Transcriptomic analysis identifies growth rate modulation as a component of the adaptation of mycobacteria to survival inside the macrophage.

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Running title: The transcriptional program of slow growing *Mycobacterium bovis* BCG

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ABSTRACT

The adaptation of the tubercle bacillus to the host environment is likely to involve a complex set of gene regulatory events and physiological switches in response to environmental signals. In order to deconstruct the physiological state of *Mycobacterium tuberculosis* *in vivo* we used a chemostat model to study a single aspect of the organism’s *in vivo* state: slow growth. *Mycobacterium bovis* BCG was cultivated at fast and slow growth rates in a carbon-limited chemostat and transcriptomic analysis performed to identify the gene regulatory events associated with slow growth. The results demonstrated that slow growth was associated with the induction of several genes of the dormancy survival regulon. There was also a striking overlap between the transcriptomic profile of BCG in the chemostat model and the response of *M. tuberculosis* to growth in the macrophage implying that a significant component of the response of the pathogen to the macrophage environment is the response to slow-growth in carbon-limited conditions. This demonstrated the importance of adaptation to slow growth rate to the virulence strategy of *M. tuberculosis* and also the value of the chemostat model for deconstructing components of the *in vivo* state of this important pathogen.

INTRODUCTION

Despite more than a century of research into tuberculosis (TB) this disease remains the number one killer due to a single infectious agent, making *Mycobacterium tuberculosis* one of the most successful human pathogens. A key to this bacterium’s success is its ability to establish and maintain a latent infection in its human host for many decades (18). The control of tuberculosis is severely impeded by the global magnitude of latent tuberculosis. One third of the world’s population are estimated to...
harbor persistent *M. tuberculosis* primed for reactivation and initiation of clinical disease (7). The bacterial response to the triggers of latency and reactivation are very poorly understood. Unraveling the mechanisms involved during the establishment, maintenance and reactivation of latent tuberculosis is an important goal for mycobacterial researchers. Such information will lead to the development of novel therapeutics, vaccines and diagnostic strategies targeted to persistent *M. tuberculosis*.

*In vitro* modelling of *M. tuberculosis* provides simple experimental approaches to studying the physiology and genetic basis of TB. The design of adequate models is however impeded by the paucity of knowledge about the biological characteristics of both the bacteria and the host environment during human TB and therefore *in vitro* modellers must make simplistic assumptions about the environmental variables within the human host. Microaerophilic adaptation, nutrient starvation, drug-persistent and extended stationary phase models of persistent TB have been established (9). All these models provide *in vitro* conditions that are intended to simulate the microenvironments of the host during persistence. Whilst these models have proved very valuable for studying persistence it is unlikely that a single model will recapitulate all the conditions experienced and the relevance of these models to the *in vivo* state remains unclear.

An alternative approach is to attempt to deconstruct the physiological state of mycobacteria into its components. It may then be possible to establish molecular fingerprints characterising the different physiological states of the pathogen that may then be compared with the molecular signature of TB cells in the host. One of the few undisputed characteristics of persistent *M. tuberculosis* is that the pathogen grows at a slower rate during persistence than during active disease. Study of the adaptation of
M. tuberculosis to slow growth is therefore a first step towards deconstructing its physiological state during TB.

Chemostat culture is a technique devised specifically to grow microbes under constant, carefully controlled conditions at a single growth rate. We initiated a study of the tubercle bacillus growing in defined media in the chemostat using the M. bovis BCG vaccine strain as a model organism (3). Our first step was to establish chemostat culture of the tubercle bacillus growing at two different growth rates in carbon-limited conditions. Detailed macromolecular analysis of steady state cultures was determined in order to compile a molecular inventory of carbon-limited BCG growing at slow and fast growth rates. These studies demonstrated that the mycobacterial cell is remodelled in response to changes in the growth rate in carbon-limited conditions (3).

Our earlier study established that the lipid, RNA and protein content of the mycobacterial cell is different in slow-growing compared to fast growing M. bovis BCG (3). The genome is of course the same in both organisms so the divergent physiological states must be maintained by differences at the level of the transcriptome, proteome and the metabolome. The chemostat system provides an ideal environment for functional genomic studies to uncover these differences, since cells can be reproducibly obtained in highly defined conditions. Microarray analysis of organisms grown in the chemostat has been shown to be more reproducible and accurate than batch culture DNA-array data (6, 12, 13, 17). A combination of chemostat culture and DNA microarrays was used to study the effects of oxygen limitation on the gene expression profile of M. tuberculosis (2). That study demonstrated the power of chemostat culture to study the response of mycobacteria to specific environmental conditions. In order to investigate the molecular mechanisms
underlying the observed shift in macromolecular composition of *M. bovis* BCG in a carbon-limited chemostat, we here describe transcriptome analysis of fast and slow-growing BCG cells.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *M. bovis* BCG strain (ATCC 35748) was cultured in a 2-litre bioreactor (Adaptive Biosystem Voyager) under aerobic conditions and at pH 6.6 as previously described (3). Chemostat cultures were grown in Roisin’s minimal medium at a constant dilution rate of 0.03 h⁻¹ (equivalent to a doubling time, *t_d* of 23 h) or 0.01 h⁻¹ (*t_d* = 69 h). Culture samples were withdrawn from the chemostat to monitor cellular dry weight, viable counts, optical density, nutrient utilisation, CO₂ and O₂ concentrations (3). Steady state conditions were assumed when the carbon dioxide evolution, OD₆₀₀ and dry weight remained constant for three consecutive volume changes. Once the steady state was reached cells were harvested for analysis. Independent triplicate chemostat cultures were run for each growth rate.

**RNA preparation.** In order to stabilise the mRNA population 20 ml of culture sample was rapidly withdrawn from the chemostat by a tube submerged in the culture broth and directly injected into a sterile bottle containing 80 ml of GTC solution (5M guanidinium thiocyanate, 0.5% sodium N-lauryl sarcosine, 25 mM tri-sodium citrate and 0.1M DTT) (20). RNA was prepared essentially as described by Stewart *et al.* (33). The extraction protocol was modified slightly to include additional chloroform extractions to remove lipid contamination.
Fluorescently labeled cDNA were produced by reverse transcription of total *M. tuberculosis* RNA (2-10 µg) with Superscript III (Invitrogen) in the presence of Cy3-dCTP or Cy5-dCTP (Amersham Pharmacia) using random hexamer oligonucleotides to prime cDNA synthesis. RNA samples prepared from chemostat cultures at a dilution rate of 0.03 h⁻¹ (td = 23 h) were directly compared to RNA samples from chemostat cultures at a dilution rate of 0.01 h⁻¹ (td = 69 h) by competitive microarray with both dye arrangements.

**Microarrays and hybridizations.** The DNA microarrays provided by the Bacterial Microarray Group at St Georges (http://bugs.sgul.ac.uk/index.php) were constructed from PCR-amplified ORF-specific DNA, representing all of the predicted open reading frames from the *M. tuberculosis* H37Rv genome robotically spotted onto a poly-L-lysine-coated glass microscope slide (33). Prehybridisation, hybridisation and washing were performed as described by Stewart *et al.* (33). All microarray experiments were technically replicated four times with cells from three independent chemostat experiments resulting in 12 sets of microarray data.

**Data processing and statistical analysis.** Microarrays were scanned using a GenePix 4000B (Axon Instruments) at a level just below saturation of the most intensely fluorescent spot on each array. Fluorescence intensity data from each array were quantified and quality control was fully automated using BlueFuse for microarrays (version 3.3) software (Bluegnome). Hybridisation intensity values were normalised using lowess local regression, followed by across array normalisation that gives the log₂ ratios on each array the same median-absolute-deviation using limma, part of the Bioconductor package of R version 2.4.0 (10). Technical replicates were averaged and filtered using a Perl script such that only genes that had a value for each dye
orientation were used: this gave 3476 genes to test for significance. Expression values were compared using an eBayes modified t-test (32) and were also subjected to the Benjamini-Hochberg multiple testing correction using the multtest package in R (26) to obtain a false positive rate of 0.05, which corresponded to an uncorrected t-test p value of approximately 0.0048. Expression data were clustered by TIGR MeV software (29) technique using Pearson correlation coefficient as a distance measure (8).

In order to identify functionally-related groups of genes that were significantly regulated three different fold thresholds were used (1.2 fold, 1.6 fold and 2 fold). Two lists were generated for each fold threshold: genes whose expression ratio (with no significance threshold) was greater than the threshold of either 1.2, 1.6 or 2 (up-regulated genes), and genes whose expression ratio was less than 0.83, 0.625 or 0.5 (down-regulated genes). Genes were divided into 101 functional categories based on the M. tuberculosis H37Rv simplified gene ontology (http://www.sanger.ac.uk/projects/M_tuberculosis/gene_list/). Any functional groups with less than eight members were excluded from further analysis. Each set of functionally related genes were then analysed as a group and the chi squared test was performed to investigate whether the difference in the distribution of up and down-regulated genes in any set of genes differed significantly from the ratio expected by chance.

**Quantitative RT-PCR analysis.** Oligonucleotides and probes to were designed using the Primer 3 software (28) and are listed in Table S2. qRT-PCR reactions were performed as described by Phillips et al, 2003 using FAM reporter dye/TAMRA quencher dye labeled probes in conjunction with specific primer sets (25). The
quantity of DNA or cDNA in each reaction was determined with reference to a standard curve generated by amplification of known amounts of a standard DNA prepared from \textit{M. bovis} BCG. Pearson’s and Spearman’s correlations were used for the comparison of qRT-PCR and microarray data.

\textbf{RESULTS/DISCUSSION}

\textbf{Chemostat culture.} BCG was inoculated into the chemostat and, after an initial phase in batch culture, continuous culture conditions were established using Roisin’s minimal media. Roisin’s is a chemically defined glycerol limited media containing only one source of carbon plus Tween 80. Steady state conditions were established for BCG cells grown at a dilution rate of 0.01 h^{-1} corresponding to a doubling time of 69 h (“slow” growth rate) and at a dilution rate of 0.03 h^{-1} corresponding to a doubling time of 23 h (“fast” growth rate). The physiological characteristics of these cells have previously been described (3). Cells were harvested from three independent chemostat cultures and RNA extracted for transcriptome analysis.

\textbf{DNA microarray analysis.} A comparison of gene expression between slow and fast growing \textit{M. bovis} BCG cells was performed in order to identify the genes most important for slow growth and carbon limitation. It was found that 338 genes representing 8.1\% of the genome were significantly differentially expressed in the slow growing cells adapting to carbon-limited conditions. Genes that were significantly altered in expression in response to slow growth in carbon-limited conditions were divided into functional categories using the gene ontology developed as part of the genome sequencing project (http://www.sanger.ac.uk/Projects/M\_tuberculosis/Gene\_list/).
We next aimed to find functional categories of genes that were significantly changed by slow growth. A chi squared test was used to calculate the probability that the distribution of up and down-regulated genes within each functional group was consistent with the null hypothesis that the group represented a random sample of the total population of genes in that dataset. For genes with known functions it was observed that at the slow growth rate genes from the PE/PPE family, IS elements and also genes associated with the biosynthesis of the cell envelope were significantly induced whereas genes involved in the synthesis and modification of macromolecules were down-regulated (Table 1). See supporting information DataSet S1 for the transcriptomic data for all genes.

**Independent validation of microarray analysis using quantitative RT-PCR.** The microarray analysis was independently validated for a subset of genes by qRT-PCR. The qRT-PCR results corroborated the microarray data for the eight genes tested (Spearman correlation = 0.87, Pearson correlation = 0.86). These data indicated that the fold-change results determined by qRT-PCR were often greater than the fold change for the same gene by microarrays (Fig.1). Ratio underestimation is a commonly recognized feature of microarray technology (40). The excellent agreement between the microarrays and the qRT-PCR substantiated the statistical approach that we utilized during this study.

**Functional Groups of Genes that respond to modulation of growth rate:**

**Synthesis and modification of macromolecules.** The observed down regulation of genes encoding ribosomal proteins during the slow growth of *M. bovis* BCG is consistent with our previous demonstration that ribosome production in *M. bovis* BCG is subject to growth rate-dependent control (3); and has also been observed in other
models of persistence (4). Despite this general downturn in ribosomal protein synthesis, four ribosomal protein genes, \textit{rpsR2}, \textit{rpsN2}, \textit{rpmG} and \textit{rpmB2} were significantly upregulated by 2.58, 6.02, 4.81 and 7.69 respectively at the slow growth rate. These genes are arranged in an operon and separated from the majority of ribosomal genes. Evidence suggests that the RpmB and RpmG proteins of \textit{E. coli} have related roles in ribosome synthesis and function (19). Ribosome synthesis is defective and not very well coordinated when these proteins are absent from \textit{E. coli} (19). The fact that these ribosomal proteins are regulated independently from their other family members in slow growing \textit{M. bovis} BCG suggests that they may also have a growth rate-related function in \textit{M. tuberculosis}. Voskuil \textit{et al} (37) speculated that RpsN, RpmB and RpmG may increase ribosomal fidelity but further work is required to establish the function of these proteins.

\textbf{Energy metabolism.} Three TCA cycle genes (\textit{sucB}, \textit{sucC}, \textit{icd2}) were down-regulated by 0.68 to 0.78 fold but we found no evidence for transcriptional activation of the glyoxylate shunt at slow growth rate; indeed, the \textit{glcB} gene encoding malate synthase was downregulated (0.67 fold) whereas the \textit{icl1} and \textit{icl2} genes encoding isocitrate lyase were unchanged. The \textit{glcB} gene was also downregulated in \textit{M. tuberculosis} cells starved in PBS (4).

Despite the oxygen levels in the chemostat vessel being continuously maintained at 70 to 100\% aerobic respiratory metabolism was down-regulated and three genes (\textit{frdA}, \textit{fdhD} and \textit{narJ}) encoding proteins involved in anaerobic respiration were upregulated by 1.35 to 1.46 fold in the slow growth rate. This is consistent with the diminished need for respiration during slow growth. Anaerobic respiration has previously been implicated in persistent \textit{M. tuberculosis} (38) and upregulation of other anaerobic
genes has been demonstrated previously in a mouse infection model (31) and also reported under oxygen sufficient conditions (11). The gene frdA was induced in macrophages residing in activated macrophages and also in the pericavity and distant lungs of humans with tuberculosis. Upregulation of 12 out of the 48 genes controlled by the DosR two component response regulator is also puzzling. Although DosR (Rv3133c) was not significantly induced in the chemostat model (1.39 fold, p value = 0.14), the gene Rv3134c, which is presumed to be a member of the same operon as dosR, was upregulated 1.8 times (p value = 0.028). The DosR system has been shown to be responsive to hypoxia in pathogenic and non-pathogenic mycobacteria (21, 24). However, it was demonstrated that dosR is also induced by nitrogen oxide and a number of other stresses in M. tuberculosis (15, 36) and upregulation of a subset of genes in the DosR regulon was also demonstrated as M. tuberculosis entered the stationary phase of growth (37). Two kinases have been identified (DosS and DosT) that phosphorylate DosR in response to reduced oxygen tension and nitric oxide. Neither of these genes were induced during slow growth in the chemostat model. An explanation of these results is that a subset of the DosR regulon is modulated by other environmental cues such as slow growth rate or nutrient limitation and then relayed through a different regulatory mechanism. Alternatively it is possible that the remaining members of the DosR regulon were upregulated in the chemostat model but were not detected in this study due to the sensitivity level of the microarrays. The qRT-PCR results presented here support this hypothesis. Irrespective of which scenario proves to be correct our data provided further evidence that in addition to hypoxia, slow growth rates and nutrient limitation modulate the expression of genes in the DosR regulon.
**Lipid Biosynthesis.** Array analysis revealed a significant down-regulation of genes involved in the modification of fatty and mycolic acids.

**Cell envelope.** *M. tuberculosis* undergoes immense changes in the cell envelope upon infection in both macrophages and in human lungs and modification of the cell wall is likely to be an important survival strategy for *M. tuberculosis*. Our study showed that slow growth rate and carbon limitation induced 22 membrane proteins suggesting some remodeling of the cell envelope.

**Chaperones and heat shock.** There was no trend observed in the transcription of chaperones and heat shock genes. The gene encoding the DosR regulon member alpha crystallin protein, *hspX*, was upregulated 3.6 fold (*p* = 0.004). Evidence suggests that HspX is an important protein in the pathogenetic strategy of *M. tuberculosis*. The *hspX* gene has been found to be upregulated during stationary phase (37), in several models of dormancy (23,37), during the growth of *M. tuberculosis* in macrophages (30), mice (31) and in lung specimens (34) from patients with TB. Recent work has demonstrated a role for HspX in slowing the growth of *M. tuberculosis* both *in vitro* and *in vivo* (14). A gene replacement mutant for *hspX* in *M. tuberculosis* exhibited increased growth in macrophages and also in a mouse model, whereas overexpression of the *hspX* gene resulted in the reduction of growth rate for *M. tuberculosis* *in vitro* (14, 39). The demonstration that *hspX* was induced during slow growth in a carbon-limited chemostat provides further evidence for the role of this chaperone in reducing the growth rate of *M. tuberculosis*.

**IS elements, repeated sequences and phage.** The expression of IS elements was significantly elevated at slow growth rate with 11 genes upregulated. Four of these
genes were also induced during the stationary phase of growth and in Wayne’s model of non-replicating persistence (37).

**PE and PPE families.** We observed a significant induction of the antigenic PE and PPE family of genes at slow growth rate under carbon limiting conditions. One of these genes, Rv0834c has been shown to be differentially expressed in the host cell (35). Localized at the cell surface PE/PPE protein participate in cell surface interactions and maybe involved in immune evasion.

**Comparisons of chemostat model with other in vitro models.**

The transcriptional response to slow growth rate in the chemostat was compared to expression profiles obtained for several *in vitro* models: (1) a PBS starvation model (4); (2) an extended stationary phase model (11); (3) Wayne’s model of persistence (23). These models were selected as they all use nutrient and/or oxygen limitation to induce slow growth rates in *M. tuberculosis*. The Wayne model was developed to investigate the effects of hypoxic conditions on mycobacteria and involves cultivating mycobacteria in sealed slowly stirred tubes exposed to limited headspace volume of air. The PBS model has been used to investigate the response of *M. tuberculosis* to nutrient starvation by resuspending mycobacterial cultures in PBS. During the extended stationary phase model of TB, *M. tuberculosis* was cultivated in a bioreactor for 100 days. The data was also compared to another chemostat model investigating the effects of 1% oxygen on *M. tuberculosis* at a constant growth rate (2). In addition, in order to establish whether the requirements for survival in the chemostat model had any similarities to the requirements for growth *in vivo*, gene expression data of *M. tuberculosis* growing in activated macrophages (after 24 hours of infection) was also compared with the data from this study (30). The comparison was performed by
downloading lists of genes significantly altered in expression in the models according
to the criteria of the original researchers that were provided as tables supplementary to
the original publications. The extraction of comparable data from these publications
was complicated by the different formats used for presenting the data and also
differences in criteria for including genes in the lists and therefore the analysis
presented (Fig. 2) is only preliminary in its nature. The problems associated with
comparing DNA expression data from different studies has been highlighted in a
review article by Kendall et al. (16).

Some similarity was observed between the slow growth rate response studied here and
the transcriptome study of Wayne’s model of persistence reported by (23). The
common response in this case was likely to be to slow growth in both models, rather
than oxygen limitation, since there was very little correlation between the
transcriptional profile of BCG in the chemostat model of TB and the response of
*M. tuberculosis* to oxygen limitation in a chemostat (2).

**Comparisons of chemostat model with adaptation to macrophage growth.**

The transcriptional response to slow growth rate in the chemostat was compared to
expression profiles for *M. tuberculosis* from infected macrophages. Surprisingly, 76
genes with altered gene expression in *M. tuberculosis* after 24 hours of macrophage
infection were similarly regulated in BCG growing in the chemostat model (30). This
result provided evidence that the chemostat model recapitulates at least some of the
conditions experienced by *M. tuberculosis* whilst growing inside the host.

To quantify the level of similarity, clustering analysis was performed using the
Pearson’s algorithm to examine the correlation between transcriptomic profiles for the
chemostat grown BCG and the datasets described by Schnappinger et al (28) including macrophage-adapted *M. tuberculosis* cells (naïve, activated and NOS2 knock-out macrophages) and also two *in vitro* conditions (hydrogen peroxide treatment or growth in palmitic acid media). The clustering algorithm separated the normalized data into two distinct clusters (Fig. 3). The first cluster included data from the chemostat model presented here, activated macrophages after 24 h and 48 hours of infection and post hydrogen peroxide treatment whilst all the other data formed a second cluster. Within this cluster the chemostat and the activated macrophage datasets were the most closely related. The clustering of the chemostat model data and post hydrogen peroxide treatment is probably a reflection of the reduction in growth rate induced by this treatment. The second cluster contained all the naïve macrophage data, activated macrophage after 4 h of infection and the NOS2 knock-out macrophage data.

To further evaluate the nature of the correlation between the chemostat and the macrophage data and to identify the level of correlated genes expression change, chi squared test significance values were calculated for the chemostat data and all datasets described by Schnappinger et al (30). This was performed by identifying genes as either up or down regulated or unchanged using three different threshold (2-fold, 1.6 fold and 1.2 fold) values. The chi squared test was then used to test the null hypothesis that the distribution of up-regulated, down-regulated and unchanged genes in the paired datasets (*e.g.* macrophage and chemostat) was compatible with the null hypothesis that the gene expression levels were independently distributed. High levels of correlation (low p values) were found between the chemostat data and macrophage data (30) at all fold changes tested (Table 2). With a 1.2-fold threshold, the macrophage data was most highly correlated with the chemostat data. However, when
a two-fold cutoff was used the highest correlation with the macrophage data was with the hydrogen peroxide-treated cells. Together, these findings indicate that the common regulatory changes between the macrophage and slow growth in the chemostat involve a large set of genes but only small deviations in gene expression (that are lost when a two-fold threshold is used); whereas the correlated changes between the macrophage and hydrogen peroxide treatment involve a smaller set of genes but larger (greater than two-fold) deviations in gene expression (Table 2).

Simple extraction of gene lists based on fold change and significance values is a useful means of revealing gross changes in gene expression. However, this strategy may miss important information that can be gained from more subtle examination of genome-wide changes in expression profile. Several methods have been developed to identify sets of functionally related genes that are significantly regulated in a particular dataset, such as Gene Set Enrichment Analysis (GSEA) (22), GO::TermFinder (5) and related methods (1). The basic principle of these methods is to use Gene Ontology terms to label genes which are then ranked according to some measure of gene expression (gene expression level or significance) and the resulting ranking order examined to discover terms which are not uniformly distributed throughout the list. Unfortunately, GO ontology is not currently available for *M. tuberculosis* and so we developed a related approach that does not require GO terms. As part of the genome sequencing project (http://www.sanger.ac.uk/Projects/M_tuberculosis/Gene_list), all *M. tuberculosis* genes have previously been divided into 101 (non-overlapping) functional groups based on a gene ontology derived for *E. coli* (27). To identify the common functional components that characterizes the response of *M. tuberculosis/M. bovis* BCG to the macrophage environment and slow growth in the chemostat, each dataset
(macrophage and chemostat) was first ranked to identify the mostly significantly regulated functional groups (using the 1.2-fold threshold that generated the highest level of correlation, as shown in Table 2). To do this, a chi squared test was used to calculate the probability that the distribution of up and down-regulated genes within each functional group was consistent with the null hypothesis that the group represented a random sample of the total population of genes in that dataset. The functional categories were then ranked on the calculated significance values for rejection of the null hypothesis. The similarity of the resulting ranking orders (data not shown) indicated that the most significantly regulated groups were common to the macrophage and chemostat. To investigate whether regulatory changes were in the same or opposite direction, the proportion of genes that were either up or down regulated or unchanged were plotted (Fig. 4) for the most significantly regulated functional categories in each dataset. The resulting plot provides a novel systems-level comparison of the transcriptional response of the tubercle bacillus to slow growth in the chemostat and to growth inside a macrophage. The similar plots obtained indicated that the response of the tubercle bacillus to either slow growth in the chemostat or adaptation to the macrophage environment (using the 1.2-fold cutoff) involves concordant changes of similar sets of functionally related genes. Concordance was observed particularly amongst genes involved in the TCA cycle (down regulated), aerobic respiration (down-regulated), ATP-proton motive force (down regulated), protein translation and modification (down-regulated), protein and peptide secretion (down-regulated), DNA replication and repair (up-regulated) and IS elements (upregulated). Some important differences were also found. For example the surface polysaccharides were down-regulated in the macrophage but mostly unchanged inside the chemostat; and the group of repressors and activators were not
significantly changed in the chemostat but upregulated in the macrophage. The results clearly demonstrate that a significant component of the tubercle bacillus’ adaptation to survival inside a macrophage is the global gene expression changes that are associated with adaptation to slow growth rate, which can be studied in the chemostat.

**Conclusions.** The transcriptional response of *M. bovis* BCG to the changes in growth rate in carbon-limited chemostats was analysed. In this system the BCG cells were actively growing at sub-optimal growth rates and in nutrient limited conditions. This system therefore differs from other *in vitro* models of mycobacterial growth in that the bacteria were neither starving nor feasting and the emphasis was therefore on the transcriptional consequences of strict carbon limitation in actively growing but ‘hungry cells’. This model also has significant advantages over these batch models in that bacteria are in a defined and constant environment allowing growth rate to be investigated independently of any other environmental parameter.

There was some overlap between the transcriptional responses of *M. bovis* BCG in the chemostat model and the responses reported for *M. tuberculosis* in other published models of persistence and therefore slow growth rates and carbon limitation are likely to be common features of all these models (4). A high level of correlated gene expression was found between BCG growing in the chemostat model and *M. tuberculosis* growing in macrophage (30), demonstrating a significant proportion of the gene regulatory changes measured for the adaptation of mycobacteria to survival in the macrophage can be accounted for by the adaptation of the bacillus to slow growth in carbon-limited conditions, as measured in the chemostat.

**SUPPLEMENTAL MATERIAL**
**Table S2.** Quantitative RT-PCR primer and probe information.

**DataSet S1.** Regulation of *Mycobacterium bovis* BCG genes in the chemostat model of tuberculosis.

**ACKNOWLEDGEMENTS**

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Table 1. Summary of the most significant functional groups of genes with significantly altered expression in *Mycobacterium bovis* BCG in a carbon limited chemostat culture of *M. bovis* BCG at a dilution rate of 0.01 h\(^{-1}\) (td = 69 h) as compared with a dilution rate of 0.03 h\(^{-1}\) (td = 23 h).

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<th>Down regulated</th>
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\(^a\)Functional category according to http://www.sanger.ac.uk/projects/M_tuberculosis/Gene_list/
\(^b\)Chi squared p value
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<th>Table 2. Chi squared test significance values$^a$</th>
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$^a$The chi squared test was used to test the null hypothesis that the distribution of genes between up and down regulated categories was independently distributed.
FIG 1. Comparison between the gene expression ratios obtained from microarray analysis (black bars) compared with quantitative RT-PCR (grey bars).

The values represent the average ratio of three independent biological replicates and the error bars indicate the standard deviation.
FIG 2. Comparison of the gene expression profiles from the chemostat model of persistence with other published microarray studies.

Bars with diagonal stripes represent the percentage of genes significantly regulated in response to slow growth in the chemostat that were also significantly regulated in the published microarray study. Approximately 9% of genes were regulated in the chemostat model so this value represents a baseline for the comparison (if there was no relationship between the paired datasets then we would expect approximately 9% of genes in any randomly generated list to be also regulated in the chemostat) and is represented as a dotted line. The grey bars represent the percentage of genes that were concordantly regulated whereas the spotted bars represent the percentage of genes discordantly regulated. The data was obtained from Betts et al (4)(starvation), Hampshire et al (11) (extended stationary), Muttucumaru et al (23) (Gradual oxygen depletion), Bacon et al, (2) (1% O₂) and Schnappinger et al (30) (macrophage).
FIG. 3. Hierarchical clustering of the transcriptional response of *Mycobacterium bovis* BCG growing in the chemostat model of TB and macrophage adapted *Mycobacterium tuberculosis* (naїve, activated and NOS2 knock-out macrophage) and also hydrogen treatment and growth in palmitic acid (30).

Expression data were clustered using TMeV data analysis software in a Pearson correlation analysis (8, 29).
FIG. 4. Proportion of either up, down or unchanged genes during growth in the chemostat model (A) and after 24 hours in activated macrophages (B) in functional groups that were significantly regulated (Chi squared p value <0.05) in one or both of the datasets. The macrophage data was obtained from Schnappinger et al. (30). The percentage of down regulated genes are represented by light grey bars, dark grey bars represent the proportion of up-regulated genes and the white bars represent the percentage of genes that were unchanged in each functional group. The gene ontology is from http://www.sanger.ac.uk/projects/M_tuberculosis/Gene_list/.


