The response of *Mycobacterium tuberculosis* to reactive oxygen and nitrogen species

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**Abstract**

The bacteriostatic and bactericidal effects and the transcriptional response of *Mycobacterium tuberculosis* to representative oxidative and nitrosative stresses were investigated by growth and survival studies and whole genome expression analysis. The *M. tuberculosis* reaction to a range of hydrogen peroxide (H₂O₂) concentrations fell into three distinct categories: (1) low level exposure resulted in induction of a few highly sensitive H₂O₂-responsive genes, (2) intermediate exposure resulted in massive transcriptional changes without an effect on growth or survival, and (3) high exposure resulted in a muted transcriptional response and eventual death. *M. tuberculosis* appears highly resistant to DNA damage-dependent, mode-one killing caused by low millimolar levels of H₂O₂ and only succumbs to overwhelming levels of oxidative stress observed in mode-two killing. Nitric oxide (NO) exposure initiated much the same transcriptional response as H₂O₂. However, unlike H₂O₂ exposure, NO exposure induced dormancy-related genes and caused dose-dependent bacteriostatic activity without killing. Included in the large shared response to H₂O₂ and NO was the induction of genes encoding iron–sulfur cluster repair functions including iron acquisition. Stress regulons controlled by IdeR, Sigma H, Sigma E, and FurA comprised a large portion of the response to both stresses. Expression of several oxidative stress defense genes was constitutive, or increased moderately from an already elevated constitutive level, suggesting that bacilli are continually primed for oxidative stress defense.

**Keywords:** *Mycobacterium tuberculosis*, reactive oxygen species, reactive nitrogen species, microarray, nitric oxide, hydrogen peroxide

**INTRODUCTION**

During the course of infection, *Mycobacterium tuberculosis* (*Mt*) must cope with a variety of host-mediated stresses, in particular, the antibacterial properties of macrophages. Macrophages produce antimicrobial reactive oxygen and nitrogen species (ROS and RNS) via NADPH oxidase (NOX2/gp91phox) and inducible nitric oxide synthase (iNOS; Ehrt and Schnappinger, 2009).

*In vitro* experiments demonstrate no effect on *Mt* intracellular growth in Phox-deficient macrophages, suggesting wild-type *Mt* is resistant to the inhibitory effects of the macrophage oxidative burst (Chan et al., 1992). Mice deficient in Phox are partially inhibited in their ability to control *Mt* growth in an aerosolized infection model before the onset of specific immunity (Cooper et al., 2000), suggesting a role for ROS in the control of *Mt* early in the infectious process. By contrast, iNOS deficient mice, which lack inducible NO production, are highly susceptible to *Mt* infection (Yang et al., 2009). In addition, treatment of *Mt*-infected mice with the iNOS inhibitors given during acute or chronic infection causes mice to rapidly succumb to the disease (Flynn et al., 1998; Nathan, 2002). Furthermore, inhibition of the iNOS modulating cytokine, TNFα, leads to reactivation of *Mt* from persistent murine infection (Mohan et al., 2001) and reactivation of latent human disease (Gardam et al., 2003). Although the role of NO in human tuberculosis remains unsettled evidence supporting its importance has come from a variety of areas (Nathan and Shiloh, 2000) including the demonstration that human granulomas contain iNOS, endothelial-NOS, and nitrotyrosine, a compound whose accumulation indicates production of NO (Nathan, 2002). Additionally, the ability of human alveolar macrophages to kill *Mt* is dependent on the activity of iNOS, and the human macrophages taken from healthy subjects latently infected with *Mt* produce NO, controlling the growth of the bacteria (Yang et al., 2009). The presence of NO within human granulomas could contribute to host resistance since *in vitro* experiments demonstrate direct RNS-mediated bacteriostatic (Firmani and Riley, 2002; Ouellet et al., 2002; Voskuil et al., 2003) and bactericidal activity (Nathan, 2002). Mice deficient in both phox and iNOS are much more susceptible to *Mt* infection than either mutant alone, which would indicate that RNS and ROS protect the host in a partially redundant fashion (Shiloh and Nathan, 2000). However, there was little overlap seen between response to either NO or H₂O₂ when cultures exposed to either stress was analyzed by 2-dimensional gel electrophoresis and compared to unexposed cultures (Garbe et al., 1996).

*Mt* utilizes a range of mechanisms to defend against ROS and RNS including direct scavenging of the reactive species and the repair and protection of proteins and DNA (Ehrt and Schnappinger, 2009). The resistance of *Mt* to ROS is partly due to the thick *Mt* cell wall containing lipoarabinomannan (LAM) and cyclopropated mycolic acids, as well as phenolic glycolipid I (PGL-1), which act as potent scavengers of oxygen radicals (Flynn and Chan, 2001). In addition, *Mt* produces the ROS scavenging enzymes catalase (KatG; Manca et al., 1999; Ng et al., 2004), superoxide dismutases...
(SodA and C; Jackett et al., 1978; Piddington et al., 2001; Tullius et al., 2001), and the peroxidase and peroxyxirite reductase complex of AhpC, AhpD, SucB(DlaT), and Lpd (Bryk et al., 2002). A dta mutant is also hyper-susceptible to RNS (Shi and Ehr, 2006), and cells with a deletion mutant in alpC are more susceptible to ROS (Springer et al., 2001; Master et al., 2002). The thioredoxin/thioredoxin reductase systems from Mtb also contributes to the reduction of peroxides (Zhang et al., 1999). The low-molecular-weight antioxidant mycothiol takes the place of glutathione found in many bacteria and is important in maintaining a reduced environment and resistance to oxidative stress (Oum et al., 2006). The sulfur assimilation pathway plays a role in oxidative and nitrosative stress as a cysM mutation deficient in cysteine and methionine synthesis is more sensitive to these stresses (Sener, 2006).

Mtb DNA is directly protected from ROS by the DNA binding protein, Lsr2 (Colangeli et al., 2009). Additional RNS resistance mechanisms include the catalytic detoxification of NO achieved by the truncated hemoglobin (trHbN) in an oxygen-dependant reaction (Ouellet et al., 2002; Puthania et al., 2002). The presence of trHbN protects aerobic respiration from inhibition by NO in Mycobacterium smegmatis (Puthania et al., 2002). Deletion of genes that encode methionine sulfoxide reductase (msra; St John et al., 2001; Lee et al., 2009), the Mtb proteasome (prcBA; Darwin et al., 2003; Gandotra et al., 2007), nucleotide excision repair (uvrB; Darwin et al., 2003), and F-420 biosynthesis (fbcG; Darwin et al., 2003) are also hyper-susceptible to RNS. Alpha crystalline (acr), and bacterioferritin (bfrB), as well as the DosR regulon are upregulated upon addition of NO (Garbe et al., 1999; Ohno et al., 2003), but the role of these proteins in NO protection is little understood.

Defense against oxidative stress in Escherichia coli is largely controlled by the OxyR and SoxR regulators (Imlay, 2008). Genes regulated by OxyR include the catalase gene, katG, for detoxification of H2O2, and the alkyl hydroperoxide reductase genes, alpCF, for detoxification of alkyl peroxides. Mtb possesses many of the same genes that encode ROS defense mechanisms in other bacteria, including katG (catalase), sodA, (superoxide dismutase), alpCDE (alkyl peroxide reductase), thioredoxin, and thioredoxin reductase genes. However, the regulation of these genes by Mtb differs markedly from their regulation in enteric bacteria. It was originally proposed that Mtb is capable of only a limited transcriptional response to oxidative and nitrosative stress compared to enteric bacteria, due to an incomplete repertoire of regulatory factors (Deretic et al., 1995; Sherman et al., 1995; Zehrt and Deretic, 2002). Previous work done by Garbe et al. (1996) would indicate that there is very little transcriptional response to H2O2 in Mtb as demonstrated on 2-dimensional gel electrophoresis, and genes involved in detoxifying oxidative species are not upregulated early in a mouse model when the oxidative burst is occurring (Shi et al., 2008). In particular, the gene encoding OxyR has sustained multiple mutations, and thus this primary regulator of the oxidative stress response in other bacteria is a pseudogene in Mtb and other closely related species in the Mtb complex (Deretic et al., 1995; Sherman et al., 1995). By contrast, a functional copy of oxyR is present in several other mycobacteria including M. leprae, M. avium, and M. marinum (Pagano-Ramos et al., 1998). SoxR regulates expression of sodA and other ROS responsive genes in E. coli, but no apparent homolog of soxR exists in the Mtb genome (Cole et al., 1998). Alternative Mtb transcriptional regulators have been implicated in ROS defense: FurA (Milano et al., 2001; Pym et al., 2001; Zehrt et al., 2001), IdeR (Dussurget et al., 1996; Rodriguez et al., 2002), CarD (Stallings et al., 2009), and Sigma H (Fernandes et al., 1999; Raman et al., 2001), as well as genes involved in iron acquisition (Jang et al., 2009).

Although NO and H2O2 both play a role in controlling Mtb infection, not much is understood on how the bacteria transcriptionally respond to different concentrations of these two stimuli. An increased understanding of the interaction between Mtb and chemicals used by the host to control it could shed light on which genes are important for bacterial persistence mechanisms. In order to determine how Mtb is differentially affected by ROS and RNS, we designed experiments to examine the concentration-dependent effects of a broad range of doses of ROS and RNS on Mtb growth and survival. We also determined the transcriptional response of Mtb to H2O2 and NO at these same doses.

MATERIALS AND METHODS

CULTURE CONDITIONS

Mtb clinical isolate isolated from human sputum, 1254 (American Type Culture Collection 51910), 7H9 medium (supplemented with bovine serum albumin, NaCl, glucose, and glycerol), 250 ml vented tissue culture flasks positioned upright, 90 rpm shaking, and a starting culture density of OD 0.15 were used, unless otherwise indicated. RNA samples isolated from OD 0.15 cultures of the Mtb strain being assayed on the same day were used for the reference sample in each experiment. Cells were collected with a 4-min centrifugation step and frozen on dry ice.

H2O2 AND NO EXPOSURE

Mtb cultures were exposed to a single doses of H2O2 or the NO donors DETA/NO (diethylenetriamine/nitric oxide adduct) or spermidine-NONOate (Sigma Co.). Doses of 0.0, 0.05, 0.5, 1.0, 5, 10, 50, and 200 mM H2O2; 0.005, 0.05, 0.5, 1.0, and 5.0 mM DETA/NO; and 5 mM spermidine-NONOate were used. Stock solutions of H2O2 were prepared in H2O and NO donors were suspended in 0.01 N NaOH. Optical density 600 nm and colony forming units (CFU) were monitored after addition of stress. Cells were sampled 40 min or 4 h following H2O2 and DETA/NO addition and total RNA was isolated and compared by microarray to RNA from untreated cells but prepared at the same time and from the same parent culture. Three independent experiments were conducted for each H2O2 and DETA/NO concentration.

RNA ISOLATION

RNA isolation and microarray analysis were performed as previously described (Wilson et al., 2001). Briefly, cell pellets were suspended in 1 ml Trizol reagent (Gibco-BRL) and transferred to 2 ml screw cap tubes containing 0.5 ml 0.1 mm diameter zirconia/silica beads (Biospec Products). Three 30 s pulses in a bead beater disrupted cells. Tubes were placed in ice between bead beating steps. Cell debris was separated by a 55-s centrifugation. The supernatant was transferred to 2 ml Heavy Phase Lock Gel I tubes (Eppendorf), containing 300 μl of chloroform, and inverted rapidly for 15 s and incubated 2 min with periodic inversion. Samples were centrifuged 5 min and the aqueous phase was added to 270 μl of isopropanol, followed by addition of 270 μl of (0.8 M Na citrate, 1.2 M NaCl). Samples were incubated 10 min to overnight at 4°C and centrifuged for 15 min at 4°C. The
RNA pellets were washed with 1 ml 75% ethanol, centrifuged 5 min and air-dried for 20 min. Following suspension of the RNA pellets in 90 μl of water, 10 μl of DNase I 10× buffer and 6 units of DNase I (Ambion) were added and the samples were incubated for 30 min. Final purification of RNA was by RNeasy column (Qiagen).

cDNA LABELING AND MICROARRAY HYBRIDIZATION
Both a PCR gene product microarray and a 70-mer oligonucleotide-based microarray (tuberculosis oligonucleotide set [Qiagen]) were used. Labeled cDNA was prepared as follows: 1.5 μg of total RNA and 4.4 μg of random oligonucleotide hexamers were incubated 2 min at 98°C, cooled on ice, combined with Stratscript RTase buffer, 0.5 mM dATP, dGTP, and dCTP, 0.02 mM dTTP, 1.5 nmol Cy3 or Cy5-dUTP (Amersham), and 1.8 μl Stratscript RTase (Stratagene) in a total volume of 25 μl and incubated 10 min at 25°C followed by 90 min at 42°C. Labeled cDNA was diluted in 400 μl TE and purified by microcon-10 (Amicon) filtration. Ten microliter hybridization solution (labeled cDNA, 5 μg tRNA, 3.8× SSC, 0.27% SDS) was sealed under a coverslip with rubber cement and hybridized overnight at 65°C for the DNA microarray. Oligonucleotide microarrays were first prehybridized for 1 h in 5X SSC, 1% BSA, and 0.1% SDS and washed with H2O and isopropanol. Following the prehybridization, 10 μl hybridization solution (labeled cDNA, 5 μg tRNA, 2× SSC, 25% formamide, 0.1% SDS) was hybridized overnight at 54°C. Slides were washed once for 2 min in 0.05% SDS, 1× SSC, washed twice for 2 min in 0.06× SSC, centrifuged at 600 rpm for 2 min, and were immediately scanned.

MICROARRAY DATA ANALYSIS
Microarrays were scanned using a GenePix 4000A (Axon Instruments). The intensities of the two dyes at each spot were quantified using ScanAlalyze (Michael Eisen1). All gene specific spots on the microarray other than those whose induction ratio was in the top or bottom 5% were used to normalize the intensities of Cy3 and Cy5 from each spot. After Cy3 and Cy5 channel normalization we eliminated large percentage fluctuations in low background spot values by adjusting low signal intensity spots to a minimum noise value. The noise value for each channel was determined by calculating the average intensity value of the 20% lowest intensity spots, and then every value below this average noise value was raised to the noise value. Microarray-determined ratios were calculated from three biological replicates and one to two microarrays for each biological replicate. Significance analysis of microarrays (SAM)2 was used to determine statistically significant regulated genes under H2O2 and NO stress (Tusher et al., 2001). Genes included in Table A1 in Appendix conformed to a highly stringent selection criteria. They exhibited at least a fourfold induction or repression ratio in at least one condition and had a SAM corresponding false discovery q value of zero.

RESULTS
EFFECr OF H2O2 AND NO ON GROWTH AND VIABILITY
Mtb clinical isolate 1254, growing in mid-logarithmic phase (optical density, OD, of 0.15), was exposed to a range of initial H2O2 concentrations from 50 μM to 200 mM (Figure 1). Culture OD was monitored for 72 h as a measure of growth, and CFU were determined at 4 and 24 h as a measure of cell viability. Exposure to 0.05, 0.5, and 5.0 mM H2O2 had no effect on culture growth or viability. A 10-mM exposure caused only a slight growth defect, while exposure to 50 and 200 mM resulted in a significant decrease in culture OD and complete sterilization of the cultures by 24 h. A small fraction of bacilli were still viable (~0.1%) 4 h post 50 mM H2O2 exposure, however, by 24 h no colonies were recovered on petri plates. OD600 measurements did not reach its minimum value until 72 h.

Mtb strain 1254 was also exposed to a range of concentrations between 5 μM and 5 mM of the slow release NO donor (DETA/NO: diethylenetriamine/nitric oxide adduct; Sigma Co.). DETA/NO is reported to release NO with a half-life of 20 h at pH 7.0. We determined the half-life of DETA/NO to be 5.5 h under our standard growth conditions (Voskuil et al., 2003). Exposure to 5 and 50 μM DETA/NO had no effect on culture growth, whereas 0.5, 1.0, and 5.0 mM DETA/NO had a dose-dependent, long-term effect on culture growth (Figure 2A). Previously, we demonstrated that exposure to intermediate levels of NO (0.5 and 1.0 mM DETA/NO) results in a 12- to 24-h temporary cessation of growth (Voskuil et al., 2003). Growth resumed after depletion of the NO donor (~18 h for 0.5 mM DETA/NO); however, the resumptive growth rate was less than maximal for the medium based on the growth rate of cultures exposed to non-inhibitory concentrations of NO.
Exposure to all NO concentrations had little effect on cell viability, ascertained by viable plate counts, assayed 4 and 24 h previously (Voskuil et al., 2003). However, this could be attributed to lower growth rates in cultures exposed to the two highest concentrations of DETA/NO rather than cell death (Voskuil et al., 2003). To further investigate the bacteriostatic activity of NO, we exposed 1-mM Spermidine-NONOate resulting in delivery of 10 mM NO to Mtb cultures at OD values of 0.01, 0.001, and 0.0001 to 0.05 mM DETA/NO by both platforms and similar results were obtained; however, the oligonucleotide platform produced on average an increased level of regulation (Table A1). The most striking difference between the H2O2 and NO expression profiles was the dramatic induction of genes controlled by the dormancy survival regulator, DosR (Figure 3A; Table S1 in Supplementary Material; Voskuil et al., 2003). The expression of many of these genes using RNA from the same experiments as those used in this paper has been previously confirmed under the same conditions via qRT-PCR (Voskuil et al., 2003). 0.05 mM of DETA/NO resulted in strong induction of the DosR regulon, but did not slow growth. Exposure to 0.05 mM DETA/NO resulted in the differential regulation of only a few genes which are not part of the DosR regulon. NO serves a signaling function which leads to the induction of the DosR regulon, even before an effect on growth (i.e., bacteriostasis) is evident. At concentrations of DETA/NO above 0.05 mM, in addition to the DosR regulon induction and bacteriostatic response seen at lower doses, a more typical oxidative stress gene expression pattern emerged. Expression data demonstrated that a large number of genes induced by NO concentration >0.05 mM were also induced by H2O2 (Table A1).

**EXPRESSION PATTERNS INDUCED BY H2O2**

Changes in mRNA expression patterns after exposure to H2O2 were monitored by oligonucleotide DNA microarray analysis. Mtb cultures were grown and exposed to H2O2 as described in Section “Materials and Methods.” The global expression patterns of genes that respond to H2O2 are depicted in Table A1. At 0.05 and 0.5 mM H2O2, few gene expression changes were observed. The furA–katG–rv1907c three gene operon and genes involved in iron acquisition were the most sensitive to low concentrations of H2O2 (Table A1). At intermediate concentrations of H2O2 (5 and 10 mM) a dramatic effect on gene expression was observed with hundreds of genes induced and repressed. Interestingly, at lethal concentrations of H2O2 (50 and 200 mM) a significantly muted transcriptional response was observed (Table A1). Many genes induced by intermediate concentrations of H2O2 were either not induced or were repressed during exposure to lethal concentrations of H2O2. Conversely, some genes were induced to a greater degree at the lethal concentrations.

**EXPRESSION PATTERNS INDUCED BY NO**

NO-induced gene expression changes were monitored by two microarray formats: a PCR product (amplicon) DNA microarray; and, an oligonucleotide microarray. Both formats produced similar qualitative regulation ratios. We examined exposure to 0.5 mM DETA/NO by both platforms and similar results were obtained; however, the oligonucleotide platform produced on average an increased level of regulation (Table A1). The most striking difference between the H2O2 and NO expression profiles was the dramatic induction of genes controlled by the dormancy survival regulator, DosR (Figure 3A; Table S1 in Supplementary Material; Voskuil et al., 2003). The expression of many of these genes using RNA from the same experiments as those used in this paper has been previously confirmed under the same conditions via qRT-PCR (Voskuil et al., 2003). 0.05 mM of DETA/NO resulted in strong induction of the DosR regulon, but did not slow growth. Exposure to 0.05 mM DETA/NO resulted in the differential regulation of only a few genes which are not part of the DosR regulon. NO serves a signaling function which leads to the induction of the DosR regulon, even before an effect on growth (i.e., bacteriostasis) is evident. At concentrations of DETA/NO above 0.05 mM, in addition to theDosR regulon induction and bacteriostatic response seen at lower doses, a more typical oxidative stress gene expression pattern emerged. Expression data demonstrated that a large number of genes induced by NO concentration >0.05 mM were also induced by H2O2 (Table A1).

**THE ROS AND RNS EXPRESSION PROFILE**

In the absence of a functional OxyR, three other transcriptional regulators (SigH, IdeR, and DosR) appear to have a large role in shaping the response to ROS and RNS in Mtb. Expression of sigH is induced up to 22-fold by 50 mM H2O2 and sixfold by 5 mM DETA/NO. ideR expression is moderately induced by both stresses as measured by the oligonucleotide microarray (3.2-fold by 10 mM H2O2 and 3.4-fold by 0.5 mM DETA/NO). However, these ideR induction ratios were below the cutoff required for inclusion in Table A1. None of the DosR regulon genes was regulated in response to H2O2 (Table S1). The DosR-regulated genes were not included in Table A1 as their induction by NO has been reported (Voskuil et al., 2003). Genes controlled by SigH, IdeR, SigE, and FurA account for a large number of the genes induced by H2O2 and NO (Figure 3B). However, 14 additional regulators are also induced by H2O2, at least fourfold and 7 regulators by NO; including the global transcription factor Sigma B (Table A1).
H₂O₂, as measured by the oligonucleotide microarray, was 12, 30, 16, 58, and 13-fold, respectively. Maximum induction of the same genes included in the amplicon microarray, was 3.2, 2.8, 3.2, 9.3, and 2.1-fold, respectively. Other genes predicted or proven to be involved with ROS and/or RNS resistance were not highly differentially expressed and thus not included in Table A1. Fortye-one of the 174 genes are members of the previously published DosR regulon (Voskuil et al., 2003) and are therefore not included in Table A1. A majority of genes were regulated by both NO and H₂O₂ and 53 genes were regulated solely by NO including the 41 the DosR regulon genes (B) 163 genes from Table A1 were induced by H₂O₂, of these 42 were not induced by NO. Four transcriptional regulators: SigH (Imlay, 2003), IdeR (Rodriguez et al., 2002), SigH (Park and Imlay, 2003), and FurA (this study and Pym et al., 2001; Zahrt et al., 2001) control genes that encompass 33% of the H₂O₂ response.

**DIFFERENTIAL EXPRESSION OF GENES WHICH CONFER RESISTANCE TO ROS AND/OR RNS**

The *Mtb* genome encodes several proteins known to be involved with or implicated in resistance to ROS and/or RNS (Imlay, 2008; Ehr et al. and Schnappinger, 2009). Expression of some of these genes was found to be highly induced in response to H₂O₂ and NO. These include the genes encoding catalase (*katG*), thioredoxin reductase (*trxB2*), and the thioredoxin genes (*thiX*, *trxB1*, and *trxC*). Maximum induction of *katG*, *trxB*, *thiX*, *trxB1*, and *trxC* by exposure to 5–50 mM H₂O₂, as measured by the oligonucleotide microarray, was 12, 30, 16, 58, and 13-fold, respectively. Maximum induction of the same genes by exposure to 0.5–5 mM DETA/NO, as measured by the ampiclon microarray, was 3.2, 2.8, 3.2, 9.3, and 2.1-fold, respectively. Other genes predicted or proven to be involved with ROS and/or RNS resistance were not highly differentially expressed and thus not included in Table A1. *ahpC* did not fit the strict criteria for inclusion in Table A1, although it had a maximum induction level of 3.2 by NO, and is important for resistance against oxidative stress in *Mtb* (Springer et al., 2001; Master et al., 2002).

Even though many genes implicated in ROS and RNS stress resistance were not highly induced by H₂O₂ or NO, their basal expression levels (as measured by the relative intensity of the microarray signal) were high even in the absence of exogenous ROS or RNS. In particular, the average microarray signal intensities from both the ampiclon and oligonucleotide microarrays were in the top 4, 12, 8, 11, 16, and 10% of all genes for *sodA*, *sodC*, *katG*, *ahpD*, *ahpE*, and *ahpC*, respectively.

**A COMMON OXIDATIVE AND NITROSATIVE STRESS RESPONSE**

The breadth of the shared transcriptional response to H₂O₂ and NO suggests that a common set of mechanisms plays a role during adaptation to ROS and RNS (Table A1). The most pronounced of these are genes controlled by the global transcription regulators IdeR and Sigma H (Figure 3B). In addition, a large number of the genes which are negatively regulated by both H₂O₂ and NO encode general housekeeping functions. The most repressed genes include 12 ribosomal protein genes and *nuoD* and *nuoE*, encoding NADH dehydrogenase subunits (Table A1), indicating a general slowing of growth.

NO and H₂O₂ both strongly induce genes from rv1460 to rv1466; the annotated functions of these genes suggest that RNS and ROS stimulate a concerted effort to replace or repair iron–sulfur clusters. Exposure to even low levels of H₂O₂ or NO-induced genes involved in iron acquisition (*mtb* cluster), while the *bfrB* gene, encoding the iron-storage protein bacterioferritin, was repressed. After exposure to higher concentrations of H₂O₂ or NO, the induction of the *mtb* cluster was consistently less than that observed during exposure to intermediate levels of these stresses.

**THE H₂O₂-SPECIFIC RESPONSE**

A notable difference between the response of *Mtb* to H₂O₂ and NO was the lack of a DNA damage response observed after initial NO exposure. By contrast, H₂O₂ induced several genes involved in DNA repair: *recA*, *radA*, and *alkA*. *alkA* encodes a methylated-DNA–protein–cysteine methyltransferase, which repairs alkylated and methylated guanine and thymine residues by a suicide reaction where the alkyl or methyl group is transferred to a cysteine in the enzyme (Samson, 1992). *rv3201c* and *rv3202c* were also induced by H₂O₂; these genes encode subunits of an ATP-dependent helicase homologous to UvrD helicases, which are also involved in DNA repair. Several transposase and resolvase genes from insertion sequences and phages, were also induced by H₂O₂ (*rv0605*, *rv0606*, *rv0829*, *rv0850*, *rv2885c*, *rv2978c*, *rv2979c*; and *rv3827c*); some of these were previously shown to respond to DNA damage (Bosshoff et al., 2003; Rand et al., 2003). Bosshoff et al. (2003) also demonstrated induction of DNA damage responsive genes (including *recA*, *radA*, *alkA*, *rv3202c*) by DNA damage from UV irradiation and mitomycin.

Although most of the above mentioned genes were not induced by a 40-min exposure to NO, most were weakly induced after 4 h of NO exposure, suggesting a moderate level of DNA damage after extended NO exposure. Interestingly, these genes were not induced (or were induced significantly less) at lethal levels of H₂O₂. For example, *recA* was induced 4.4- and 4.1-fold by 5 and 10 mM H₂O₂ but only 0.8 and 0.5-fold at 50 and 200 mM H₂O₂; and *radA* was induced 9.3 and 9.5-fold at 5 and 10 mM H₂O₂, but only 1.0- and 1.1-fold at 50 and 200 mM H₂O₂.

Another striking difference between the H₂O₂ and NO expression profiles is the apparent exclusive induction of several PE and PPE genes by H₂O₂. Four such genes reside between *rv1806*–*rv1809* (PE20, PPE31, PPE32, PPE33); the first two in this group are induced over 40-fold. A fifth gene in the PE/PPE families, *PE33* (*rv3650*), was found to be induced 9-fold. By contrast to several of the DNA repair genes, these genes were not induced after 40 min or 4 h of NO exposure and even appeared to be slightly repressed. We previously demonstrated that regulation of genes in the PE and PPE families is highly variable under a variety of conditions and largely independent of other family members (Voskuil et al., 2004).

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**FIGURE 3** | Distribution of genes regulated in response to NO and H₂O₂.

(A) 174 were induced at least 2-fold after 40 min exposure to 0.005, 0.05, 0.5, 1.0, or 5.0 mM DETA/NO and fulfilled the criteria for inclusion in Table A1. A majority of genes were regulated by both NO and H₂O₂ and 53 genes were regulated solely by NO including the 41 the DosR regulon genes (B) 163 genes from Table A1 were induced by H₂O₂, of these 42 were not induced by NO. Four transcriptional regulators: SigH (Imlay, 2003), IdeR (Rodriguez et al., 2002), SigH (Park and Imlay, 2003), and FurA (this study and Pym et al., 2001; Zahrt et al., 2001) control genes that encompass 33% of the H₂O₂ response.
Genes encoding a variety of other functions were highly regulated by \( \text{H}_2\text{O}_2 \), but not by NO, including genes involved in propionate metabolism \([\text{rv1130} \text{ and } \text{rv1131} (\text{gltA1})] \) and the \( \text{leuD} \) and \( \text{leuC} \) genes involved in leucine biosynthesis, which were induced 20–40-fold. Interestingly, the resuscitation-promoting factor gene \( \text{rpfA} \) was highly repressed at 5 and 10 mM \( \text{H}_2\text{O}_2 \), but not at higher concentrations of \( \text{H}_2\text{O}_2 \) or after short-term NO exposure. However, after 4 h of 0.5 mM DETA/NO exposure, \( \text{rpfA} \) was also repressed.

**THE NO-SPECIFIC RESPONSE**

Most genes that respond to high, but not to low levels of NO (i.e., NO-responsive genes other than the DosR regulon, which uniquely responds to low levels of NO) were also regulated in response to \( \text{H}_2\text{O}_2 \) (Table A1). One hundred seventy-four genes were induced in response to DETA/NO (Figure 3A). Forty-one of these genes are members of the DosR regulon (not included in Table A1). Of the remaining 133 NO-induced genes, 121 were also induced by \( \text{H}_2\text{O}_2 \). Accordingly, only 12 genes \([\text{cyp138, rv0330c, rv0967, sigE, rv1684, rv2033c, rv2036, rv2038c, rv2125, rv2620c, rv2621c, and lqfA} ] \) were selectively induced by NO but not \( \text{H}_2\text{O}_2 \). Thus, other than the DosR regulon, 121 of the 133 genes which were differentially regulated by higher concentrations of NO were also regulated by \( \text{H}_2\text{O}_2 \). Taken together, these results reflect the oxidative stress capacity of NO at high levels.

**DISCUSSION**

A large number of genes have been identified as important in resistance to oxidative and nitrosative stress (Ehrt and Schnappinger, 2009). Interestingly, many of these genes are not induced by \( \text{H}_2\text{O}_2 \) or NO. The presence in the \( \text{Mtb} \) genome of a disrupted copy of \( \text{oxyR} \) predicts that genes normally repressed by OxyR in other species will exhibit higher basal expression in \( \text{Mtb} \). In turn, higher basal expression levels would correlate with reduced induction ratios in response to oxidative stress. The lack of genes that respond to oxidative stress has led to the conclusion that \( \text{Mtb} \) is not capable of a robust response to this stress (Sherman et al., 1995; Garbe et al., 1996; Zahrt and Deretic, 2002; Shi et al., 2008). We find this to be the case for several genes that are commonly associated with oxidative stress resistance. This exhibit a relatively high basal level of expression even in the absence of exogenous ROS. Expression of \( \text{katG} \) and \( \text{ahpC} \) was maintained at a high basal level, although it was also further induced by addition of stress. In the absence of ROS generated by activated macrophages, a basal level of expression by ROS detoxification genes might be warranted to cope with oxidative stress emanating from metabolism in the presence of molecular oxygen. Most aerobic organisms, including some mycobacteria species (Pagan-Ramos et al., 2006; Imlay, 2008), employ OxyR to keep transcription at a low basal level until exogenous stress is encountered. The success of \( \text{Mtb} \) as a pathogen suggests that the loss of OxyR and the resulting constitutive expression of a portion of the oxidative stress response is not debilitating, but instead is likely to have been a propitious event that conferred a selective advantage. However, even in the absence of OxyR-mediated regulation, \( \text{Mtb} \) is capable of a large concerted transcriptional response to \( \text{H}_2\text{O}_2 \) as demonstrated in Table A1.

Several studies have indicated significant \( \text{Mtb} \) resistance to oxidative stress in vitro and in vivo. Our data confirm the high tolerance of \( \text{Mtb} \) for \( \text{H}_2\text{O}_2 \), \( \text{H}_2\text{O}_2 \) exposure resulted in no appreciable effect on \( \text{Mtb} \) growth or viability until exposures reached levels greater than 10 mM; suggesting \( \text{Mtb} \) is resistant to all but extreme levels. Low levels of \( \text{H}_2\text{O}_2 \) (0.5 mM) initiated the regulation of only the \( \text{furA–katG} \) and the \( \text{mtb} \) clusters, which are repressed by the regulators FurA and IdeR, respectively. Both of these regulators appear to be highly sensitive to the presence of \( \text{H}_2\text{O}_2 \), a consequence of their oxidative-sensitive metal-centers. At 5 and 10 mM \( \text{H}_2\text{O}_2 \), growth and viability were still largely unaffected. However, the large number and amplitude of the genes induced at these concentrations indicates at least a transient stress from \( \text{H}_2\text{O}_2 \) exposure. The response includes genes controlled by four transcriptional regulators previously implicated in ROS resistance [FurA (Zahrt et al., 2001), IdeR (Dussurget et al., 1996; Rodriguez et al., 2002), Sigma H (Fernandes et al., 1999; Raman et al., 2001), and Sigma E (Wu et al., 1997)] as well as the induction of 14 additional transcriptional regulators by \( \text{H}_2\text{O}_2 \). Thus, the response of \( \text{Mtb} \) to stress-inducing levels of \( \text{H}_2\text{O}_2 \) is broad and involves many regulatory networks. At very high doses (50 and 200 mM), \( \text{H}_2\text{O}_2 \) is lethal and the transcriptional response is significantly reduced and altered in comparison to effects mediated by non-lethal levels of \( \text{H}_2\text{O}_2 \). At first glance, the reduced response to lethal concentrations of \( \text{H}_2\text{O}_2 \) would seem to be most plausibly attributed to a collapse of the transcriptional machinery in dying bacilli. However, some genes are induced, or are more highly induced, in response to lethal levels of \( \text{H}_2\text{O}_2 \) compared to their expression during exposure to intermediate (non-lethal) concentrations of \( \text{H}_2\text{O}_2 \). Additionally, results from the study of high millimolar concentrations of \( \text{H}_2\text{O}_2 \) may not be physiologically relevant, unless the high concentration mimics the accumulation of oxidative damage after prolonged exposure to lower levels, which is likely observed under lethal conditions. Taken together, our results demonstrate that \( \text{Mtb} \) exhibits three, concentration-dependent \( \text{H}_2\text{O}_2 \) transcriptional responses: (1) a low dose response marked by induction of the most sensitive systems (\( \text{H}_2\text{O}_2 \) scavenging and iron acquisition); (2) a massive response to high, but sub-lethal levels of \( \text{H}_2\text{O}_2 \); and (3) a muted response to lethal levels of \( \text{H}_2\text{O}_2 \). The amount of peroxynitrite (formed by the reaction between equimolar amounts of NO and superoxide within the phagosome) produced per hour per macrophage is approximately 6 fmol (Ischiropoulos et al., 1992), which is approximately the same as the 5-fmol experienced by each cell of \( \text{Mtb} \) when 0.5 mM \( \text{H}_2\text{O}_2 \) was added, although it is still difficult to determine what specific level of ROS and RNS \( \text{Mtb} \) experiences in vivo over time. It is informative that many of the genes listed in Table A1 upregulated under mid-range concentrations of NO (0.5–5 mM) or \( \text{H}_2\text{O}_2 \) (5–10 mM) were previously found to be induced by \( \text{Mtb} \) during infection of activated macrophages (Schnappinger et al., 2003), indicating that the levels of ROS and RNS used in these experiments are most likely similar to what is experienced by the bacteria within the macrophage.

The second and third responses of \( \text{Mtb} \) to \( \text{H}_2\text{O}_2 \) are reminiscent of the two modes of \( \text{E. coli} \) killing by \( \text{H}_2\text{O}_2 \), first described by Imlay and Linn (1986). They demonstrated that \( \text{E. coli} \) killed by 1–2 mM \( \text{H}_2\text{O}_2 \)
is due to DNA damage and was termed mode-one killing. Mode-
two killing occurs at levels above 20 mM where multiple cellular
components are targeted and killing is dependent on exposure time,
and occurred when other cellular damage accumulated to a lethal
level. Our data are partially in agreement with the results from E. coli
indicating that high levels of \( \text{H}_2\text{O}_2 \) interfere with a \( \text{H}_2\text{O}_2 \)-mediated
mechanism that damages DNA. One of the main differences in the
\( \text{H}_2\text{O}_2 \) susceptibility of \( \text{E. coli} \) and \( \text{Mtb} \), however, appears to be the
resistance of \( \text{Mtb} \) to mode-one killing. Induction of \( \text{Mtb} \) DNA damage
repair mechanisms indicates that DNA damage occurs at 5 and
10 mM \( \text{H}_2\text{O}_2 \). However the pathogen is not killed at these concen-
trations and thus the damage does not result in DNA-dependent
mode-one killing which typifies the effects of \( \text{H}_2\text{O}_2 \) on \( \text{E. coli} \). We
hypothesize that one mechanism by which \( \text{Mtb} \) avoids mode-one
killing is by maintaining a very slow growth rate. An \( \text{Mtb} \) doubling
time of 12–24 h (compared to 20 min for \( \text{E. coli} \)) allows significantly
more time for DNA repair to occur before a DNA lesion has a chance
to disrupt DNA replication and exert its lethal effects.

Previous work has indicated that the response to \( \text{H}_2\text{O}_2 \) shares
very little in common with the response to NO in \( \text{Mtb} \) (Garbe et al.,
1996). This study, however, used 2-dimensional gel electrophore-
sis to analyze response to these stresses, which is much less sensi-
tive than microarray technology. It is interesting that much of the
transcriptional response of \( \text{Mtb} \) to \( \text{H}_2\text{O}_2 \) and NO was remarkably
similar at higher concentrations of and longer exposure times to
DETA/NO (Table A1), but their effects on growth and survival are
markedly different. \( \text{Mtb} \) either quickly adapts to \( \text{H}_2\text{O}_2 \) stress and
continues to grow, or it dies. NO slowed or stopped \( \text{Mtb} \) growth but
had little effect on survival even at high NO to bacilli ratios. RNS
are known to kill under acidic conditions (Darwin and Nathan,
2005; Rhee et al., 2005). Our data indicate that exposure to NO is
not lethal under standard laboratory conditions of aerobic growth
and medium composition. However, sub-optimal conditions, such
as those found in a phagosome, may well be more relevant to dis-
ease than growth in optimal laboratory medium. A recent study
also indicates NO is lethal under anaerobic conditions and our
unpublished data support this finding (Singh et al., 2008). It is well
established that NO has both short-term reversible and long-term
irreversible effects on respiratory components (Brown, 2001). NO
will reversibly inhibit cytochrome \( c \) oxidase by competing with oxy-
gen for binding, but after several hours of exposure NO irreversibly
inactivates cytochrome \( c \) oxidase and other respiratory proteins
including the NADH reductase. Immediate cessation of growth
following NO exposure is most likely a result of reversible inhibition
of cytochrome \( c \) oxidase and thus, of aerobic respiration (Voskuil
et al., 2003), while the longer term effects on growth result from
less reversible damage to the bacteria or medium. Another possible
mechanism could involve the DosR regulon, which is induced by
NO, but not \( \text{H}_2\text{O}_2 \) (Voskuil et al., 2003). DosR regulon proteins
may modulate the growth rate, directly or indirectly.

Similar to what was found in the present study, iron acquisition
genes were induced in \( \text{M. bovis} \) BCG cultures upon addition of
\( \text{H}_2\text{O}_2 \) (Jang et al., 2009). Induction by \( \text{H}_2\text{O}_2 \) and NO of several
genes and clusters of genes, point to an effect by ROS and RNS
on iron–sulfur metabolism and iron–sulfur cluster synthesis or
repair. Iron–sulfur cluster prosthetic groups \( \text{Fe}_4\text{S}_4 \), \( \text{Fe}_3\text{S}_4 \), and
\( \text{Fe}_5\text{S}_6 \) undergo oxidation–reduction reactions. Therefore, they
are often part of electron transfer proteins in addition to fulfill-
ing \( \text{Fe}_3\text{O}_4 \), \( \text{O}_2 \), and NO sensing roles (Beinert et al., 1997; Beinert
and Kiley, 1999). Due to their sensitivity to oxidation, iron–sulf-
ur cluster containing proteins are highly susceptible to oxidative
and nitrosative stress (Beinert and Kiley, 1999). Restoration of an
oxidatively damaged iron–sulfur center entails the synthesis of
separate units and their insertion into the corresponding protein.
\( \text{H}_2\text{O}_2 \) and NO strongly induce the SUF six gene operon \( (rvl460–
rvl466) \) that encodes the alternative mycobacterial iron–sulfur
cluster machinery (Huet et al., 2005). Pathways for the accumu-
lation of the iron and sulfur precursors of iron–sulfur clusters
are also upregulated. Two genes involved in cysteine biosynthesis
\( \text{cysK2} \) and \( \text{cysM} \) were also induced. Cysteine provides the sulfur for
iron–sulfur center construction. Two other genes highly induced
by \( \text{H}_2\text{O}_2 \) but not NO, are also involved in cysteine metabolism.
These are \( \text{cysD} \) and \( \text{cysN} \), which encode sulfate adenylytrans-
ferase subunits involved in the activation of sulfate for cysteine
biosynthesis (Pinto et al., 2004). Iron acquisition is induced by
the induction of the \( \text{mbt} \) gene cluster \( (rv2377c-b4 \) and \( rv2386c) \),
which encodes the biosynthesis of the iron-chelating siderophore
mycobactin. Repression of \( \text{bfrB} \), encoding the iron-storage protein
bacterioferritin, is also consistent with the need to acquire and use,
not store, available iron. IdeR negatively regulates the \( \text{mbt} \)
cluster and positively regulates \( \text{bfrB} \) when iron levels are high
(Gold et al., 2001; Rodriguez et al., 2002). The oxidation of the
iron-center of IdeR by \( \text{H}_2\text{O}_2 \) or NO appears to mimic low-iron
conditions by affecting a “chemical” knockout of this protein,
resulting in the expression of IdeR-repressed genes, and repression
of IdeR-induced genes.

Despite the need to acquire iron to repair iron–sulfur centers,
the powerful expression of iron scavenging genes during oxidative
stress was unexpected as free ferrous \([\text{Fe(II)}]\) iron reacts with \( \text{H}_2\text{O}_2 \)
to form extremely reactive and damaging hydroxyl radicals owing
to the Fenton reaction (Imlay, 2003). Indeed, derepression of iron
acquisition genes in \( \text{E. coli} \) results in iron overload and an increase
in oxidative stress (Imlay, 2003). Cysteine also promotes DNA
damage by favoring the conversion of intracellular ferric \([\text{Fe(III)}]\) iron
to ferrous iron thus making it available to react with \( \text{H}_2\text{O}_2 \) to pro-
duce hydroxyl radicals (Park and Imlay, 2003). Consequently, both
the uptake of iron and the synthesis of cysteine should promote
DNA damage via Fenton chemistry. However, the need for iron and
cysteine to repair damaged iron- and cysteine-containing proteins
appears to super-cede the negative effects of hydroxyl radical forma-
tion at intermediate levels of \( \text{H}_2\text{O}_2 \) and NO stress.

**CONCLUSION**

Exposure to \( \text{H}_2\text{O}_2 \) or NO initiated an overlapping robust transcrip-
tional response marked by the induction of a number of genes
including those involved in iron–sulfur cluster synthesis and other
cellular repair mechanisms. NO exposure was bacteriostatic, while
\( \text{H}_2\text{O}_2 \) exposure was not bacteriostatic at concentrations below
50 mM while at or above this concentration \( \text{H}_2\text{O}_2 \) was bactericidal.
It appears \( \text{Mtb} \) is resistant to DNA damage-mediated killing by \( \text{H}_2\text{O}_2 \),
as no effect on growth or viability was observed after exposure to
\( \text{H}_2\text{O}_2 \) levels that highly induced DNA repair mechanisms. We are
currently investigating the mechanisms utilized by \textit{Mtb} to resist DNA damage-mediated killing by \textit{H}_2\textit{O}_2. The high basal resistance of \textit{Mtb} to oxidative and nitrosative stress seems to be a combination of the intrinsic resistance of the \textit{Mtb} cell wall, the constitutive expression of genes that encode ROS and RNS scavenging functions, induction of genes that encode for repair of oxidized proteins, and induction of DNA repair mechanisms.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at http://www.frontiersin.org/search.php?journals=mic&article=10.3389/fmicb.2011.00105/abstract

Complete microarray expression data can be obtained from the TB database (TBDB: http://www.tbdb.org; Reddy et al., 2009) or the NCBI Gene Expression Omnibus series number GSE16146.

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## APPENDIX

Table A1 | Fold change of H$_2$O$_2$ and NO responsive genes (untreated vs. treated).

| Gene          | Oligonucleotide microarrays | Amplicon microarrays |
|---------------|-----------------------------|----------------------|
|               | mM H$_2$O$_2$               | mM DETA/NO           |
|               | 40 min                      | 40 min               |
|               | 0.05 | 0.5 | 5 | 10 | 50 | 200 | 5.0 (4 h) | 0.5 | 0.005 | 0.05 | 1.0 | 5.0 | 0.5 (4 h) | Gene product |
| Rv0324        | 6.3  | 4.8  | 4.2  | 1.6 | 1.6 | 1.5 | P transcriptional regulator |
| Rv0353 hspR   | 1.6  | 5.0  | 0.6  | 0.7  | 3.8 | 1.7 | P heat shock repressor |
| Rv0823c       | 1.6  | 7.9  | 3.7  |     |     |     | P transcriptional regulator |
| Rv1049        | 3.2  | 11   | 19   | 2.8  | 20  | 2.0  | P transcriptional regulator |
| Rv1129c       | 0.7  | 6.6  | 7.2  | 2.1  | 2.3 | 0.6  | P transcriptional regulator |
| Rv1460        | 1.6  | 2.7  | 9.6  | 12   | 7.6 | 8.4  | P transcriptional regulator |
| Rv1674c       | 1.6  | 5.1  | 19   | 38   | 3.1 | 21   | P transcriptional regulator |
| Rv1909c furA  | 2.7  | 4.8  | 16   | 17   | 8.1 | 18   | 4.1  | P transcriptional regulator |
| Rv2374c hrcA  | 2.9  | 4.2  | 1.9  |     | 0.4 |     | P heat-inducible repressor |
| Rv2621c       | 1.8  | 1.8  | 1.7  |     | 0.6 |     | P transcriptional regulator |
| Rv2710 sigB   | 5.2  | 8.3  | 5.3  |     |     | 12   | Sigma factor |
| Rv2745c       | 4.5  | 6.0  | 2.8  | 0.6  |     | 4.7  | P transcriptional regulator |
| Rv2989        | 39   | 31   | 2.5  |     | 3.3 | 0.1  | P transcriptional regulator |
| Rv3173c       | 3.2  | 3.9  | 4.5  |     |     | 2.7  | P transcriptional regulator |
| Rv3223c sigH  | 8.0  | 17   | 22   | 7.4  | 23  | 3.4  | Sigma factor |
| Rv3334        | 2.9  | 5.1  | 8.2  | 2.1  |     | 4.4  | P transcriptional regulator |
| Rv3583c       | 2.5  | 2.5  | 0.2  | 0.6  | 0.4 |     | P transcriptional regulator |
| Rv3840        | 10   | 13   | 5.7  | 1.8  | 11  |     | 2.6  | P transcriptional regulator |
| Rv3383c       | 1.8  | 5.3  |     | 3.5  |     | 2.4  | 5.1  | 2.7  | 4.9  | 5.9  | 10  | CHP |
| Rv3223c sigH  | 1.8  | 5.3  |     | 3.6  |     | 6.6  | 3.4  | 9.1  | 8.9  | 2.5 | 12 | P ABC-type transporter |

### RESPONSE REGULATORS

| Gene          | Oligonucleotide microarrays | Amplicon microarrays |
|---------------|-----------------------------|----------------------|
|               | mM H$_2$O$_2$               | mM DETA/NO           |
|               | 40 min                      | 40 min               |
|               | 0.05 | 0.5 | 5 | 10 | 50 | 200 | 5.0 (4 h) | 0.5 | 0.005 | 0.05 | 1.0 | 5.0 | 0.5 (4 h) | Gene product |
| Rv0324        | 6.3  | 4.8  | 4.2  | 1.6 | 1.6 | 1.5 | P transcriptional regulator |
| Rv0353 hspR   | 1.6  | 5.0  | 0.6  | 0.7  | 3.8 | 1.7 | P heat shock repressor |
| Rv0823c       | 1.6  | 7.9  | 3.7  |     |     |     | P transcriptional regulator |
| Rv1049        | 3.2  | 11   | 19   | 2.8  | 20  | 2.0  | P transcriptional regulator |
| Rv1129c       | 0.7  | 6.6  | 7.2  | 2.1  | 2.3 | 0.6  | P transcriptional regulator |
| Rv1460        | 1.6  | 2.7  | 9.6  | 12   | 7.6 | 8.4  | P transcriptional regulator |
| Rv1674c       | 1.6  | 5.1  | 19   | 38   | 3.1 | 21   | P transcriptional regulator |
| Rv1909c furA  | 2.7  | 4.8  | 16   | 17   | 8.1 | 18   | 4.1  | P transcriptional regulator |
| Rv2374c hrcA  | 2.9  | 4.2  | 1.9  |     | 0.4 |     | P heat-inducible repressor |
| Rv2621c       | 1.8  | 1.8  | 1.7  |     | 0.6 |     | P transcriptional regulator |
| Rv2710 sigB   | 5.2  | 8.3  | 5.3  |     |     | 12   | Sigma factor |
| Rv2745c       | 4.5  | 6.0  | 2.8  | 0.6  |     | 4.7  | P transcriptional regulator |
| Rv2989        | 39   | 31   | 2.5  |     | 3.3 | 0.1  | P transcriptional regulator |
| Rv3173c       | 3.2  | 3.9  | 4.5  |     |     | 2.7  | P transcriptional regulator |
| Rv3223c sigH  | 8.0  | 17   | 22   | 7.4  | 23  | 3.4  | Sigma factor |
| Rv3334        | 2.9  | 5.1  | 8.2  | 2.1  |     | 4.4  | P transcriptional regulator |
| Rv3583c       | 2.5  | 2.5  | 0.2  | 0.6  | 0.4 |     | P transcriptional regulator |
| Rv3840        | 10   | 13   | 5.7  | 1.8  | 11  |     | 2.6  | P transcriptional regulator |
| Rv3383c       | 1.8  | 5.3  |     | 3.5  |     | 2.4  | 5.1  | 2.7  | 4.9  | 5.9  | 10  | CHP |
| Rv3223c sigH  | 1.8  | 5.3  |     | 3.6  |     | 6.6  | 3.4  | 9.1  | 8.9  | 2.5 | 12 | P ABC-type transporter |

### IRON RESPONSIVE/IRON–SULFUR CLUSTER REPAIR

| Gene          | Oligonucleotide microarrays | Amplicon microarrays |
|---------------|-----------------------------|----------------------|
|               | mM H$_2$O$_2$               | mM DETA/NO           |
|               | 40 min                      | 40 min               |
|               | 0.05 | 0.5 | 5 | 10 | 50 | 200 | 5.0 (4 h) | 0.5 | 0.005 | 0.05 | 1.0 | 5.0 | 0.5 (4 h) | Gene product |
| Rv0338c       | 0.3  | 0.2  | 0.3  |     | 0.5 | 0.7 | P iron–sulfur reductase |
| Rv0816c thiX  | 1.9  | 4.1  | 16   | 2.0  | 21  | 3.0  | P thioredoxin |
| Rv0848 cysK2  | 3.7  | 12   | 6.6  | 28   | 11  | 18   | P cysteine synthase |
| Rv1285 cysD   | 1.7  | 34   | 52   | 9.1  | 26  | 20   | P cysteine synthase B |
| Rv1286 cysN   | 17   | 24   | 3.5  | 2.5  | 13  | 1.9  | P ABC-type transporter |
| Rv1336 cysM   | 2.0  | 4.0  | 3.2  |     | 21  | 1.9  | P cysteine synthase B |
| Rv1461        | 2.1  | 8.7  | 9.9  | 2.0  | 6.6 | 2.1  | CHPE  |
| Rv1462        | 1.8  | 5.9  | 7.3  | 2.4  |     | 5.1  | CHPE  |
| Rv1463        | 1.8  | 5.0  | 5.3  | 3.6  |     | 6.6  | CHPE  |

(Continued)
### Table A1 | Continued

| Rv No. | Gene | Ref vs ref | Oligonucleotide microarrays | Amplicon microarrays |
|--------|------|------------|-----------------------------|----------------------|
|        |      |            | mM H₂O₂                    | mM DETA/NO           |
|        |      |            | 40 min | 0.05 | 0.5 | 5 | 10 | 50 | 200 | 5.0 | (4 h) | 0.5 | 0.005 | 0.05 | 1.0 | 5.0 | 0.5 | Gene product |
| Rv1464 | csd  |            | 1.9 | 6.8 | 6.8 | 2.2 | 5.4 | 1.8 | 3.4 | 4.3 | 1.8 | 7.1 | P cysteine desulferase |
| Rv1465 |      |            | 2.0 | 6.4 | 5.8 | 2.0 | 2.8 | 5.1 | 2.1 | 4.1 | 3.7 | 8.0 | P nitrogen fixation protein |
| Rv1466 |      |            | 2.3 | 7.6 | 7.0 | 0.6 | 3.3 | 2.0 | 4.9 | 4.1 | 9.5 | CHP |
| Rv1471 | trxB |            | 1.7 | 11  | 23  | 58  | 19  | 16  | 5.2 | 2.3 | 3.5 | 9.3 | 1.8 | P thioredoxin |
| Rv2377c| mbtH | 2.8        | 8.0 | 3.1 | 1.6 | 1.8 | 16  | 8.1 | 6.6 | Lysine-N-oxygenase |
| Rv2378c| mbtG | 2.0        | 6.0 | 2.9 | 1.7 | 1.5 |     | 16  | 6.6 |          |
| Rv2379c| mbtF | 0.6        | 1.7 | 6.0 | 4.5 | 2.4 | 2.0 | 16  | 3.3 | 9.9 | Peptide synthetase |
| Rv2380c| mbtE | 0.7        | 1.6 | 6.1 | 8.7 | 6.7 | 18  | 2.3 | 15 | 4.1 | 12 | Peptide synthetase |
| Rv2381c| mbtD | 1.5        | 4.7 | 12 | 12 | 16 | 3.8 | 1.7 | 5.6 | 3.8 | 15 | Polyketide synthetase |
| Rv2382c| mbtC | 4.0        | 16 | 15 | 2.5 | 16 | 17 | 9.6 | 4.1 | 2.3 | 7.0 | Polyketide synthetase |
| Rv2383c| mbtB | 3.7        | 17 | 19 | 4.6 | 2.3 | 4.8 | 3.9 | 2.1 | 4.3 | P isochorismate synthase |
| Rv2384c| mbtA | 1.9        | 4.5 | 2.8 | 1.8 | 15 | 4.6 | 2.3 | 4.8 | 3.9 | 2.1 | 4.3 | P isochorismate synthase |
| Rv2386c| mbtl | 3.4        | 14 | 12 | 8.7 | 1.5 | 1.8 | 15 | 6.6 | Lysine-N-oxygenase |
| Rv3841c| bfrB | 0.3        | 0.2 | 0.3 | 0.2 | 0.2 | 0.4 | 0.3 | 0.3 | 0.3 | P bacterioferritin |

**ROS OR RNS DAMAGE RESPONSE**

| Rv No. | Gene | Ref vs ref | Oligonucleotide microarrays | Amplicon microarrays |
|--------|------|------------|-----------------------------|----------------------|
|        |      |            | mM H₂O₂                    | mM DETA/NO           |
|        |      |            | 40 min | 0.05 | 0.5 | 5 | 10 | 50 | 200 | 5.0 | (4 h) | 0.5 | 0.005 | 0.05 | 1.0 | 5.0 | 0.5 | Gene product |
| Rv0816c| thiX |            | 1.9 | 4.1 | 16 | 2.0 | 7.1 | 3.0 | 18 | 2.6 | 2.7 | 3.2 | 1.6 | P thioredoxin |
| Rv1471 | trxB |            | 1.7 | 11 | 23 | 58 | 19 | 16 | 5.2 | 2.3 | 3.5 | 9.3 | 1.8 | P thioredoxin |
| Rv1908c| katG | 2.1        | 4.1 | 11 | 12 | 16 | 3.9 | 7.4 | 3.2 | 2.9 | 2.5 | 2.5 | Catalase |
| Rv3913c| trxB2| 1.5        | 4.2 | 10 | 30 | 2.0 | 24 | 3.7 | 2.6 | 2.8 | 2.0 | 1.6 | P thioredoxin reductase |
| Rv3914c| trxC | 5.9        | 13 | 12 | 18 | 27 | 2.9 | 1.8 | 2.1 | 1.6 | 1.9 | Thioredoxin |

**DNA DAMAGE RESPONSE**

| Rv No. | Gene | Ref vs ref | Oligonucleotide microarrays | Amplicon microarrays |
|--------|------|------------|-----------------------------|----------------------|
|        |      |            | mM H₂O₂                    | mM DETA/NO           |
|        |      |            | 40 min | 0.05 | 0.5 | 10 | 50 | 200 | 5.0 | (4 h) | 0.5 | 0.005 | 0.05 | 1.0 | 5.0 | 0.5 | Gene product |
| Rv1317c| alkA |            | 6.3 | 8.4 | 1.5 | 1.6 | 6.5 | 2.9 | 1.5 | P DNA alkyltransferase |
| Rv2737c| recA |            | 4.4 | 4.1 | 0.5 | 0.4 | 0.7 | 1.5 | 1.5 | P DNA helicase |
| Rv3201c| mbtF |            | 6.4 | 4.7 | 6.9 | 2.5 | 2.6 | 1.5 | 1.7 | P DNA helicase |
| Rv3202c| mbtB |            | 8.3 | 6.7 | 1.5 | 1.6 | 3.4 | 3.3 | DNA repair protein |

**P values**

- **P**: probable
- **HP**: hypothetical protein
- **CHP**: conserved hypothetical protein

**Table Notes**

- Genes in bold are genes which are induced 4-fold or more.
- To be included in the table genes had an induction or repression ratio in at least one condition of at least fourfold and had a corresponding SAM q value of 0. Ratios below a 1.5-fold change were not included in the table.
- Ref vs. Ref indicates experiments in which RNA from untreated samples from independent experiments was compared.
- 0.5 oligo indicates a set of 0.5 mM DETA/NO experiments in which oligonucleotide microarrays were used instead of amplicon microarrays in order to determine if major differences resulted from using the amplicon platform versus the oligo platform.
- P, probable; HP, hypothetical protein; CHP, conserved hypothetical protein.