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Mycobacterial Bacilli Are Metabolically Active during Chronic Tuberculosis in Murine Lungs: Insights from Genome-Wide Transcriptional Profiling▽†

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Chronic tuberculosis represents a major health problem for one-third of the world's population today. A key question relevant to chronic tuberculosis is the physiological status of *Mycobacterium tuberculosis* during this important stage of infection. To examine the molecular bases of chronic tuberculosis and the role of host immunity in mycobacterial growth, we determined the mycobacterial transcriptional profiles during chronic and reactivation phases of murine tuberculosis using in vivo microarray analysis (IVMA). Following 28 days of aerosol infection, mycobacterial counts remained stable, although the bacilli were metabolically active with a 50% active transcriptome. The expression of genes involved in lipid and carbohydrate pathways was significantly enriched during the middle stage of chronic tuberculosis, suggesting a nutrient-rich microenvironment. A total of 137 genes were significantly regulated in mid-chronic tuberculosis (45 and 60 days) compared to an early stage (14 days) of infection. Additional sets of genes, including the virulence regulator *virS*, were up-regulated during the reactivation stage, indicating their possible roles in mycobacterial resurgence. Interestingly, a set of potential transcriptional regulators was significantly induced at the late stage of chronic tuberculosis. Bioinformatic analysis identified a large number of genes that could be regulated by one of the potential transcriptional regulators encoded by rv0348, including the *sigF* operon. Taken together, IVMA provided a better definition of the transcriptional machinery activated during chronic and reactivation stages of tuberculosis and identified a novel transcriptional regulator. A similar approach can be adopted to study key stages of intracellular pathogens.

One-third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, with an annual death rate of more than 2 million (8). Treatment regimens are effective in only 60 to 90% of patients with persistent infection (14). In humans, tuberculosis can be divided into three phases (28): an active phase, characterized by initial replication of *M. tuberculosis* that triggers host cell-mediated immunity; a chronic phase, in which infected individuals are not infectious, with undetectable levels of tuberculous bacilli; and finally, a reactivation phase, which occurs in 10% of patients with an intact immune system. The reactivation rate is even higher for immune-suppressed individuals (19). Both in vitro and in vivo models have been used to investigate tuberculosis during the dormancy and reactivation phases in humans. One of the first in vitro models of tuberculosis dormancy was established by Wayne (49, 50) and is based on culturing *M. tuberculosis* under decreasing concentrations of oxygen levels, creating a microaerophilic environment where mycobacteria transform into a nonreplicating persistent form. More in vitro models were developed to study chronic tuberculosis, and they have shown that the host microenvironment is rich in reactive nitrogen intermediates (29, 48) but deficient in nutrients necessary for bacterial survival (5), suggesting that tuberculous bacilli persist in a metabolically inactive form. For in vivo studies, the mouse model of chronic and reactivation stages of tuberculosis was used to profile the immunological responses activated during both phases (9, 34). Using an artificial-granuloma implant in mice, the DosR regulon was shown to play a role in mycobacterial entry into the dormant phase, with the involvement of *relA* (20). Unfortunately, the main genetic bases of the mycobacterial conversion from the "dormant" to the "replicating" phase in the lungs has remained largely unknown. In this report, we communicate our efforts to investigate both the chronic and reactivation phases of tuberculosis.

A key question relevant to tuberculosis is the physiological status of *M. tuberculosis* during different stages of infection. The work of different groups (27, 39) using quantitative PCR on both the genomic and transcriptional levels indicated that mycobacterial bacilli persist at a constant level with a very low growth rate. However, many questions related to chronic tuberculosis remain unanswered. Does the low growth rate mean that the bacilli are metabolically inactive? Can the mycobacterial bacilli sense the surrounding microenvironment? Also, do mycobacterial bacilli adapt to the change in their microen-

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vironment following the change in host immunity? Previously, the low growth rate of *M. tuberculosis*, the need to conduct experiments under strict biosafety conditions, and its intractable genetic system made deciphering chronic tuberculosis problematic. Fortunately, the availability of the *M. tuberculosis* whole-genome sequence and the development of several protocols designed to examine *M. tuberculosis* during in vivo growth (20, 33, 42) have allowed great strides in tuberculosis research. A recent analysis of the early stages of tuberculosis on a genome-wide level revealed several sets of genes that are specifically activated during lung infection of immune-competent mice, while other sets of genes were activated in immune-compromised mice (43). Another transcriptional profiling of infected human lungs portrayed a bacillus transcribing hundreds of unique genes, depending on the site of sample isolation (31).

To address some of the questions raised above, we adapted a DNA microarray-based protocol (43) to profile the transcriptome of *M. tuberculosis* during the chronic and reactivation stages of tuberculosis in mouse lungs. Our analysis indicated that despite the stability of mycobacterial colonization levels during chronic tuberculosis, the bacilli remained metabolically active, especially during the middle phase of chronic tuberculosis. A set of potential transcriptional regulators were identified throughout the examined times, with much higher levels of induction during the late stage of chronic infection. One of the transcriptional regulators (encoded by rv0348) was also shown to bind to its own putative promoter sequence and potentially to regulate several other genes that could be involved in establishing chronic tuberculosis. Finally, the analysis employed identified a set of genes ($n = 174$) that was specifically regulated during the reactivation stage of tuberculosis, including a well-studied virulence regulator, *virS* (17).

MATERIALS AND METHODS

Mouse infection. BALB/c mice (21 to 28 days old) purchased from Harlan (Indianapolis, IN) were infected with $\sim 10^2$ CFU/animal via intranasal instillation or inhalation using the Glas-Col inhalation system (Glas-Col, LLC, Terre Haute, IN). Within 4 h postinfection (p.i.), lung tissues were collected from two mice to verify the exact infectious dose, and the two methods of infection yielded comparable colony counts. At designated times p.i. (28, 45, and 60 days), lung tissues were harvested from groups of infected mice ($n = 50$) using established protocols (43). For the reactivation experiment, some groups were injected daily with dexamethasone (0.1 mg/day) for 7 or 15 days starting at 45 days p.i. according to earlier protocols (34, 47). At 52 and 60 days p.i., lung tissues were harvested and subjected to RNA extraction and subsequent DNA microarrays. For each examined time, histopathology and colony counts of the harvested tissues (lung, liver, and spleen) were performed as described previously (43, 44).

RNA extraction. To isolate mycobacterial RNA, lung tissues used for DNA microarrays were harvested and placed directly onto dry ice. The lungs from each group of four mice were rapidly homogenized in 20 ml of chilled 0.01% sodium dodecyl sulfate (SDS), followed by filtration through a nylon wool column to remove large tissue particles. The filtrates were collected in 50-ml Falcon tubes (chilled in ice) and centrifuged at 4,000 rpm and 4°C for 10 min to collect bacterial pellets released from disrupted cells. Additionally, mycobacterial cultures started from the same seed (1:100 dilutions of bacteria frozen at mid-log phase) were allowed to grow in Middlebrook 7H9 broth supplemented with 10% albumin-dextrose complex. Culture samples were collected after 28, 45, and 60 days postinoculation for total RNA extraction to serve as comparison points for the in vivo-growing mycobacteria. Total RNA was extracted from bacterial pellets (harvested from cultures or lungs) using Tri-Zol reagent (Gibco) and zirconium beads in a bead beater unit (three pulses for 30 seconds each) (BioSpec Products, Inc., Bartlesville, OK) (42). For real-time quantitative PCR analysis, lungs from a small number of infected animals ($n = 10$) were harvested and placed directly onto RNA*Later* (Ambion, Austin, TX), an RNA stabilizer, before homogenization and RNA extraction as described above.

Microarray hybridization. Extracted total-RNA samples were used in a standard reverse transcriptase reaction in the presence of *M. tuberculosis* genome-directed primers (mtGDPs) (41). Briefly, RNA samples (10 to 15 μg) were labeled using CyScribe post-labeling kits (GE Healthcare Life Sciences, Piscataway, NJ) as described in the manufacturer's manual, with the exception of using 6 μl of mtGDPs (250 ng/μl) to prime the transcription reactions instead of using random primers. Mycobacterial genomic DNA (gDNA) was labeled using a nick translation kit from Promega (Madison, WI) according to the manufacturer's protocol. The unincorporated nucleotides were removed from the labeled samples using QiaQuick PCR purification kits (QIAGEN, Inc., Valencia, CA). The Cy3-labeled cDNA samples were cohybridized with the Cy5-labeled gDNA to oligonucleotide DNA microarrays (Operon Technologies; Tuberculosis set version 1.1) (41) designed from the genome sequence of *M. tuberculosis* H37Rv (11). Following incubation at 67°C for 12 to 16 h, the hybridized slides were washed for 5 min at room temperature in low-stringency wash buffer (1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]-0.1% SDS), followed by a 3-min wash in high-stringency buffer (0.1× SSC). Centrifuge-dried slides were scanned using a GenPix 4.0B laser scanner (Molecular Devices Corp., Sunnyvale, CA) to generate 16-bit TIFF images representing signal intensities of both cDNA and gDNA. Hybridization signals from cDNA samples were compared to the hybridization signals generated from gDNA samples to estimate the relative gene expression levels (41). Employing such a protocol for normalizing gene expression levels (termed genomic normalization) proved to be superior to other protocols that use RNA or spiked-sample normalization (41, 51). Simple ratios from single hybridizations were converted to logarithmic scale before a subsequent Lowess normalization protocol was employed (GeneSpring 7.0; Agilent Technologies, Santa Clara, CA). A total of five to eight hybridizations were analyzed for each RNA sample, extracted from two repeated biological samples. The significance of differences in gene expression levels was tested using a nonparametric test, such as Kruskall-Wallis ($P < 0.05$), after applying the false-discovery rate correction protocol to normalize for multiple-comparison testing. Normalized gene expression levels were analyzed using a hierarchical cluster algorithm (12) to identify the temporal changes in transcripts associated with samples collected at different times p.i. (The Institute for Genomic Research; MeV 4.0 [http://www.tm4.org/]).

Real-time quantitative PCR. For real-time quantitative PCR, total-RNA samples were treated with RNase-free DNase I (Ambion, Austin, TX) for 1 h at 37°C before undergoing a standard reverse transcription reaction in the presence of mtGDP as outlined previously (41). The generated cDNA served as a template for quantitative PCR in the presence of gene-specific primers (see Table S1 in the supplemental material) and SYBR Green dye (35, 36). For each amplification run (iCycler thermocycler; Bio-Rad), the calculated threshold cycle (C_T) for each gene amplicon was normalized to the C_T of the 16S rRNA gene (amplified from the same sample) before calculating the change from in vivo/in vitro samples at different times. The following formula was used to estimate change: change (n-fold) $= 2^{-\Delta\Delta C_T}$, where $-\Delta\Delta C_T$ for gene j was equal to $(C_{T,j} - C_{T,16srRNA})_{\text{in vivo}} - (C_{T,j} - C_{T,16srRNA})_{\text{in vitro}}$. The melting curves for all reactions were examined to identify primer-dimer formation and to ensure the uniformity of the amplicons of all the genes.

Sequence analysis. The BLAST algorithm (2) was used to search the nonredundant GenBank protein database for proteins similar to Rv0348. An E score of <0.05 was used as the cutoff value for similar sequences. To identify potential binding sites for the Rv0348 protein, the pattern search algorithm provided by the TubercuList website (http://genolist.pasteur.fr/TubercuList/) was used, allowing for two or three mismatches for either −35 or −10 regulatory sequences. Searches included the first 900 bp upstream of each gene, in addition to the first 100 bp following the start codon. The search strategy included a 1,000-bp cutoff limit to accommodate genes that were members of operons.

Protein purification and electrophoresis mobility shift assay (EMSA). Cloning, protein production, and purification were described in detail previously (4). Briefly, a maltose binding protein (MBP) fusion of *M. tuberculosis* Rv0348 was constructed in *Escherichia coli* using the pMAL-c2 vector (New England Biolabs, Beverly, MA). The MBP-Rv0348 protein was then overexpressed by induction with 0.3 mM isopropyl-β-D-thiogalactopyranoside (Sigma, St. Louis, MO) and purified by affinity chromatography using an amylose resin supplied by New England Biolabs. The expression of mycobacterial fusion proteins was monitored with GelCode Blue (Pierce Biotechnology Inc., Rockford, IL)-stained SDS-polyacrylamide gel electrophoresis gels. Selected fractions eluted from the amylose resin column were pooled and dialyzed using Slide-A-Lyzer cassettes (Pierce Biotechnology Inc.) in 1.5-liter phosphate-buffered saline (150 mM NaCl, 10 mM

$NaPO_4$, pH 7.4), with three exchanges at 4°C. The purified protein aliquots were stored at −20°C.

For EMSA, probes were generated by PCR amplifications. PCR primers were designed with Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) by providing the upstream probable regulatory sequences of selected genes. PCR amplicons were analyzed on 2% agarose gels and then purified with the Wizard SV Gel and PCR Clean-Up System (Promega). The purified DNA fragments (3.5 pmol) were end labeled with 10 U of T4 polynucleotide kinase (Promega) and 10 μCi of [γ^{32}-P]ATP (Perkin-Elmer, Wellesley, MA) at 37°C for 10 min. Various amounts of the purified proteins and probes were incubated in EMSA binding buffer (20 mM KCl, 5% glycerol, 25 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 10 ng/ml herring sperm DNA) at 25°C for 10 min in a total volume of 10 μl. The mixtures were resolved on 4% native polyacrylamide gels that were subsequently dried and exposed to X-ray films (Kodak, Rochester, NY).

RESULTS

Persistent infection with *M. tuberculosis*. Our working definition of chronic tuberculosis is the presence of a constant level of mycobacterial bacilli in the host organs while the host appears relatively healthy, reflecting an equilibrium between the host and *M. tuberculosis* (13). Generally, mice infected with a low dose of aerosolized *M. tuberculosis* develop a persistent infection by 28 days p.i., which continues for prolonged times (>300 days) (21). Before conducting microarray analysis of *M. tuberculosis*-infected mice, we examined organ colonization levels to test the feasibility of isolating enough mycobacterial RNA for microarray analysis. A protocol for a low-dose aerosolization (32) with the virulent strain H37Rv was used to mimic natural infection in humans. As expected, colony counts of *M. tuberculosis* in organs were higher at 21 and 28 days p.i. than at later times (60, 120, and 140 days p.i.) (see Fig. S1 in the supplemental material). Analysis of the bacterial load of lung tissues following the initial infection indicated the presence of sufficient mycobacterial bacilli (~10^6 CFU/g) suitable for transcriptional analysis. Generally, the level of bacterial colonization remained almost unchanged in all organs (liver, spleen, and lung) from 60 days onward, indicating the transition from active to chronic stages of infection. Histological examination of *M. tuberculosis*-infected mouse tissue collected at different times p.i. showed signs of tuberculosis progression for aggregates of lymphocytes and macrophages (at early stages, i.e., 21 and 28 days p.i.) to a more organized inflammatory response with the presence of foamy macrophages (at later times, e.g., 140 days p.i.). At all times examined, acid-fast bacilli were seen in lung sections, while their numbers per microscopic field declined over time. Both the colony counts and the histological findings proved that persistent tuberculosis was established in mice following low-dose (10^2 CFU) aerosolization. Because this stage of infection spans a long time, generally between 20 and >300 days p.i., we and others (16, 32) further divide the chronic phase of tuberculosis into early, middle, and late stages based on histological changes observed in animals (see Fig. S1 in the supplemental material). Subsequent analyses described here are focused on the middle stage of chronic tuberculosis (45 and 60 days p.i.), using DNA microarrays. The late stage (140 days p.i.) of chronic tuberculosis was analyzed using real-time PCR to accommodate the small amount of mycobacterial RNA isolated at that stage.

IVMA during chronic tuberculosis. To profile the expression levels of mycobacterial genes as the bacilli enter the chronic stage of tuberculosis, total RNA was isolated from *M. tuberculosis* directly following its harvesting from lungs of infected mice (43). Using genome-directed primer technology (42), RNA samples collected at 28, 45, and 60 days p.i. were processed for DNA microarrays in comparison to mycobacterial cultures grown in Middlebrook 7H9 broth, which were sampled 28, 45, and 60 days after inoculation. The quality of the hybridization signals was assessed using scatter plot analysis of sample replicates and by calculating the coefficient of variance (CV) as suggested previously (46) (Fig. 1A and B). Sample hybridizations with high correlation levels ($P > 0.9$) among replicates were used for downstream analysis. Almost 18% of the hybridization experiments (out of 56 hybridizations used throughout this project) were excluded from analysis because of low correlation levels among replicates. Additionally, genes with a CV of >1 within each group of replicates (0.3 to 1% of the genes), indicating low reproducibility, were also excluded from downstream analysis. Finally, to examine the overall performance of DNA microarrays to estimate gene expression levels from BALB/c samples, a randomly chosen set of genes ($n = 21$) were selected for real-time PCR analysis (36). Such analysis included genes with either induced or repressed levels. An agreement in induction or repression of 86% of the genes was obtained when IVMA and real-time PCR expression levels were compared (see Table S1 in the supplemental material).

To compare gene transcripts in a large number of replicates (six to eight replicates/time point) among several times following infection, we employed a genomic normalization protocol that was able to accurately profile the transition in mycobacterial transcripts during growth from log to stationary phases (41). In this protocol, each Cy3-labeled sample derived from RNA was cohybridized with Cy5-labeled gDNA extracted from *M. tuberculosis* H37Rv, the same strain used for animal infections. Generally, genes were considered transcribed only when they passed all of the filters described above and when the normalized signal intensity generated from the labeled cDNA was equal to or more than that generated by gDNA hybridization (i.e., the primary cDNA/gDNA ratio was ≥1). Also, to compute the change in gene transcripts, the ratios of the primary ratios were compared among different samples.

Analysis of the overall change in the number of mycobacterial transcripts identified a unique global pattern that needs to be highlighted. Contrary to the IVMA of active tuberculosis, where transcription levels were detected in >75% of the genes at 14 days (43), IVMA of the middle phase of chronic tuberculosis (45 and 60 days p.i.) detected hybridization signals in ~50% of the genes transcribed during growth in BALB/c mice compared to only 43% for in vitro cultures (Fig. 1C and D). The change in gene transcripts did not reach statistical significance for all genes with detectable levels. In fact, only 299 genes (7.6%) were significantly changed among in vivo (BALB/c) compared to in vitro (7H9 broth) samples during the progression to the middle phase of chronic tuberculosis (see Table S2 in the supplemental material). The main goal of such analysis is to identify genes activated solely during infection but not during in vitro growth. Compared to the in vitro cultures at 45 days p.i., the significantly up-regulated genes in BALB/c mice included genes involved in the biosynthesis of peptidoglycan (*glmU*) and lipid hydrolysis (*lipM*), as well as stringent stress response (*lytB2*). At 60 days p.i., mycobacterial genes

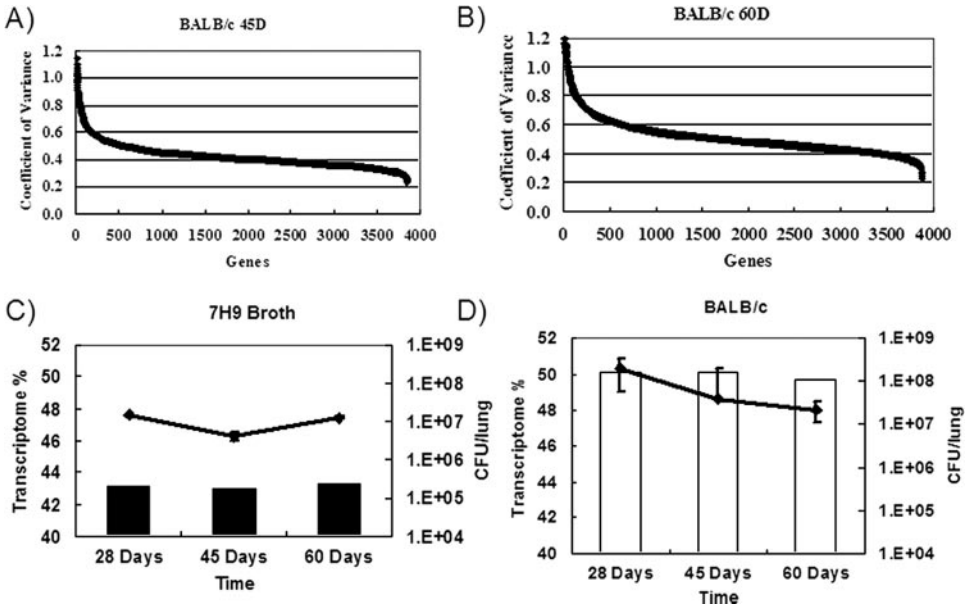

FIG. 1. Transcriptional profile of *M. tuberculosis* during early and middle stages of chronic tuberculosis. (A) A sorted dot plot displaying the CV of genes with detectable levels of gene expression in BALB/c mice at 45 days p.i. (B) A sorted dot plot displaying the CV of genes with detectable levels of gene expression in BALB/c mice at 60 days p.i. (C) Transcriptional profile of *M. tuberculosis* growing in 7H9 broth (bars) and CFU counts (line graph) at 28, 45, and 60 days p.i. (D) Transcriptional profile of *M. tuberculosis* growing in BALB/c mouse lungs (bars) and CFU counts (line graph) at 28, 45 and 60 days p.i. The error bars indicate standard deviations.

involved in glycolysis (*fba*), thiamine biosynthesis (*thiE*), and base excision repair (*ung*) were up-regulated in mice.

A unique transcriptional profile for chronic tuberculosis. To further identify unique patterns of gene expression associated with mycobacterial growth in the murine lung, we employed a hierarchical clustering algorithm (12). The applied algorithm portrayed a distinctive pattern of gene expression of *M. tuberculosis* growing in mouse lungs compared to those bacilli growing in vitro (Fig. 2A; see Table S3 in the supplemental material). Additionally, transcripts from bacilli growing at later times (45 and 60 days) within each cluster (in vitro or in vivo) were closely related to each other compared to the profile of 28-day samples, implying the transition of the tuberculous bacilli to the chronic stage. Again, this pattern confirms the overall similarity between 45- and 60-day samples. Some of the clusters included groups of genes with higher expression levels during survival in BALB/c mice than during growth in vitro. One of these clusters included genes involved in the Krebs cycle (*icd2*) and arginine metabolism (*rocD2* and *adi*) as well as peptidoglycan metabolism (*glmU*). In another cluster, the potential transcriptional regulator rv0348 was coexpressed with other genes, such as those involved in lipid transfer across the mycobacterial membrane (*ltp1*), sialic acid transfer (*nanT*), aerobic respiration (*glpD1*), and lipid degradation (*fad16*).

The metabolic state of *M. tuberculosis* during chronic infection. A major objective of the IVMA of chronic tuberculosis is to assess the physiological status of mycobacterial bacilli as they enter this important phase of the infection. We considered bacilli metabolically active when the majority of the genes in key metabolic pathways, such as carbohydrate, ATP synthesis, and energy pathways, were transcribed. Such criteria were suggested earlier for *M. tuberculosis* (18, 40). Accordingly, we analyzed the list of transcribed genes (primary ratio, ≥ 1) to identify their participation in metabolic pathways already known for *M. tuberculosis* compiled by the KEGG encyclopedia (http://www.genome.ad.jp/kegg/kegg2. html). Using Fisher's exact test (1, 22), the significant enrichment of certain pathways was identified among the total number of transcribed genes (Table 1). Among the significantly enriched pathways ($P < 0.05$) are those involved in carbohydrate metabolism (e.g., the citrate cycle and starch and sucrose groups), lipid metabolism (e.g., fatty acid metabolism), and energy metabolism (e.g., oxidative phosphorylation and sulfur metabolism). Other significantly enriched pathways included those responsible for processing genetic information (e.g., DNA polymerase) or responding to environmental stimuli (e.g., two-component signal transduction). Overall, more than 50% of the genes involved in the metabolic pathways listed in Table 1 were represented in samples analyzed in the middle phase of chronic tuberculosis (45 or 60 days p.i.). The active transcription of genes represented by these pathways indicates metabolically active bacilli that can utilize both carbohydrate and lipid sources to generate the necessary energy for carrying on cellular processes or responding to the surrounding microenvironment.

Transcriptional profile of the middle stage of chronic tuberculosis. Since we previously analyzed the transcriptional profile of early chronic tuberculosis (28 days p.i.) in comparison to active tuberculosis (43), we decided to focus further analysis on the middle and late stages. To identify unique genes important for the mid-chronic stage of tuberculosis, we compared the expression profile of the 45- and 60-day-p.i. samples to the expression profile of samples collected during the active stage of tuberculosis (14 days p.i.) that was reported earlier (43). Although we did not expect significant change in the transcriptional machinery of *M. tuberculosis* during progression from 45

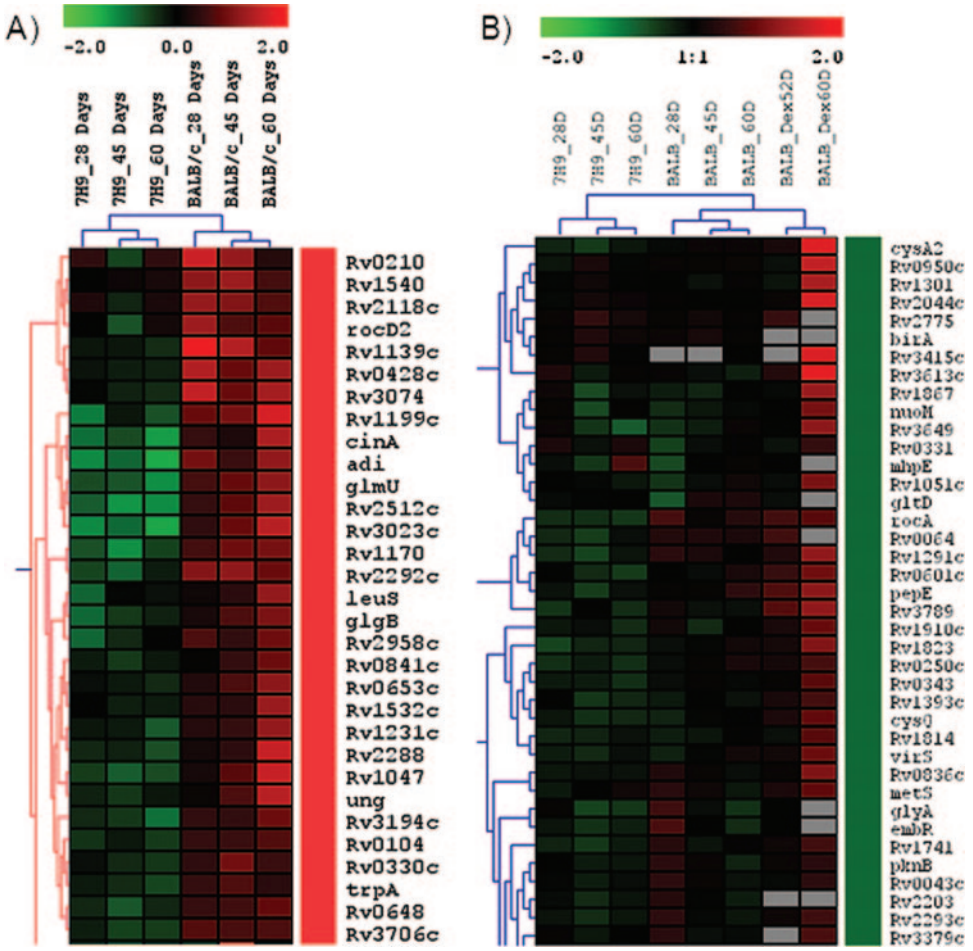

FIG. 2. Expression profile of *M. tuberculosis* in mice during chronic and reactivation stages of infection. (A) A two-dimensional hierarchical cluster analysis of expression levels of *M. tuberculosis* H37Rv genes growing in 7H9 Middlebrook broth or in BALB/c mice at different times after aerosol infection (28, 45, and 60 days p.i.). One example of a gene cluster with down-regulated genes in all in vivo samples is presented. (B) A two-dimensional hierarchical cluster analysis of gene expression levels of *M. tuberculosis* growing in 7H9 Middlebrook broth or in BALB/c mice at 28, 45, 52, and 60 days p.i. One example of a cluster with up-regulated genes in all in vivo samples is presented. The red and green boxes denote up- or down-regulation, respectively.

to 60 days, we included both times to provide a higher-resolution analysis of the middle stage of chronic tuberculosis. In fact, only five genes (*glf*, *narK2*, rv0188, rv1738, and rv2672) were significantly changed between 45- and 60-day samples, confirming that both times could represent the middle stage of chronic tuberculosis. However, a total of 88 and 49 mycobacterial genes were significantly changed when 14-day samples were compared to samples collected from mice infected for 45 or 60 days p.i., respectively. Interestingly, most of the significantly changed genes (>90%) were up-regulated at later times compared to the 14-day-p.i. samples (see Table S2 in the supplemental material). Among the significantly up-regulated genes at later times were those genes encoding the glycoxolate shunt functions (*icl* and *aceAa*) (26) and histidine biosynthesis (*hisS*), as well as the transcriptional regulator *sigK* (24). Correlation analysis of gene expression levels further identified a small group of genes that displayed consistent transcription profiles at all time points examined (Table 2). Some of these genes were always induced in mice compared to 7H9 broth (e.g., *mprA*, *lppJ*, and rv0348) or always repressed (e.g., *accD4* and rv3830c). The *mprA* gene encodes a transcriptional regulator that plays a role in establishing chronic infection that was described earlier using a deletion mutagenesis approach (52, 53), as well as using IVMA (43). The rv3830c gene encodes another putative transcriptional regulator that could play a role as a transcriptional repressor (http://genolist.pasteur.fr/TubercuList/).

Transcriptional profile of *M. tuberculosis* during immune suppression. An important phase of human tuberculosis is the reactivation of the tuberculous bacilli after exposure to immune suppression. To profile the change in gene expression levels of *M. tuberculosis* undergoing reactivation, groups of BALB/c mice were inoculated daily with dexamethasone for 7 or 15 days starting at 45 days p.i., i.e., during the middle phase of chronic tuberculosis. Colony counts from lung tissues at 52 and 60 days p.i. displayed an increase in mycobacterial counts in the lungs of the dexamethasone-treated group (5.6×10^7 CFU/lung) compared to the untreated control group (1×10^7 CFU/lung). To identify the overall change in the mycobacterial expression profile following immune suppression, hierarchical

TABLE 1. Metabolic pathways transcribed in BALB/c mice at 45 or 60 days post-aerosol infection with *M. tuberculosis* H37Rv

Metabolic pathway[a]	No. of genes/ pathway	Total no. of transcripts	*P* value[b]
Whole genome	762	2,855	
1- and 2-methylnaphthalene degradation	57	44	**0.04289**
ABC transporters	107	79	**0.00037**
Alanine and aspartate metabolism	17	12	0.05764
Aminoacyl-tRNA biosynthesis	22	16	0.05462
Aminosugar metabolism	10	9	1.00000
Arginine and proline metabolism	28	24	0.77532
ATP synthesis	8	8	0.60675
Biosynthesis of siderophore, nonribosomal peptides	9	8	1.00000
Biotin metabolism	14	11	0.40938
Carbon fixation	13	10	0.22790
Citrate cycle (tricarboxylic acid cycle)	30	22	**0.04761**
DNA polymerase	11	5	**0.00119**
Fatty acid biosynthesis	13	10	0.22790
Fatty acid elongation	2	2	1.00000
Fatty acid metabolism	39	28	**0.01288**
Folate biosynthesis	20	15	0.16698
Glycolysis gluconeogenesis	31	23	0.05211
Glyoxylate and dicarboxylate metabolism	15	13	1.00000
Nucleotide sugar metabolism	18	12	**0.02135**
One-carbon pool by folate	12	10	0.65930
Oxidative phosphorylation	37	23	**0.00013**
Peptidoglycan biosynthesis	13	11	0.67817
Phenylalanine, tyrosine, and tryptophan biosynthesis	26	22	0.56691
Ribosome	55	48	1.00000
Starch and sucrose metabolism	25	18	**0.03443**
Sulfur metabolism	11	9	0.64181
Tryptophan metabolism	65	50	**0.02336**
Two-component system	54	35	**0.00003**
Urea cycle and metabolism of amino groups	16	12	0.14037

[a] Based on KEGG website (http://www.genome.ad.jp/kegg/pathway.html).
[b] Fisher's test (http://home.clara.net/sisa/fisher.htm). Significant values are in boldface.

cluster analysis was used to organize expression levels into groups based on their levels of induction or repression. Although colony counts of the dexamethasone-treated groups displayed a modest increase in bacterial load, IVMA was able to identify clusters of genes that were differentially expressed in dexamethasone-treated samples (Fig. 2B). Moreover, samples collected from in vitro cultures or BALB/c mice and dexamethasone-treated mice occupied distinct nodes of the cluster, indicating the sensitivity of the IVMA to profile the overall change in gene expression. Some of the genes up-regulated during the dexamethasone treatment were also up-regulated in samples collected from infected but untreated mice (e.g., *glmU* and *ung*). However, some genes were activated only during dexamethasone treatment, including *virS* (Fig. 2B) and members of the *mce* family of proteins. Overall, a total of 174 genes (see Table S4 in the supplemental material) occupied the same cluster and were up-regulated during the reactivation phase of tuberculosis.

An interesting feature of the genes activated during reactivation is the inclusion of several transcriptional regulators other than VirS. Some of these transcriptional regulators are members of the signal transduction protein kinase (STP-K) family involved in signal transduction through phosphorylation (*pknB* and *pknG*). It is possible that *pknB* is involved in cell division, while *pknG* could be involved in regulating amino acid uptake and stationary-phase metabolism (7). Another transcriptional regulatory protein (*phoY1*), responsible for transporting phosphate ions, was also activated during dexamethasone treatment. Another group of reactivation-specific genes included those responsible for iron-sulfur transport (*fdxC*), sulfur transferase (*cysA2*), and sulfite synthesis (*cysQ*), indicating the importance of both iron and sulfur metabolism to *M. tuberculosis* during reactivation. Interestingly, genes expected to be activated during the mycobacterial active stage of growth were also differentially activated during dexamethasone treatment, such as those involved in hydrolysis of peptide bonds (*pepB* and *pepE*), redox reactions (*trxB1*), and trehalose and mycolic acid biosynthesis (*glgY* and *pcaA*). Overall, a unique set of genes were activated as a consequence of mouse exposure to immunosuppressant, reflecting the surge in mycobacterial growth during the reactivation phase of tuberculosis.

TABLE 2. Change in expression levels of mycobacterial genes with similar expression patterns in samples collected from mice relative to 7H9 broth

Gene	Product	Annotation[a]	Change (*n*-fold) ± SD at (days):		
			28	45	60
rv0156	PntAB	Nicotinamide nucleotide transhydrogen subunit	2.72 ± 0.27	1.87 ± 0.22	1.57 ± 0.31
rv0348	Rv0348	Possible transcriptional regulator, TetR/AcrR family	1.59 ± 0.25	1.49 ± 0.11	1.67 ± 0.36
rv0981	MprA	Two-component response regulator	4.83 ± 2.25	2.26 ± 0.39	9.51 ± 5.17
rv1062	Rv1062	Unknown conserved protein	3.67 ± 0.2	2.77 ± 0.15	1.99 ± 0.31
rv2080	LppJ	Possible lipoprotein	3.43 ± 0.33	2.74 ± 0.22	2.13 ± 0.37
rv2326c	Rv2326c	Putative ABC transporter	3.36 ± 0.26	2.95 ± 0.19	2.67 ± 0.56
rv3079c	Rv3079c	Unknown conserved protein	−2.51 ± 0.57	−2.58 ± 0.6	−3.24 ± 1.19
rv3103c	Rv3103c	Proline-rich proteins of unknown function	−3.15 ± 0.96	−3.42 ± 0.96	−3.08 ± 0.8
rv3685c	Rv3685c	Probable cytochrome P-450	−2.3 ± 0.55	−2.17 ± 0.4	−2.98 ± 0.84
rv3799c	AccD4	Probable propionyl-coenzyme A carboxylase	−3.49 ± 0.49	−2.96 ± 0.4	−3.58 ± 1.07
rv3830c	Rv3830c	Transcriptional regulator protein	−1.87 ± 0.38	−1.92 ± 0.42	−1.74 ± 0.29
rv3871	Rv3871	Unknown conserved protein	−2.6 ± 0.48	−2.44 ± 0.58	−2.85 ± 0.71

[a] Tuberculist website (http://genolist.pasteur.fr/TubercuList/).

TABLE 3. Expression of a selected list of mycobacterial genes in mouse lungs using samples collected at 20 weeks compared to 20 days p.i.

Gene	Reason for inclusion[a]	Change (*n*-fold) ± SD at:	
		20 wk/20 days	20 wk/60 days
rv0967	Potential transcriptional regulator and iVEGI member	6.7 ± 0.11	ND[b]
rv0990c	Potential transcriptional regulator and iVEGI member	16.1 ± 0.34	ND
rv1873	Induced in BALB/c mice compared to 7H9 broth at 60 days	122.8 ± 0.69	84.6 ± 0.66
mutT1	Induced in BALB/c mice compared to 7H9 broth at 45 days	3.0 ± 0.28	24.9 ± 0.45
rv0348	Potential transcriptional regulator activated at 60 days	200.8 ± 1.00	68.4 ± 0.01
cobI	Significant between groups of BALB/c mice vs. 7H9 at 60 days	12.9 ± 0.79	36.9 ± 0.49
clpX	Significant between BALB/c mice at 14 and 45 days	−2.8 ± 1.00	13.9 ± 1.20
adi	Significant between groups of BALB/c mice vs. 7H9 at 45 days	2.8 ± 1.10	−1.8 ± 1.00

[a] Reason for inclusion based on this study and the bioinformatics analysis of the gene sequence.
[b] ND, not done.

Expression analysis of selected genes at the late stage of chronic tuberculosis. The IVMA of mycobacterial transcripts identified genes with unique profiles that could be responsible for mycobacterial survival during the middle stage of chronic tuberculosis. We reasoned that if these genes are important for establishing mycobacterial persistence, they will continue their trend of activation at later times of tuberculosis beyond 60 days p.i. (the late chronic stage). To examine this hypothesis, the transcriptional levels of selected mycobacterial genes were analyzed using real-time quantitative PCR in samples collected from a small number of mice infected for 20 weeks (140 days p.i.) compared to samples collected following 20 and 60 days of infection. For real-time quantitative PCR, genes were selected based on information gleaned from their sequence analysis or because they represented groups of significantly changed genes. The list of selected genes included representative members of the in vivo-expressed genomic island (iVEGI) gene group (rv0967 and rv0990c) (43), genes identified in unique clusters (rv0348, rv1873, and *mutT1*), or those that were significantly regulated among in vivo and in vitro samples (*cobI*, *clpX*, and *adi*) at 45 and 60 days p.i. (see Table S7 in the supplemental material). As expected, most of the selected genes were up-regulated in 20-week (140-day-p.i.) samples compared to 20- and 60-day-p.i. samples (Table 3), indicating a difference in the bacterial transcripts at the active compared to the middle and late stages of chronic tuberculosis. Interestingly, the putative transcriptional regulator rv0348 was up-regulated at a much higher level at 20 weeks p.i.

Transcriptional control of chronic tuberculosis. Because of the high induction of rv0348 in samples collected in late tuberculosis and based on the preliminary annotation of this gene as a transcriptional regulator, we analyzed the rv0348 gene sequence to examine its potential role during chronic tuberculosis. Analysis of the secondary sequence structure of amino acid residues encoded by rv0348 indicated the presence of a helix-turn-helix domain of 21 residues among the 217 amino acid residues (Fig. 3A). This helix-turn-helix domain usually indicates DNA binding ability (25). BLAST analysis of Rv0348 indicated the highly conserved nature of the protein in mycobacterial species of the *M. tuberculosis* complex (100% identity) and to a much lesser degree (55%) in the nonpathogenic strain of *Mycobacterium smegmatis*. Additionally, the only significant similarity ($P < 6E-19$) between the Rv0348 sequence and other proteins outside the *Mycobacteriaceae* was found in *Nocardia farcinica*, with 33% identity. Analysis of rv0348 flanking sequences identified rv0348 as a member of an operon (Fig. 3A) with unknown function.

We reasoned that if rv0348 encodes a potential transcriptional regulator, it may encode a DNA-binding protein that can bind to its own promoter. To test this hypothesis, we expressed and purified the recombinant Rv0348 protein (rRv0348) in *E. coli*. The binding ability of the purified protein was tested against several probe constructs amplified from different sites upstream of the rv0348 sequence by using EMSA (Fig. 3B). As expected, EMSA analysis identified a stretch of DNA for protein binding (148 bp) that was actually within 150 bp of the rv0348 operon, i.e., within the predicted rv0347 open reading frame. Additionally, several concentrations of the rv0347 probes were able to bind competitively to rRv0348, but not to a putative promoter region in *Mycobacterium paratuberculosis*, confirming the specificity of rRv0348-DNA binding (Fig. 3C). Using a promoter recognition algorithm, BPROM (http://www.softberry.com/berry.phtml), we were able to predict a potential promoter binding site (TGGGTA-N[12]-GGG TACAT) within the first 148-bp fragment from the start codon of rv0347. The putative promoter sequence was used to search the *M. tuberculosis* genome for other genes that could also be under the control of Rv0348, using criteria similar to those used to predict genes under the control of SigD (6). The search pattern algorithm identified a list of 130 genes with a DNA recognition sequence similar to the motif identified upstream of the rv0348 coding sequence. Most of the motifs were present in the 500 bp upstream of each identified gene (see Table S5 in the supplemental material). Interestingly, some of the genes identified in this analysis included the *sigF* operon, which is known to orchestrate entry into the chronic stage of tuberculosis (10). Moreover, the *sigF* promoter consensus sequence (15) was found to overlap with the putative promoter sequence identified for rv0348, indicating possible regulation of rv0348 by the SigF protein itself.

DISCUSSION

The chronic phase of tuberculosis remains an understudied stage of infection because of the inherent problems in isolating enough bacteria for molecular analysis. For example, whether mycobacterial bacilli survive in dormant or replicative forms is not completely understood and remains a controversial issue (30). In this report, we employed a novel protocol for IVMA to profile the metabolic states of *M. tuberculosis* during the mid-

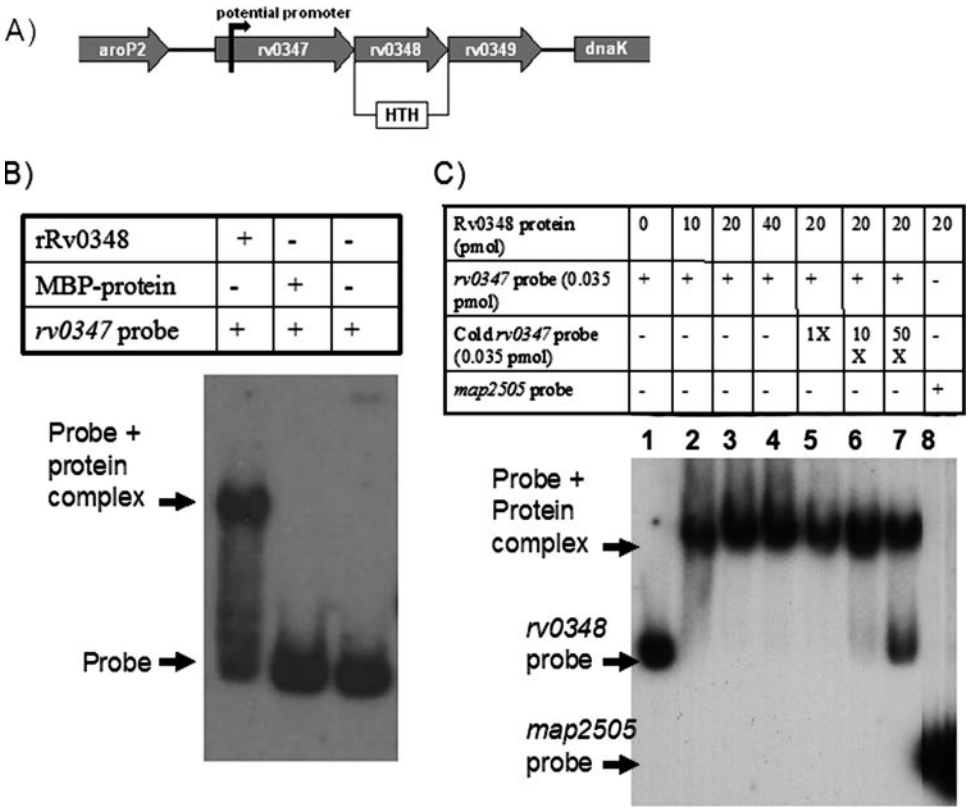

FIG. 3. Transcriptional activities of in vivo-activated genes during chronic tuberculosis. (A) Organization of the rv0348 operon with the secondary structure of the Rv0348 protein. (B) EMSA of the binding ability of rRv0348 or the MBP to an upstream region within the rv0347 sequence. (C) EMSA of the binding ability of rRv0348 in the presence of both specific (rv0347) and nonspecific (map2505) probes. ^{32}P-labeled probes were prepared by end labeling using T4 polynucleotide kinase. Protein-DNA complexes were resolved in 4% SDS-polyacrylamide gel electrophoresis gels and exposed to X-ray films for 2 to 6 h before development. Probe names and concentrations are listed above each gel image.

dle and late stages of murine tuberculosis. Interestingly, IVMA revealed metabolically active bacilli despite a constant level of mycobacterial colonization of the lung. The analyses presented identify bacilli actively transcribing 50% of their encoded genes, including those involved in several metabolic pathways important for mycobacterial survival (e.g., the citrate cycle, DNA polymerase, ATP synthesis, and nucleotide sugar metabolism). Such a finding suggests bacilli that are not metabolically dormant, at least during the middle stage of chronic tuberculosis, the target stage for IVMA. Earlier studies used similar criteria to assess mycobacterial metabolic activity (18, 40). Unfortunately, it is difficult to decipher whether pathways that are not induced are actually repressed in mouse lungs or whether they are beyond detectable levels because of the technical limitations of IVMA. Among the genes activated during mid-chronic tuberculosis are those that were also regulated during active tuberculosis (43), including members of the iVEGI region. Some of these genes (e.g., rv0967 and rv0970) were also activated in the artificial-granuloma model of tuberculosis (20). Additional genes activated in murine lungs included those encoding metalloregulatory proteins, such as rv0763c (for iron metabolism) and *mog* (for molybdenum metabolism), implying an important role played by metal ions in the pathogenesis of chronic tuberculosis. Such a role for metals in mycobacterial pathogenesis has just been illustrated by our group's delineation of a novel transcriptional regulator (*csoR*) (23). Because of the unique features of the mouse model of chronic tuberculosis (maintenance of a constant but relatively high level of *M. tuberculosis*) compared to natural infection, further studies are needed before drawing conclusions on human tuberculosis based on the identified transcriptional profiles. For example, genes activated inside the typical human granuloma, where *M. tuberculosis* encounters caseation and anaerobic conditions, will be difficult to study in murine lungs, which lack such conditions (45).

An interesting aspect of the IVMA reported here is the identification of a group of genes that was coregulated at all times examined during in vivo and in vitro growth of *M. tuberculosis*, including *mprA*, a known transcriptional regulator involved in bacterial persistence (52, 53). This unique pattern of expression implies an important role of these genes in regulating mycobacterial entry into the chronic stage of infection. Bioinformatics and protein analyses of one of the identified genes, rv0348, indicated its potential regulation of a large number of genes, including *sigF*, the well-characterized sigma factor responsible for establishing chronic tuberculosis (15). It will be interesting to decipher the interplay between proteins encoded by *sigF* and rv0348 during chronic infection. Experimental protocols (e.g., protein-DNA assays) are also needed to confirm the role of Rv0348 in regulating genes identified by bioinformatics analysis. Clearly, the IVMA approach also helped to gain insights into the reactivation phase of tubercu-

losis. Once the host immunity was repressed, a different profile of *M. tuberculosis* emerged, characterized by the up-regulation of several transcriptional regulators, such as the *virS* system and several mammalian cell entry operons (the *mce* family). The *virS* gene was shown to be involved in transcriptional regulation of *M. tuberculosis* virulence (17), while the other genes (*lprK*, *mce2A*, and *mce4A*) were shown to be involved in cell invasion (3). Both functions could be necessary for the reactivation phase of tuberculosis. It is possible that VirS could be one of the master regulators involved in the reactivation of tuberculosis. It is also possible that a different reactivation profile would emerge if the time of immune suppression was delayed beyond 45 days p.i. or if another model of reactivation was attempted.

We also took advantage of the growing number of publications on mycobacterial expression profiling to compare expression levels obtained from the IVMA of mouse lungs to those generated by in vitro models when *M. tuberculosis* was grown under variable but defined stress conditions. The goal of this comparative analysis is to "sketch" the main features of the microenvironments where tuberculous bacilli reside. A number of significantly regulated genes inside mouse lungs were also regulated during in vitro stimulation of *M. tuberculosis*, using stress conditions, such as hypoxia (38), nutrient starvation (5), exposure to nitric oxide (48), or survival inside the macrophage microenvironment (37). Genes encoding proteins involved in aerobic/anaerobic respiration (e.g., *nuoC* and *nuoL*) and homologues for isocitrate lyase (*aceAa*) or isocitrate dehydrogenase (*icd*) were the only genes activated during nutrient starvation, as well as during survival in mouse lungs. Additionally, a small number of genes (rv0079, *fdxA*, rv2028c, and rv2629) were shared with the exposure to nitric oxide or hypoxia, implying that at the times examined so far in mice, the host microenvironment is only partially anaerobic and partially deficient in nutrients. On the other hand, a relatively high number of genes identified by IVMA were shared with macrophage survival (see Table S6 and Fig. S2 in the supplemental material).

To gain more understanding of the chronic stage of human tuberculosis, we compared the transcriptional levels of *M. tuberculosis* profiled in murine lungs to that profiled in human lungs (31). However, such analysis could be complicated by the clinical variations observed in murine compared to human tuberculosis, as well as the differences in sampling strategies from patients with multiple-drug regimens and unknown stages of infection compared to the precise time points examined in mice by IVMA. Generally, a surprising number of genes (n = 58) regulated in mice were shared with the human samples (see Table S6 and Fig. S2 in the supplemental material). Overall, the comparative analysis of IVMA in murine lungs and in vitro and other models of infection revealed a microenvironment that is not limited in nutrients or oxygen, consistent with earlier analysis of mouse lungs (45), but definitely rich in immune cells, such as macrophages. In conclusion, the IVMA reported here provided a dynamic view of the changes in mycobacterial bacilli during both chronic and reactivation stages of infection. Transcriptional profiling further assisted the dissection of chronic tuberculosis into early, middle, and late chronic stages, with a unique transcriptional profile for each stage. The IVMA approach has begun to identify gene groups activated only during the reactivation stage of infection, a stage for which little information is available. Currently, experiments are under way to inactivate specific genes identified during this investigation (e.g., rv0348) to better characterize their roles in the chronic and reactivation phases of tuberculosis. The knowledge gained from such an approach will allow investigators to target novel pathways for chemotherapies to treat chronic tuberculosis.

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