogenase	1.6
Rv0285	PE5	PE family protein	1.6
Rv0286	PPE4	PPE family protein	1.6
Rv0312		Conserved hypothetical proline- and threonine-rich protein	1.6
Rv0477		Possible conserved secreted protein	1.7
Rv0478	*deoC*	Probable deoxyribose-phosphate aldolase	1.7
Rv0508		Conserved hypothetical protein	2.1
Rv0509	*hemA*	Probable glutamyl-tRNA reductase	2.1
Rv0510	*hemC*	Probable porphobilinogen deaminase	1.6
Rv0554	*bpoC*	Possible peroxidase	1.8
Rv0695		Conserved hypothetical protein	2.3
Rv0696		Probable membrane sugar transferase	2.6
Rv0983	*pepD*	Probable serine protease	1.7
Rv0984	*moaB2*	Possible pterin-4-α-carbinolamine dehydratase	1.7
Rv1064c	*lpqV*	Possible lipoprotein	1.6
Rv1065		Conserved hypothetical protein	1.6
Rv1071c	*echA9*	Possible enoyl-coA hydratase	1.8
Rv1140		Probable integral membrane protein	1.5
Rv1141c	*echA11*	Probable enoyl-coA hydratase	1.5
Rv1162	*narH*	Probable respiratory nitrate reductase	1.7
Rv1181	*pks4*	Polyketide β-ketoacyl synthase	3.8
Rv1262c		Hypothetical HIT-like protein	1.7
Rv1263	*amiB2*	Probable amidase	1.7
Rv1524		Probable glycosyltransferase	1.9
Rv1604	*impA*	Probable inositol-monophosphatase	1.7
Rv1605	*hisF*	Probable cyclase	1.7
Rv1759c	*wag22*	PE-PGRS family protein	2.0
Rv1857	*modA*	Probable molybdate-binding lipoprotein	1.6
Rv1858	*modB*	Probable molbdenum-transport integral membrane protein	1.6
Rv2041c		Probable sugar-binding lipoprotein	1.9
Rv2042c		Hypothetical protein	1.9
Rv2199c		Possible conserved integral membrane protein	1.8
Rv2200c	*ctaC*	Probable transmembrane cytochrome C oxidase	1.8
Rv2258c		Possible transcriptional regulatory protein	1.6
Rv2259	*adhE2*	Probable zinc-dependent alcohol dehydrogenase	1.6
Rv2309A		Hypothetical protein	2.0
Rv2310		Possible excisionase	2.0
Rv2345		Possible conserved transmembrane protein	2.0
Rv2346c	*esxO*	Putative ESAT-6 like protein	2.0
Rv2350c	*plcB*	Probable membrane-associated phospholipase C 2	1.7
Rv2448c	*valS*	Probable valyl-tRNA synthase protein	1.9
Rv2449c		Conserved hypothetical protein	1.9
Rv2504c	*scoA*	Probable succinyl-coA : 3-ketoacid-coenzyme A transferase	1.5
Rv2505c	*fadD35*	Probable fatty-acid-coA ligase	1.5
Rv2739c		Possible alanine-rich transferase	1.9
Rv2812		Probable transposase	2.5
Rv2956		Conserved hypothetical protein	2.0
Rv2957		Possible glycosyl transferase	2.0
Rv2972c		Possible conserved membrane or exported protein	1.7
Rv2973c	*recG*	Probable ATP-dependent DNA helicase	1.7

Table 1. cont.

Locus	Annotated gene	Product	Fold change
Rv3263		Probable DNA methylase	2.3
Rv3304		Conserved hypothetical protein	2.4
Rv3331	*sugI*	Probable sugar-transport integral membrane protein	1.8
Rv3334		Probable transcriptional regulatory protein	1.8
Rv3342		Possible methyltransferase	1.9
Rv3343c	PPE54	PPE family protein	1.9
Rv3472		Conserved hypothetical protein	1.7
Rv3473c	*bpoA*	Possible peroxidase	1.7
Rv3498c	*mce4B*	MCE-family protein	2.0
Rv3560c	*fadE30*	Probable acyl-coA dehydrogenase	1.8
Rv3725		Possible oxidoreductase	1.6
Rv3771c		Conserved hypothetical protein	1.5
Rv3772	*hisC2*	Probable histidinol-phosphate aminotransferase	1.5
Rv3868		Conserved hypothetical protein	1.7

has been reported (Clemens & Horwitz, 1995; Piddington *et al.*, 2000); however, their response to oxygen- and pH-induced stress is unclear. To evaluate the influence of an acidic pH and low oxygen levels on gene expression in *M. tuberculosis* H37Rv, bacilli were cultured under the following conditions: normal conditions (pH 7.2 and 21 % O_2), normal pH with hypoxia (pH 7.2 and 13 % O_2), normal pH with anoxia (pH 7.2 and 0 % O_2), mildly acidic pH with hypoxia (pH 6.0 and 13 % O_2) and mildly acidic pH with anoxia (pH 6.0 and 0 % O_2). Mycobacterial RNA extracted from the five cultures grown under the different conditions was then reverse transcribed and hybridized with the CAL. The CAL-binding cDNAs were amplified by PCR and used as probes for dot blot hybridization with the *M. tuberculosis* H37Rv library (Fig. 1, step 2).

Identification of differentially expressed genes under mild acidic and hypoxic conditions

Those genes that were differentially expressed under acidic and/or hypoxic conditions were identified by analysing the differential hybridization patterns of the probes prepared from the total RNA and CAL hybridization (Fig. 2). The fold changes were calculated in comparison to the results from a normal culture (pH 7.2, 21 % O_2). We then selected the clones that showed at least a 1.5-fold change in expression in the five different cultures for further analysis.

The sequences of the differentially expressed clones were analysed using the BLAST program from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). All sequences with at least 100 bp of alignment with the *M. tuberculosis* genome were considered to be specifically amplified.

Bioinformatic analysis of induced and repressed genes

Of the 144 genes identified by screening the *M. tuberculosis* H37Rv library, 101 were induced under acidic and/or hypoxic conditions, while 43 were repressed. Among the 101 induced genes, 71 were induced by both an acidic pH and hypoxia (Table 1), whereas 14 were induced only by hypoxia (see Supplementary Table S2 available with the online journal). Sixteen genes were induced by an acidic pH condition (see Supplementary Table S2 available with the online journal). Among the 43 downregulated genes, 8 were repressed by both an acidic pH and hypoxia (Table 2),

Table 2. *M. tuberculosis* H37Rv genes with decreased expression under mild acidic and hypoxic conditions

Locus	Annotated gene	Product	Fold change
Rv0456A		Conserved hypothetical protein	−1.4
Rv0457c		Probable peptidase	−1.4
Rv0556		Probable conserved transmembrane protein	−1.5
Rv1393c		Probable monoxygenase	−2.0
Rv2576c		Possible conserved membrane protein	−1.9
Rv2577		Conserved hypothetical protein	−1.9
Rv3249c		Possible transcriptional regulatory protein	−1.8
Rv3250c	*rubB*	Probable rubredoxin	−1.8

whereas 33 were repressed only by hypoxia and 2 genes were repressed only by acidic pH condition (see Supplementary Table S3 available with the online journal). The induced and repressed genes were then subjected to bioinformatic analysis.

To characterize the roles of the differentially expressed genes, the genes were divided into functional categories based on information obtained from the Clusters of Orthologous Groups of proteins (COGs) website (http://ncbi.nlm.nih.gov/COG/; Table 3). Interestingly, the number of induced genes was greater than the number of repressed genes, even though *M. tuberculosis* was not actively growing under either condition.

Those 71 genes with a mean-fold change in expression >1.5 under acidic pH and hypoxic conditions in comparison to expression in a normal culture (pH 7.2, 21 % O_2) are listed in Table 1. *Rv1181*, also known as *pks*4, was the gene most highly induced by an acidic pH and hypoxia. This gene encodes a polyketide β-ketoacyl synthase involved in the biosynthesis of cell wall lipid (Dubey *et al.*, 2002). The second most highly induced gene was *Rv0696*, which encodes a membrane sugar transferase that functions in cell wall biosynthesis. *Rv0211* (*pckA*), which encodes phosphoenolpyruvate carboxykinase required for growth on fatty acids as the major carbon source (Liu *et al.*, 2003), was also induced under acidic and hypoxic conditions. *Rv2350c* (*plcB*), which encodes a phospholipase involved in the virulence of *M. tuberculosis* (Raynaud *et al.*, 2002), was upregulated under acidic and hypoxic conditions. It has been proposed that lipids serve as the carbon source for growing bacteria. The storage of lipids *in vivo* may therefore be crucial to the survival of the bacteria and allow them to adapt to the host cell environment.

Under conditions of oxygen stress, the most highly induced gene was *Rv2934*, also known as *ppsD*. The protein encoded by *ppsD* is a phenolpthiocerol synthesis type-I polyketide synthase, which is involved in the synthesis of the lipid core common to phthiocerol dimycocerosates and phenolglycolipids, which have been studied because of their key roles in the pathogenesis of TB and tubercle bacillus–host interactions (Reed *et al.*, 2004).

Rv0006, also known as *gyr*A, was the most highly induced gene under acidic conditions. *Rv2456c*, which encodes a probable conserved integral membrane transport protein, and *Rv3731*, which encodes a DNA ligase required for the growth of *M. tuberculosis*, were induced under acidic pH conditions. Also, *Rv1750c* (*fadD1*), which encodes fatty acid-CoA ligase, which catalyses the formation of fatty acyl-CoA (Weimar *et al.*, 2002), was highly induced under pH stress.

Forty-three genes were significantly downregulated by acidic pH and/or hypoxia, including five genes involved in energy production and conversion. Among these five

Table 3. List of functional categories of 71 induced and 8 repressed genes identified in this study

Genes with a mean fold change in expression of >1.5 or <0.5 are classified.

Functional category	No. of genes in H37Rv genome	No. of induced genes in library	No. of repressed genes in library
Translation	121	2	0
RNA processing and modification	1	0	0
Transcription	200	1	1
Replication, recombination and repair	204	3	0
Cell cycle control, mitosis and meiosis	40	1	0
Defence mechanisms	39	0	0
Signal transduction mechanisms	117	0	1
Cell wall/membrane biogenesis	112	4	0
Cell motility	11	1	0
Intracellular trafficking and secretion	21	0	0
Post-translational modification, protein turnover, chaperones	98	4	0
Energy production and conversion	210	7	1
Carbohydrate transport and metabolism	111	4	0
Amino acid transport and metabolism	184	2	1
Nucleotide transport and metabolism	64	2	0
Coenzyme transport and metabolism	121	4	0
Lipid transport and metabolism	257	5	0
Inorganic ion transport and metabolism	124	3	1
Secondary metabolites biosynthesis, transport and catabolism	234	5	0
General function prediction only	436	5	1
Function unknown	208	2	0
Not in COGs website	1480	16	2

genes one gene was downregulated by acidic pH and hypoxia, and four genes were downregulated by hypoxia only. It is possible that a slow metabolism occurs in regard to energy production or conversion under limited oxygen supply. The *rubB* gene, which encodes rubredoxin, was downregulated, as were *sucD*, which encodes succinyl-CoA synthetase, TCA cycle enzyme, and *pdhB* and *pdhC*, which encode subunits of the pyruvate dehydrogenase (PDH) enzyme complex, which functions to convert pyruvate to acetyl-CoA and CO_2. During the chronic phase of infection, *M. tuberculosis* switches its metabolism to use fatty acids as its major carbon and energy source (McKinney *et al.*, 2000; Timm *et al.*, 2003). Under these conditions, glycolysis is decreased and PDH activity is not essential. *pca*, which encodes pyruvate carboxylase, was repressed under hypoxic conditions. Those eight genes with a mean fold change in expression <1.5 under acidic pH and hypoxic condition are listed in Table 2.

The differential expression of these genes at the level of transcription under each set of conditions was confirmed by RT-PCR using *rpoB* as an internal standard. As shown in Fig. 3, a single band of the expected molecular size was detected for each of the transcripts from *M. tuberculosis* H37Rv. *rpoB* varied little during the bacterial growth cycle *in vitro*. Based on the intensities of the bands, the results are expressed as the fold induction relative to the control value of normal culture (pH 7.2, 21 % O_2).

Six induced and three repressed genes involved in the TCA cycle and the glyoxylate cycle pathways, and cell wall lipid synthesis, were identified in this study. *M. tuberculosis* can survive in macrophages; thus, it must assimilate carbon and produce energy in the phagosome (Höner zu Bentrup & Russell, 2001). Schnappinger *et al.* (2003) demonstrated that *M. tuberculosis* switched its carbon source *in vitro* from glucose and glycerol to fatty acids in the phagosome. These fatty acids may be derived from triglycerides by lipase. The breakdown products of fatty acids are metabolized via the citric acid and glyoxylate cycles, as suggested by the induction of genes such as *fadD1*. The induction of *pckA* suggests that fatty acids are in part converted to sugars via gluconeogenesis. These data support the view that *M. tuberculosis* might use glyoxylate bypass through degradation of fatty acid during metabolism under conditions of acidic pH and hypoxia. The glyoxylate cycle bypasses the steps in which CO_2 is generated by the TCA cycle and allows acetyl-CoA and glyoxylate to form succinate, a precursor for the synthesis of sugars. Lipids are a source of acetyl-CoA through the degradation of fatty acids via the β-oxidation cycle. Consequently, the glyoxylate bypass allows *M. tuberculosis* and other bacteria to synthesize carbohydrates from fatty acids, and it supplies the intermediates necessary to support the TCA cycle. This is of obvious importance when fatty acids are the main source of carbon and energy, as has been suggested for *M. tuberculosis* and *Mycobacterium leprae* in chronically infected tissues (Bloch & Segal, 1956; Wheeler & Ratledge, 1988).

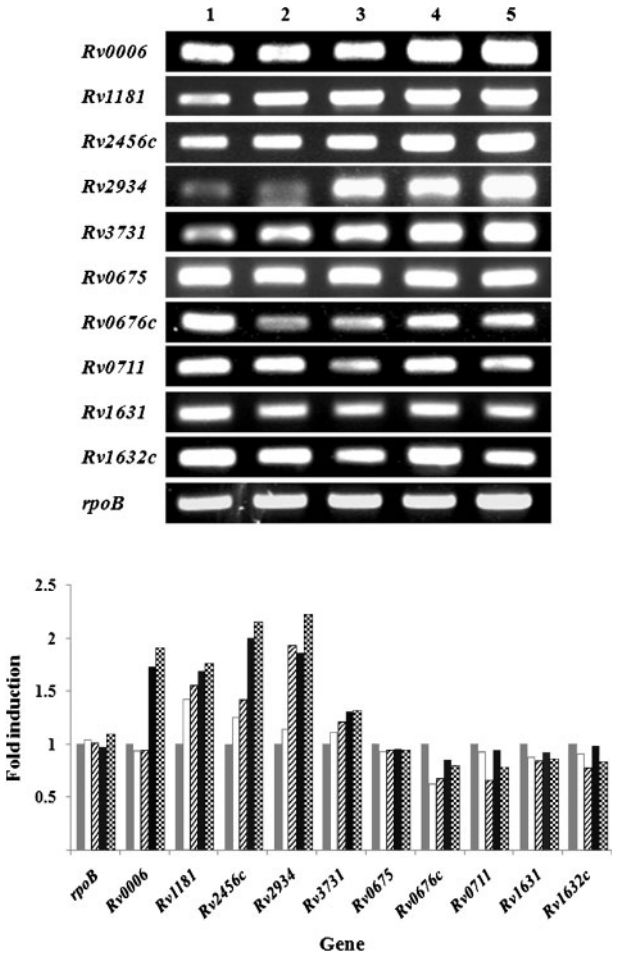

Fig. 3. RT-PCR analysis of the induced and repressed genes of *M. tuberculosis* H37Rv cultured under an *in vitro* dormancy model. The five induced and five repressed genes were selected according to their order of increase and decrease in fold change. The transcription level of each *M. tuberculosis* H37Rv gene cultured at different oxygen atmospheres with normal or acidic pH was analysed by RT-PCR. 1, normal atmosphere with pH 7.2 (grey bars); 2, hypoxic atmosphere with pH 7.2 (white bars); 3, hypoxic atmosphere with pH 6.0 (hatched bars); 4, anoxic atmosphere with pH 7.2 (black bars); 5, anoxic atmosphere with pH 6.0 (bars with spots).

Furthermore, the upregulation of genes such as *pks4*, *Rv0696*, *plcB* and *ppsD* involved in the synthesis of selected cell wall lipids using fatty acids was also apparent during survival under acidic pH and hypoxia. Therefore, *M. tuberculosis* might avoid producing acetyl-CoA from pyruvate by downregulation of genes such as *pdhB* and *pdhC* and prefer glyoxylate bypass through degradation of fatty acid in metabolism.

The abundance of genes encoding enzymes involved in fatty acid degradation supports the idea that *M. tuberculosis* uses host lipids to support its growth *in vivo*. Bloch & Segal (1956) showed that bacteria grown *in vitro* had a preference

for carbohydrates, whereas bacteria grown *in vivo* preferred fatty acids. *M. tuberculosis* probably feeds on the lipid-rich host-cell debris in mature granulomas and on degraded fatty acids in the endosomal milieu of nascent granulomas. In this setting, fatty acid biosynthesis is repressed and cell wall constituents such as mycolic acids arise preferentially by the elongation of internalized fatty acids, as has been shown for other mycobacterial species (Bloch & Segal, 1956; Wheeler *et al.*, 1990, 1991).

The limited growth of *M. tuberculosis* H37Rv under acidic pH and oxygen stress parallels the survival of *M. tuberculosis* in the acidic vacuoles of macrophages. The identification of *M. tuberculosis* genes that contribute to bacterial survival will ultimately enhance the development of new treatments for the latent TB patient.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Korea Health 21 R&D project, Ministry of Health and Welfare, Republic of Korea (01-PJ10-PG6-01GM03-002), the Chungnam National University Hospital Research Fund, 2005, and the Korea Science & Engineering Foundation through the Infection Signaling Network Research Center (R13-2007-020-01000-0) at Chungnam National University.

REFERENCES

Alland, D., Kramnik, I., Weisbrod, T. R., Otsubo, L., Cerny, R., Miller, L. P., Jacobs, W. R., Jr & Bloom, B. R. (1998). Identification of differentially expressed mRNA in prokaryotic organisms by customized amplification libraries (DECAL): the effect of isoniazid on gene expression in *Mycobacterium tuberculosis. Proc Natl Acad Sci U S A* **95**, 13227–13232.

Belisle, J. T. & Sonnenberg, M. G. (1998). Isolation of genomic DNA from mycobacteria. *Methods Mol Biol* **101**, 31–44.

Bloch, H. & Segal, W. (1956). Biochemical differentiation of *Mycobacterium tuberculosis* grown in vivo and in vitro. *J Bacteriol* **72**, 132–141.

Clemens, D. L. & Horwitz, M. A. (1995). Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med* **181**, 257–270.

Dubey, V. S., Sirakova, T. D. & Kolattukudy, P. E. (2002). Disruption of *msl3* abolishes the synthesis of mycolipanoic and mycolipenic acids required for polyacyltrehalose synthesis in *Mycobacterium tuberculosis* H37Rv and causes cell aggregation. *Mol Microbiol* **45**, 1451–1459.

Höner zu Bentrup, K. & Russell, D. G. (2001). Mycobacterial persistence: adaptation to a changing environment. *Trends Microbiol* **9**, 597–605.

Kochi, A. (1991). The global tuberculosis situation and the new control strategy of the World Health Organization. *Tubercle* **72**, 1–6.

Koul, A., Herget, T., Klebl, B. & Ullrich, A. (2004). Interplay between mycobacteria and host signalling pathways. *Nat Rev Microbiol* **2**, 189–202.

Liu, K., Yu, J. & Russell, D. G. (2003). *pckA*-deficient *Mycobacterium bovis* BCG shows attenuated virulence in mice and in macrophages. *Microbiology* **149**, 1829–1835.

McKinney, J. D., Höner zu Bentrup, K., Muñoz-Elías, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchettini, J. C., Jacobs, W. R., Jr & Russell, D. G. (2000). Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**, 735–738.

Mustafa Abu, S. & Al-Attiyah, R. (2003). Tuberculosis: looking beyond BCG vaccines. *J Postgrad Med* **49**, 134–140.

Payton, M. & Pinter, K. (1999). A rapid novel method for the extraction of RNA from wild-type and genetically modified kanamycin resistant mycobacteria. *FEMS Microbiol Lett* **180**, 141–146.

Piddington, D. L., Kashkouli, A. & Buchmeier, N. A. (2000). Growth of *Mycobacterium tuberculosis* in a defined medium is very restricted by acid pH and Mg^{2+} levels. *Infect Immun* **68**, 4518–4522.

Raynaud, C., Guilhot, C., Rauzier, J., Bordat, Y., Pelicic, V., Manganelli, R., Smith, I., Gicquel, B. & Jackson, M. (2002). Phospholipases C are involved in the virulence of *Mycobacterium tuberculosis. Mol Microbiol* **45**, 203–217.

Reed, M. B., Domenech, P., Manca, C., Su, H., Barczak, A. K., Kreiswirth, B. N., Kaplan, G. & Barry, C. E., III (2004). A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature* **431**, 84–87.

Russell, D. G. (2001). *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat Rev Mol Cell Biol* **2**, 569–577.

Salkin, D. & Wayne, L. G. (1956). The bacteriology of resected tuberculous pulmonary lesions. I. The effect of interval between reversal of infectiousness and subsequent surgery. *Am Rev Tuberc* **74**, 376–387.

Sambrook, J. & Russel, D. R. (2001). *Molecular Cloning; a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D. & other authors (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J Exp Med* **198**, 693–704.

Timm, J., Post, F. A., Bekker, L. G., Walther, G. B., Wainwright, H. C., Manganelli, R., Chan, W. T., Tsenova, L., Gold, B. & other authors (2003). Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. *Proc Natl Acad Sci U S A* **100**, 14321–14326.

Wayne, L. G. (1994). Dormancy of *Mycobacterium tuberculosis* and latency of disease. *Eur J Clin Microbiol Infect Dis* **13**, 908–914.

Wayne, L. G. & Hayes, L. G. (1996). An in vitro model for sequential study of shiftdown of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect Immun* **64**, 2062–2069.

Weimar, J. D., DiRusso, C. C., Delio, R. & Black, P. N. (2002). Functional role of fatty acyl-coenzyme A synthetase in the transmembrane movement and activation of exogenous long-chain fatty acids. Amino acid residues within the ATP/AMP signature motif of *Escherichia coli* FadD are required for enzyme activity and fatty acid transport. *J Biol Chem* **277**, 29369–29376.

Wheeler, P. R. & Ratledge, C. (1988). Use of carbon sources for lipid biosynthesis in *Mycobacterium leprae*: a comparison with other pathogenic mycobacteria. *J Gen Microbiol* **134**, 2111–2121.

Wheeler, P. R., Bulmer, K. & Ratledge, C. (1990). Enzymes for biosynthesis de novo and elongation of fatty acids in mycobacteria grown in host cells: is *Mycobacterium leprae* competent in fatty acid biosynthesis? *J Gen Microbiol* **136**, 211–217.

Wheeler, P. R., Bulmer, K. & Ratledge, C. (1991). Fatty acid oxidation and the β-oxidation complex in *Mycobacterium leprae* and two axenically cultivable mycobacteria that are pathogens. *J Gen Microbiol* **137**, 885–893.