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Transcriptional characterization of the antioxidant response of *Mycobacterium tuberculosis in vivo* and during adaptation to hypoxia *in vitro*

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Summary

Transcriptional profiling of antioxidant genes of *M. tuberculosis* was performed by real-time RT-PCR during mouse lung infection and during adaptation to gradual oxygen depletion *in vitro. M. tuberculosis* genes involved in major detoxification pathways of oxidative stress were not up-regulated during chronic mouse lung infection, which is established in response to expression of host adaptive immunity. This result suggests that a major function of bacterial antioxidant enzymes is to protect from oxidants generated during the early, acute phase of infection. *In vivo* transcription profiles of bacterial antioxidant enzymes differed from those seen under adaptation to low oxygen *in vitro*, indicating differences between growth arrest *in vivo* and that induced by hypoxia *in vitro*.

Keywords

Reactive oxygen species; Reactive nitrogen species; Mouse lung infection; Reverse transcription polymerase chain reaction; bacterial persistence; bacterial growth arrest

Introduction

The interaction between the intracellular pathogen *Mycobacterium tuberculosis* and mononuclear phagocytes typically leads to induction of host adaptive immune responses, which control infection without sterilizing the host [1, 2]. Survival of *M. tuberculosis* to expression of adaptive immunity is presumably associated with entry into a “dormant” state, in which tubercle bacilli exhibit no or slow growth and low metabolic activity. As a result, a latent infection is established. When immunity fails to control infection, tubercle bacilli exit dormancy, resume growth and cause disease. Thus, adaptation to the conditions found inside the phagocytic cell is key to the survival and persistence of *M. tuberculosis in vivo*.

Characteristic of the intracellular environment of the activated macrophage is high-output generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) [3, 4]. ROS are also produced during bacterial aerobic metabolism [5, 6]. It is well established that *M.*

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tuberculosis has evolved multiple ways to detoxify RNS and ROS. First, superoxide can be directly detoxified by the sequential activity of superoxide dismutases (SODs) and catalase (KatG); peroxynitrite can be detoxified by KatG [7]. Second, hydrogen peroxide and peroxynitrite can also be detoxified by thiol-specific redox systems, which also maintain the intracellular thiol-disulfide balance. These include alkyl hydroperoxide reductases (AhpC and AhpD) [8], thioredoxin and thioredoxin reductase (TrxB2) [9–11], and methionine and methionine sulfoxide reductase (MsrA) [12]. Third, NO can be directly detoxified by hemoglobins. *M. tuberculosis* produces two small, truncated hemoglobins (trHbs), encoded by *glnO* and *glbN* [13, 14], and another hemoglobin (Hmp) [15] that is homologous to the flavohemoglobin of *Escherichia coli*. Although progress has been made toward characterizing the transcriptional response of *M. tuberculosis* to oxidative and nitrosative stress in culture and in infected cultured macrophages [16–18], more limited is our understanding of the antioxidant responses of tubercle bacilli at different stages of lung infection.

In this paper, we report results of transcriptional profiling of the nitrosative and oxidative stress responses of tubercle bacilli during murine infection. In the lung of mice infected by the respiratory route, tubercle bacilli multiply exponentially for ~ 20 days (acute infection) and then stop growing in response to expression of adaptive, Th1-mediated immunity. Stabilization of bacterial numbers characterizes a much longer, second phase of infection (chronic infection) [19]. Since bacteriostasis is primarily induced by the generation of NO from activated macrophages (mice incapable of producing iNOS die rapidly from acute infection [20]), this animal model is particularly appropriate to characterize the antioxidant response of *M. tuberculosis* during growth and persistence *in vivo*. For comparative purposes, we also characterized the bacterial antioxidant response of *M. tuberculosis* in cultures gradually starved for oxygen. This *in vitro* model, which was developed by L. Wayne [21], reflects some aspects of the *in vivo* situation, since it too causes bacterial growth arrest and induction of the "dormancy" regulon, a set of ~50 genes that is upregulated when tubercle bacilli stop (or slow down) growth *in vivo* and *in vitro* [16, 18, 22].

When quantitative RT-PCR was used to measure the abundance of *M. tuberculosis* transcripts involved in the detoxification of ROS and RNS during mouse lung infection, it was found that the transition from bacterial replication to growth arrest was characterized by decreased transcript levels for all genes tested. It was also found that some genes were selectively induced at specific stages of O_2 starvation *in vitro*, thus providing the first example of key transcriptional differences between a mouse model and the Wayne model.

Materials and Methods

Mouse infection

C57BL/6 mice at the age of 8 to 10 weeks were infected with ~2×10^2 CFU of *M. tuberculosis* strain H_{37}Rv via the respiratory route, as described previously [23]. At selected times, lungs from three to four mice were harvested. Half of the lung was used to monitor bacterial growth by standard plating [24], and the other half was snap-frozen in liquid nitrogen for subsequent RNA extraction.

Bacterial culture under gradual oxygen depletion

M. tuberculosis H_{37}Rv was grown in liquid culture at 37°C in Dubos Tween-albumin broth as described [21]. Gradual oxygen depletion in the Wayne model was obtained by culturing bacteria in sealed tubes with a headspace ratio (HSR) of 0.5 with slow magnetic stirring [21]. The density of the starting culture was 10^6 cells/ml. At selected times, cells from 2-ml cultures were harvested by rapid centrifugation and snap-frozen in liquid nitrogen for subsequent RNA

extraction. CFU were enumerated by plating 10-fold serial dilutions of liquid cultures on Dubos oleic albumin agar.

Copy number measurement of bacterial transcripts

RNA extraction, reverse transcription (RT) and real time PCR were performed as per our published protocol [22] and at http://www.phri.org/research/res_pigennaro.asp. Briefly, infected mouse lungs or bacterial cultures were homogenized in guanidinium thiocyanate buffer in a Mini-Beadbeater (Biospec Products, Bartlesville, OK), followed by phenol extraction of total nucleic acids. Total RNAs were purified with TRI reagent (Molecular Research Center, Cincinnati, OH). RT was performed using ThermoScript™ Reverse Transcriptase (Invitrogen, Carlsbad, CA), and quantification of cDNA was carried out using real time PCR with molecular beacons [25]. RT primers, PCR primers and molecular beacons are listed in Table 1. Copy numbers of *M. tuberculosis* 16S rRNA were used as normalization factor to enumerate bacterial transcripts per cell, since 16S rRNA copy numbers correlate well with CFU in culture (26 and Fig. 2) and during the course of lung infection [22].

Results

Transcriptional profiles of *M. tuberculosis* genes encoding antioxidant enzymes in the mouse lung

To investigate the antioxidant response of *M. tuberculosis* during mouse lung infection by transcriptional profiling, total lung RNA was extracted at various times post-infection (the course of mouse lung infection is shown in Fig. 1A), and specific bacterial transcripts were enumerated by quantitative RT-PCR. As a reference gene we selected *cydA*, which encodes a subunit of cytochrome *bd* oxidase, an alternative terminal oxidase that is transiently upregulated at the transition from acute to chronic infection [27] (Fig. 1A). The first set of antioxidant genes probed included *sodA*, *sodC* and *katG*. These genes encode, respectively, the secreted, iron-dependent SodA [28, 29], the membrane bound, copper/zinc-dependent SodC [30–32] and catalase (KatG) [33]. The two SODs and KatG sequentially convert superoxide into molecular oxygen and water [33]. KatG also detoxifies peroxynitrite that arises from the reaction of nitric oxide (NO) and superoxide [7]. Levels of *sodA* and *sodC* mRNAs were highest at day 12 post-infection and decreased starting at day 15, when the Th1 cytokine IFNγ is upregulated [22]. At day 30 mRNA levels for *sodA* and *sodC* were 9- and 6-fold lower than at day 12 (Fig. 1B). The *katG* transcript levels were essentially stable during acute infection and showed a significant drop only at day 21. At day 30 *katG* mRNA was 2.5-fold lower than at day 12 and remained low thereafter (Fig. 1B).

We also measured transcript levels for key enzymes in the thio-redox systems, which are involved in detoxification of H_2O_2 and peroxynitrite. The alkyl hydroperoxide reductase (AhpC and AhpD) system and the thioredoxin/thioredoxin reductase (TrxB2) system participate in detoxification of peroxide and peroxynitrite via oxidation-reduction of a conserved NH_2-terminal cysteine [8–11]. Similarly, the methionine and methionine sulfoxide reductase (MsrA) system scavenges ROS and RNS via cycles of oxidation and reduction of methionine residues in surface-exposed proteins [12]. The *ahpC* transcript levels were essentially stable during acute infection and decreased after day 21 (Fig. 1C). At day 30 *ahpC* mRNA levels were 6-fold lower than at day 12. The transcript levels for *trxB2* and *msrA* were highest at day 12 and decreased with bacterial growth arrest. By day 30 the transcript levels of these two transcripts were 3- to 4-fold lower than at day 12 and remained low thereafter (Fig. 1C).

From among enzymes that directly detoxify NO, we measured mRNA transcripts for the truncated hemoglobin TrHbN encoded by *glbN*. TrHbN exhibits an oxygen-dependent NO consumption activity [13, 14] and protects heterologous hosts from growth inhibition by NO

[34]. The copy number of the *glbN* transcript was highest at day 12 and decreased 18-fold by day 30 (Fig. 1D). At day 50 the *glbN* transcript levels showed a transient, 6-fold increase relative to day 30 and then decreased further by day 100 (Fig. 1E). Levels of the transcript encoding flavohemoglobin (*hmp*), were below detection (data not shown).

Transcriptional profiles of *M. tuberculosis* genes encoding antioxidant enzymes during gradual oxygen depletion *in vitro*

Gradual depletion of oxygen *in vitro* also causes bacterial growth arrest *of M. tuberculosis,* with similar transcriptional changes as seen *in vivo* [22, 27]. In the Wayne model [21], tubercle bacilli stop growing at ~78 h, when the dissolved oxygen approaches 1% of saturation (the microaerophilic stage of non-replicating persistence, NRP-1) [21]. Anaerobiosis (NRP-2) commences at ~200 hrs, when the dissolved oxygen content decreases below 0.06% saturation (Fig. 2A).

To compare the transcriptional profiles of genes encoding antioxidant enzymes in O_2-starved cultures of *M. tuberculosis* with the *in vivo* data, *M. tuberculosis* transcripts characterized in the mouse lung were also enumerated in hypoxic cultures (Fig. 2B). We found that *ahpC* was induced during NRP-1 (up to 8-fold), while *msrA* and *glbN* were induced in NRP-2 (7.5-fold and 4-fold, respectively). All other transcripts showed no or modest ($\leq$ 2-fold) increase. During the late stages of anaerobiosis, i.e., hour 460 through 578, all transcripts decreased drastically (Fig. 2B), when *M. tuberculosis* has very low metabolic activity but remains viable (26 and Fig. 2A).

Discussion

The antioxidant response of *M. tuberculosis in vivo* was characterized during the course of mouse respiratory infection by measuring levels of seven bacterial transcripts encoding enzymes involved in the oxidative and nitrosative stress responses. Transcript levels for *sodA, sodC, glbN, trxB2,* and *msrA* decreased starting at day 15, coincident with expression of host adaptive immunity [22], whereas mRNA levels for *ahpC* and *katG* began to decrease a few days later (day 21), when bacterial growth was halted. Thus the response of *M. tuberculosis* to host adaptive immunity in the mouse lung is characterized by the downregulation of antioxidant enzymes. These mouse data are consistent with earlier observations that the same bacterial transcripts were not induced either by IFNγ-activation of cultured macrophages infected with *M. tuberculosis* or by exposure of bacterial cultures to bacteriostatic concentrations of NO [16]. Indeed, the data suggest the alternative possibility that the main function of the antioxidative pathways investigated in the present work is to detoxify harmful products produced by the bacterial aerobic metabolism of growing bacilli or by host defense mechanisms implemented during acute infection.

Several observations agree with the hypothesis proposed above. One is that a *sodA* mutant is attenuated for growth both *in vitro* and in mice [35]. Another observation is that *katG* is essential for bacterial survival only between 2 and 4 weeks post-infection [36], that is, the same time interval in which respiratory-burst-deficient mice experience an increased load of wild-type *M. tuberculosis* in their lungs [37]. Accordingly, the growth defect of an *M. tuberculosis sodC* mutant in murine macrophages is abolished when macrophages are derived from respiratory-burst-deficient mice but not from $NOS2^{-/-}$ mice [38]. Further, none of *M. tuberculosis* mutants deleted for genes investigated in the present work shows decreased ability to persist during chronic infection of the mouse lung [39–42].

The downregulation of antioxidative pathways in growth-arrested tubercle bacilli in the mouse lung suggests that the bacterial defense against ROS and RNS produced by the host adaptive immunity may be carried out by enzymes other than those investigated in the present paper.

One potential candidate is cytochrome *bd* oxidase, encoded by *cydABDC* gene cluster. This gene cluster is transiently upregulated when tubercle bacilli stop growing in the mouse lung (Fig. 1A), and it is required for the transition from acute to chronic mouse lung infection [27]. Due to the high affinity of cytochrome *bd* oxidase for oxygen, this enzyme may act as oxygen scavenger and thus prevent cellular damage from potential excessive production of ROS, as postulated for other bacteria [43, 44].

Several considerations derive from the transcriptional profiles associated with gradual oxygen depletion *in vitro*. First, the upregulation of *ahpC* during NRP-1 may protect tubercle bacilli from oxidative stress and also prevent protein misfolding or unfolding [45] under microaerobic conditions. Second, the elevated levels of *msrA* during NRP-2 may help maintain cellular redox balance and deal with damage under anaerobic conditions. Third, the high expression of *glbN* during NRP-2 suggests the possibility that homodimeric hemoglobin, which displays extremely high oxygen binding affinity and cooperativity, facilitates oxygen diffusion to the terminal oxidase [13, 46]. Since no upregulation of these genes was observed in the mouse lung, these pathways have presumably evolved in tubercle bacilli to adapt to hypoxic/anoxic conditions that are not found in the lung of infected mice. Transcriptome microarray analysis of *M. tuberculosis* in human lungs of tuberculosis patients also reveals no upregulation of *M. tuberculosis* antioxidant genes investigated in this study [47], suggesting that the physiological status of tubercle bacilli is similar in the murine model and in granulomas from human tuberculosis.

In conclusion, immunity-induced arrest of *M. tuberculosis* growth in the mouse lung is associated with an overall decrease of the levels of transcripts encoding antioxidant enzymes. These data imply that a major function of these genes is to defend tubercle bacilli from ROS and RNS produced by the host during acute infection and from ROS produced by the bacterial aerobic metabolism. The data add to existing evidence that the antioxidant response is not essential for *M. tuberculosis* persistence *in vivo* and lend further credence to the idea that the key survival strategy of this pathogen lies in its ability to enter a state of slow growth or no growth accompanied by decreased metabolic activity rather than to implement active defense mechanisms against the adaptive host immune response.

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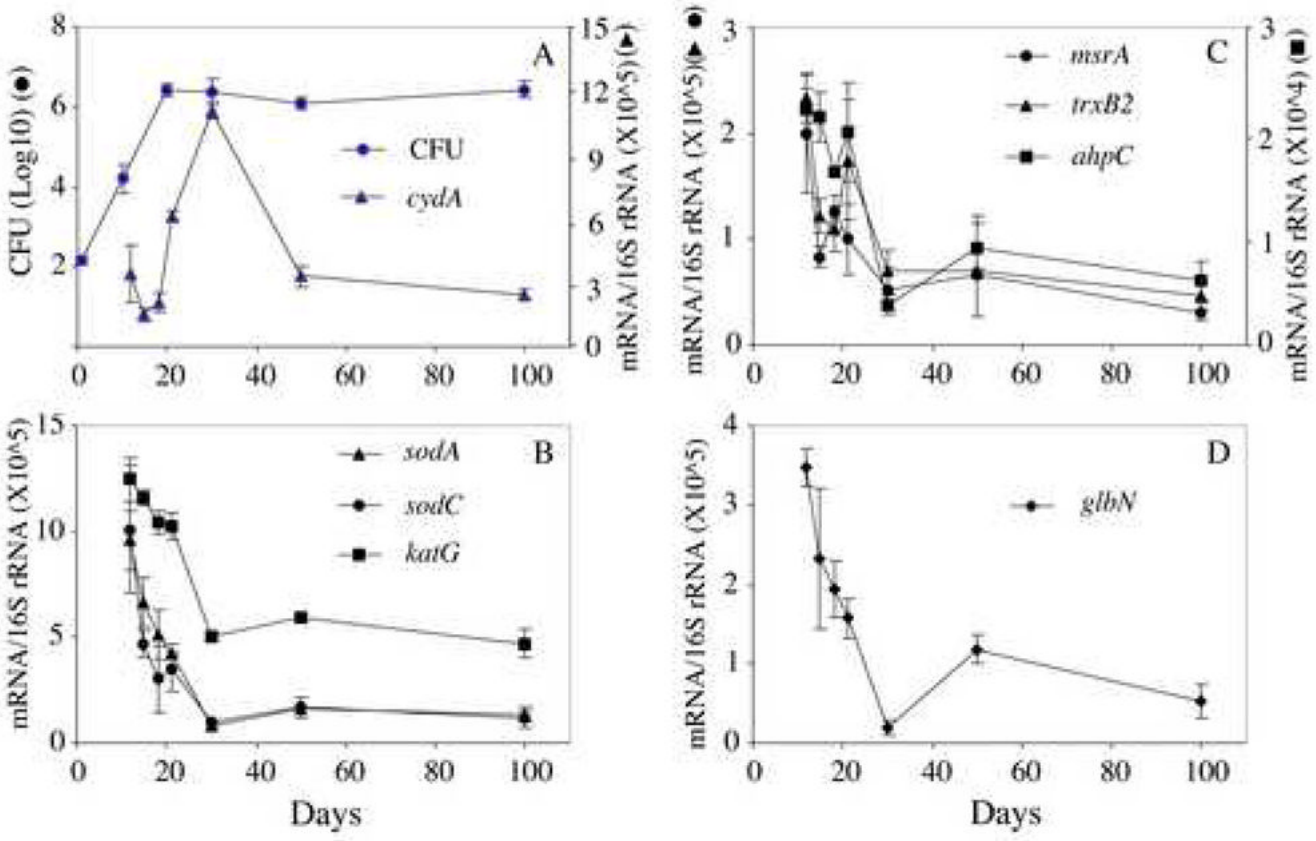

Figure 1. *M. tuberculosis* growth and normalized copy number of bacterial transcripts encoding antioxidant enzymes in infected mouse lung

Panel A: *M. tuberculosis* growth and copy numbers of cytochrome *bd* oxidase (*cydA*) during infection. In lungs of C57BL/6 mice infected with *M. tuberculosis* H_{37}Rv via the respiratory route, *M. tuberculosis* grew exponentially for about 20 days followed by chronic phase characterized with stabilization of bacterial counts (filled circles). Copy numbers of *cydA* transcripts were determined by real time RT-PCR and normalized against 16S rRNA (filled triangles, data from [27]). Panels B–D: Copy numbers of bacterial transcripts encoding antioxidant enzymes. Normalized copy numbers of *M. tuberculosis* mRNAs against 16S rRNA were expressed as mean ± SD of data obtained from lungs of 3 or 4 mice per time point.

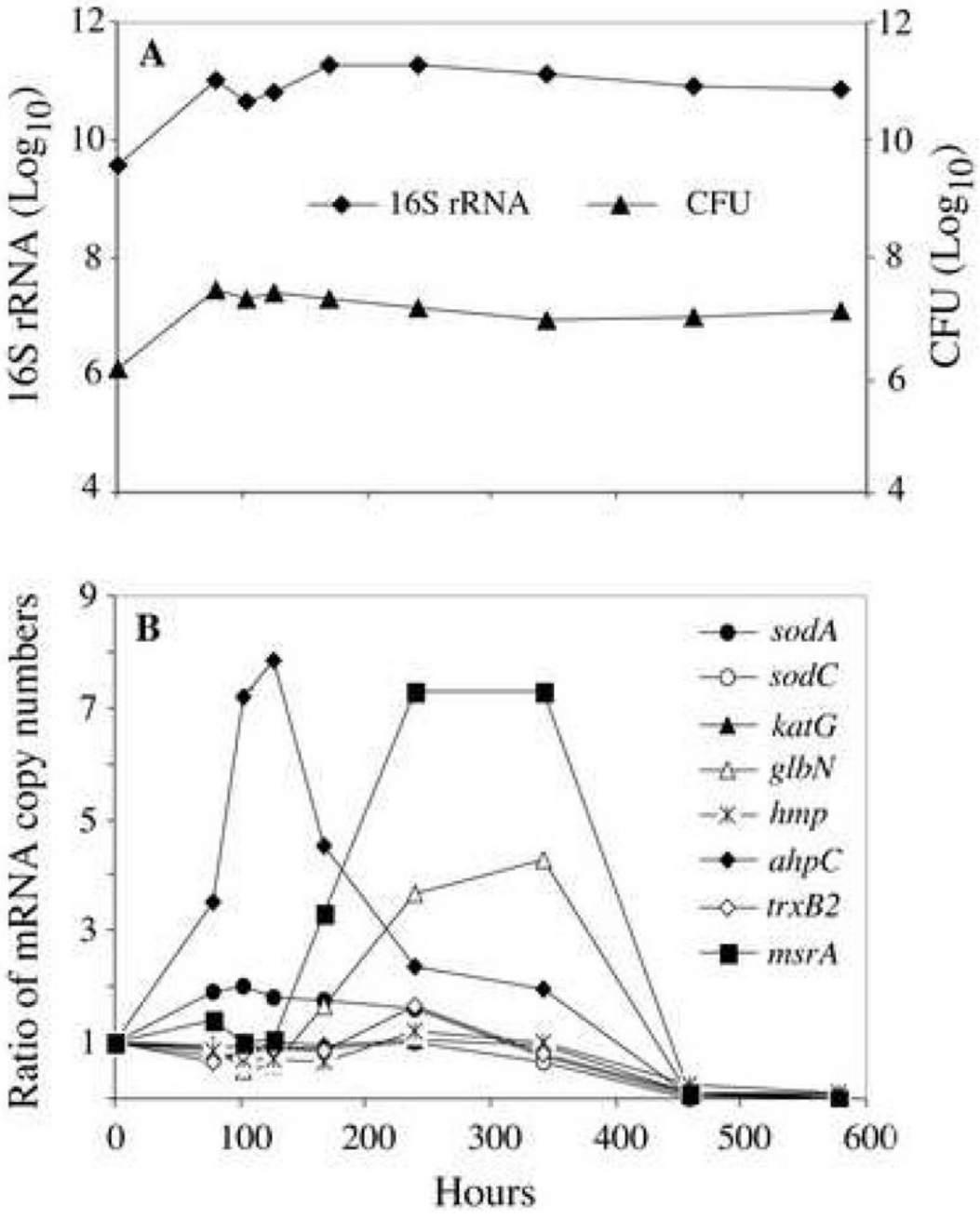

Figure 2. *M. tuberculosis* growth and changes of bacterial transcripts encoding antioxidant enzymes in liquid cultures during adaptation to gradual oxygen depletion

M. tuberculosis was cultured under conditions of gradual O_2 depletion established by Wayne and Hayes [21]. Bacterial RNA was extracted, and transcripts were enumerated by real-time RT-PCR with molecular beacons. Panel A: *M. tuberculosis* growth curve and 16S rRNA levels. Panel B: Changes of *M. tuberculosis* transcripts encoding antioxidant enzymes during gradual oxygen depletion. Levels of transcripts per cell were obtained by dividing mRNA copy number by the corresponding 16S rRNA copy number. Shown are ratios of normalized mRNA copy numbers determined at hour 78, 102, 126, 168, 240, 342, 460 and 578 relative to aerated mid-log cultures (hour 0). Replicate experiments produced similar results.

Table 1

Nucleotide sequences of RT primers, PCR primers, and molecular beacons for bacterial gene expression measurements.

Gene	RT primers	PCR primers	Molecular beacons
katG (*Rv1908*)	tgacctcccacccgacttgtg	catgggtcccgttgcgagata cccggatctggctcttaaggc	FAMagcgcgatccggtccctgcg gtcagcgcgctDabcyl
glbN (*Rv1542c*)	caggctgaagtggtgcatggtaa	caaacgtgagccgatcagcat cggcaagcacacgaacataga	FAMccgcggcatgaggccatcga agtcgtcgcggDabcyl
ahpC (*Rv2428*)	tgttggggtcgacgataaaggtc	ggcgttcagcaagctcaatgac agcatcgggaagggtaacgtttt	FAMccggcggcccagatcctggg ggtttcgcgccggDabcyl
trxB2 (*Rv3913*)	tcagcggcccgtgaagtgatac	ctggtcttcgagggcacgtct cccgcatctcatccatcaactc	FAMacgcgcgacgtggagaacta cccgggagcgcgtDabcyl
msrA (*Rv0137c*)	tggggtagcgctgcaggtagt	cttccagatccacgacccgacaac gatccgcttttgctgctcatcgaa	FAMgcgcgaacgaccgggggga ccagctaccgcgcDabcyl
hmp (*Rv3571*)	gatccgattccagcgacttgaa	ctcgttgtgcagttcgccctac gagggttgtggggacgaagtt	FAMcgggccaccgccgacgggt acgcctcggcccgDabcyl

Nucleotide sequences were obtained from http://genolist.pasteur.fr/TubercuList/[48]. RT and PCR primers (direction: 5′ to 3′, one primer per line) were designed by using the software Oligo 6.6 (Molecular Biology Insights, Cascade, CO) and were purchased from Integrated DNA Technologies (Coralville, Iowa). Molecular beacons were synthesized by Biosearch Technologies (Novato, CA). The nucleotide sequences of primers and molecular beacons for *M. tuberculosis* 16S rRNA, *sodA* and *sodC* were published previously [22]. FAM, iodoacetamide derivative of fluorescein (5-iodoactamidofluorescein); Dabcyl, 4-(4′-dimethylaminophenylazo)-benzoic acid) succinimidyl ester.