Inhibition of Respiration by Nitric Oxide Induces a *Mycobacterium tuberculosis* Dormancy Program

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Abstract

An estimated two billion persons are latently infected with *Mycobacterium tuberculosis*. The host factors that initiate and maintain this latent state and the mechanisms by which *M. tuberculosis* survives within latent lesions are compelling but unanswered questions. One such host factor may be nitric oxide (NO), a product of activated macrophages that exhibits antimycobacterial properties. Evidence for the possible significance of NO comes from murine models of tuberculosis showing progressive infection in animals unable to produce the inducible isoform of NO synthase and in animals treated with a NO synthase inhibitor. Here, we show that O_2 and low, nontoxic concentrations of NO competitively modulate the expression of a 48-gene regulon, which is expressed in vivo and prepares bacilli for survival during long periods of in vitro dormancy. NO was found to reversibly inhibit aerobic respiration and growth. A heme-containing enzyme, possibly the terminal oxidase in the respiratory pathway, likely senses and integrates NO and O_2 levels and signals the regulon. These data lead to a model postulating that, within granulomas, inhibition of respiration by NO production and O_2 limitation constrains *M. tuberculosis* replication rates in persons with latent tuberculosis.

Key words: *M. tuberculosis* • latency • nitric oxide • cytochrome oxidase • microarray

Introduction

Current clinical models of tuberculosis postulate a three-stage pathogenic sequence. Inhalation of an infectious aerosol begins the process leading to unimpeded replication of *Mycobacterium tuberculosis* in alveolar macrophages. The onset of cell-mediated immunity causes bacteriostasis and initiates a period of bacterial persistence in the granulomatous lesions of asymptomatic hosts. Finally, in ~10% of infected hosts, declining immune competence permits resumption of bacterial replication leading to overt disease. The second of these stages, clinical latency, is of surpassing importance for the epidemiology and control of tuberculosis. An estimated one-third of the world's population is latently infected (1) and most active cases of tuberculosis arise from this vast reservoir. The eradication of bacilli in latent lesions by antimicrobials is notoriously inefficient.

Little is known about the nature of the persistent state in vivo or the host factors that induce and maintain it. Control of bacterial replication in murine models of latency requires interferon-γ, tumor necrosis factor-α, and nitric oxide (NO; reference 2). Long-term survival in the murine lung requires that *M. tuberculosis* express isocitrate lyase (3), and in vitro studies show that a persistent or dormant state can be generated by nutrient deprivation (4) and O_2 depletion (5). Of these, O_2 depletion has been the most comprehensively studied. Furthermore, it provides a mechanistic link between the pathology of latent lesions in humans (which are often in avascular or poorly aerated foci of the lung)

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Abbreviations used in this paper: CcO, cytochrome *c* oxidase; DETA/NO, diethylenetriamine/nitric oxide adduct; DosR, dormancy survival regulator; GSNO, nitrosoglutathione; NO, nitric oxide; qRT, quantitative real-time reverse transcriptase.

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and the capacity of *M. tuberculosis* to adapt to hypoxic conditions. Seminal studies by L.G. Wayne et al. (5) with an in vitro O_2 depletion model of latency demonstrate the following. A gradual depletion of O_2 leads to a nonreplicating persistent state characterized by bacteriostasis and metabolic, chromosomal, and structural changes of the dormant bacteria. Further reductions in O_2 tension lead to a more quiescent state, characterized in part by the onset of sensitivity to metronidazole and resistance to other antimicrobials. Provision of O_2, even after long periods of hypoxia-induced bacteriostasis, results in resuscitation of the dormant bacteria.

In this work, we have focused on NO as an immune factor that could restrain mycobacterial replication rates in vivo and play a role, in conjunction with low tissue concentrations of O_2, in the initiation and maintenance of the latent state. NO from activated murine macrophages or exogenous sources exhibits antimycobacterial properties, and can irreversibly damage bacteria (6). However, NO also acts as a potent reversible inhibitor of aerobic respiration in mitochondria and in bacteria (7) and is a versatile signaling agent in eukaryotic systems (8). In view of these properties, we designed experiments to test the hypothesis that NO controls *M. tuberculosis* growth by inhibiting aerobic respiration. During the course of these studies, we corroborated this hypothesis and, in addition, showed that NO induces an *M. tuberculosis* genetic program that is expressed in vivo and adapts the organism for survival during extended periods of in vitro dormancy.

Materials and Methods

Culture Conditions. Clinical isolate 1254, 7H9 medium (supplemented with BSA, NaCl, glucose, and glycerol), 250-ml vented tissue culture flasks, 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 *M. tuberculosis* 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.

RNA Isolation. 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. Cell debris was separated by a 45-s centrifugation. The supernatant was transferred to 2-ml Heavy Phase Lock Gel I tubes (Eppendorf) containing 300 μl chloroform, inverted rapidly for 15 s, and incubated 2 min. Samples were centrifuged for 5 min, and the aqueous phase was added to 270 μl isopropanol, followed by addition of 270 μl of the following mixture: 0.8 M sodium citrate and 1.2 M NaCl. Samples were incubated for 10 min 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. After suspension of the RNA pellets in 90 μl water, 10 μl DNase I 10× buffer, and 6 U DNase I (Ambion) were added, and the samples were incubated for 30 min. Final purification of RNA was by RNeasy column (QIAGEN).

For the murine infection experiments, groups of 15 6–8-wk-old C57BL/6 mice were infected by the low dose aerosol method (87 cfu) with the wild-type H37Rv strain of *M. tuberculosis*. 21 d later, the mice were killed, and lung tissue was obtained for quantitative culture and RNA. Viable plate counts were conducted with serial dilutions of the left lung homogenized in PBS/0.5% NP-40, plated onto 7H10 agar, and incubated at 37°C. Colonies were counted 2–3 wk after inoculation, giving a value of 2.8×10^6 cfu of *M. tuberculosis* per lung. Quantitative cultures at later time points (6, 12, and 21 wk) showed a persistent steady-state infection with viable plate counts averaging 5.8×10^5 cfu/lung. For the preparation of RNA, mouse lungs were ground with a tissue homogenizer in a mixture of phenol, chloroform, and guanidinium thiocyanate, and RNA was isolated as described in the previous paragraph.

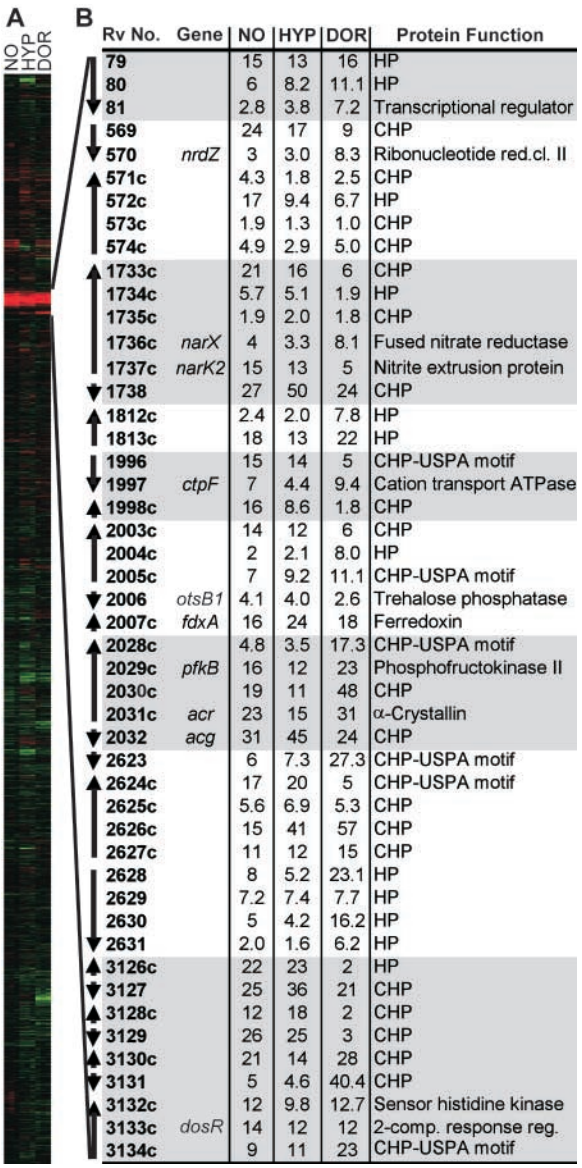
cDNA Labeling and Microarray Hybridization. Both a PCR gene product microarray and a 70-mer oligonucleotide-based microarray (tuberculosis oligonucleotide set; QIAGEN) were used. Labeled cDNA was prepared as follows: 2 μg total RNA and 4.4 μg of random oligonucleotide hexamers were incubated for 2 min at 98°C, cooled on ice, combined with Stratascript RTase buffer, 0.5 mM dA,G,CTP, 0.02 mM dTTP, 1.5 nmol Cy3 or Cy5-dUTP (Amersham Biosciences), and 1.8 μl Stratascript RTase (Stratagene) in a total volume of 25 μl, and incubated for 10 min at 25°C and 90 min at 42°C. cDNA was purified by microcon-10 (Amicon) filtration. 10 μl of 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 5× SSC, 1% BSA, and 0.1% SDS and washed with H_2O and isopropanol. After the prehybridization, 10 μl of hybridization solution (labeled cDNA, 5 μg tRNA, 2× SSC, 25% formamide, and 0.1% SDS) was hybridized overnight at 54°C.

Data Analysis. Microarrays were scanned using a GenePix 4000A (Axon Instruments, Inc.). The intensities of the two dyes at each spot were quantified using ScanAlyze (M. Eisen, Lawrence Berkeley National Lab, Berkeley, CA). 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 for the 20% lowest intensity spots, and every value below this average noise value was raised to the noise value. Microarray-determined ratios were calculated from three biological replicates and two microarrays for each biological replicate, except for Fig. 2 (A and B), where data was from two biological replicates and four microarrays (8a).

Online Supplemental Material. Table S1 includes induction ratios for dormancy regulon genes after exposure of wild-type and mutant *M. tuberculosis* strains to NO, hypoxia, and 4 d into the low O2–induced dormancy model of latency. Fig. S1 illustrates that growth of the Rv3134c dormancy regulon mutant and its parent were equally impaired in growth after NO exposure. The online supplemental material is available at at http://www.jem.org/cgi/content/full/jem.20030205/DC1.

Results

Low (Nontoxic) Concentrations of NO Signal M. tuberculosis to Induce a Set of 48 Genes. Microarray expression profiling was used to capture the transcriptional response of *M. tuberculosis* to low and high concentrations of NO. A



Rv No.	Gene	NO	HYP	DOR	Protein Function
79		15	13	16	HP
80		6	8.2	11.1	HP
81		2.8	3.8	7.2	Transcriptional regulator
569		24	17	9	CHP
570	*nrdZ*	3	3.0	8.3	Ribonucleotide red.cl. II
571c		4.3	1.8	2.5	CHP
572c		17	9.4	6.7	HP
573c		1.9	1.3	1.0	CHP
574c		4.9	2.9	5.0	CHP
1733c		21	16	6	CHP
1734c		5.7	5.1	1.9	HP
1735c		1.9	2.0	1.8	CHP
1736c	*narX*	4	3.3	8.1	Fused nitrate reductase
1737c	*narK2*	15	13	5	Nitrite extrusion protein
1738		27	50	24	CHP
1812c		2.4	2.0	7.8	HP
1813c		18	13	22	HP
1996		15	14	5	CHP-USPA motif
1997	*ctpF*	7	4.4	9.4	Cation transport ATPase
1998c		16	8.6	1.8	CHP
2003c		14	12	6	CHP
2004c		2	2.1	8.0	HP
2005c		7	9.2	11.1	CHP-USPA motif
2006	*otsB1*	4.1	4.0	2.6	Trehalose phosphatase
2007c	*fdxA*	16	24	18	Ferredoxin
2028c		4.8	3.5	17.3	CHP-USPA motif
2029c	*pfkB*	16	12	23	Phosphofructokinase II
2030c		19	11	48	CHP
2031c	*acr*	23	15	31	α-Crystallin
2032	*acg*	31	45	24	CHP
2623		6	7.3	27.3	CHP-USPA motif
2624c		17	20	5	CHP-USPA motif
2625c		5.6	6.9	5.3	CHP
2626c		15	41	57	CHP
2627c		11	12	15	CHP
2628		8	5.2	23.1	HP
2629		7.2	7.4	7.7	HP
2630		5	4.2	16.2	HP
2631		2.0	1.6	6.2	HP
3126c		22	23	2	HP
3127		25	36	21	CHP
3128c		12	18	2	CHP
3129		26	25	3	CHP
3130c		21	14	28	CHP
3131		5	4.6	40.4	CHP
3132c		12	9.8	12.7	Sensor histidine kinase
3133c	*dosR*	14	12	12	2-comp. response reg.
3134c		9	11	23	CHP-USPA motif

Figure 1. Dormancy regulon. Comparison of microarray expression results from *M. tuberculosis* strain 1254 exposed for 40 min to 50 μM of the NO donor DETA/NO (NO), and strain H37Rv exposed for 2 h to 0.2% O_2 (hypoxia-HYP) and at day 4 during the gradual adaptation, to low O_2 as described previously (11), resulting in an in vitro dormant state (DOR). DNA microarrays were used for the NO and low O_2 experiments, whereas oligonucleotide microarrays were used for the DOR experiments. (A) All *M. tuberculosis* genes are represented and organized based on their regulatory profile by average linkage clustering, using the Cluster and Treeview programs. Red indicates induced, green indicates repressed, and black indicates no change in gene expression. (B) The initial selection of genes in the dormancy regulon was based on their coinduction by NO, low O_2, and adaptation to an in vitro dormant state, as well as their failure to be induced in the presence of KCN (see Fig. 3 A). Rv numbers designate genes in genomic order, and arrows indicate transcript direction. Annotations are from TubercuList (http://genolist.pasteur.fr/TubercuList; reference 34). CHP and HP indicate conserved hypothetical and hypothetical proteins.

50-μM pulse of diethylenetriamine/nitric oxide adduct (DETA/NO) generated nanomolar concentrations of NO and rapidly induced a set of 48 genes (Fig. 1; and Fig. 2, A and B). The expression kinetics of all the 48 genes appears to be the same. Time course studies showed that induction of the genes was rapid with accumulated mRNA detectable by microarray within 5 min of NO exposure. As NO dissipated from the reaction vessel, expression levels declined to basal levels (Fig. 2 B; and see Fig. 4 B). An additional low dose pulse of DETA/NO elicited renewed induction, indicating that exposure to low concentrations of NO does not diminish the response to a subsequent dose (Fig. 2 B). This NO-induced gene set was also up-regulated 13.2 ± 3.6-fold by 0.5 mM of the nitrosothiol compound, nitrosoglutathione (GSNO). However, when coincubated with 1 mM carboxy-PTIO, a NO-specific trap, induction of the NO-responsive genes by GSNO was prevented, showing that gene induction was not due to S-nitrosation via GSNO, but rather to NO release from GSNO. None of the genes induced by low concentrations of NO (Fig. 1) was found to be regulated by several other in vitro conditions believed to simulate the intraphagosomal environment of the macrophage, including oxidative stress (0.05–200 mM H_2O_2), low iron (9), and nutrient deprivation (4) or exposure to the detergent sodium dodecyl sulfate (10). These data, and the absence of desensitization to repeated doses of NO, show that the induction of these genes by low concentrations of NO is not part of a general stress response. By contrast, exposure of *M. tuberculosis* to high concentrations of NO, generated by ≥1 mM pulse of DETA/NO, induces ~400 genes (Fig. 2 A). Thus, higher concentrations of NO cause a pleiotropic reaction, including an oxidative stress response, which is fundamentally different than the circumscribed induction of 48 genes by low concentrations of NO. Genes induced by high concentrations of NO that are responsible for oxidative stress defense include *katG* (catalase), *ahpC* (alkyl hydroperoxide reductase), *trxC* (thioredoxin), *trxB2* (thioredoxin reductase), and *sigB* and *sigH*, which code for two alternative sigma factors. Microarray expression–derived induction values provide a relative measure of transcript abundance. Therefore, quantitative real-time reverse transcriptase PCR (qRT-PCR) was used to measure the induction magnitude of five sentinel NO-induced genes after exposure to low concentrations of NO. Induction ratios ranged from 2,000 to 15,000 (Fig. 2 C) and mRNA levels reached up to 140 times the highly transcribed *sigA* control transcript.

Dormancy Gene Regulation by NO and Hypoxia. NO has been demonstrated to inhibit mitochondrial and bacterial respiration (7). Therefore, experiments were conducted to determine if low concentrations of NO initiate an adaptive response that is similar to the response induced by the gradual reduction of O_2 in the in vitro dormancy model of latency. Expression profiles were obtained of *M. tuberculosis* at day 4 during adaptation to a low O_2 state, produced by bacterial consumption of the limited O_2 within a sealed, stirred culture tube (11). A common set of 48 genes was induced during entry into the low O_2 dormant state and by low concentrations of NO. Moreover, most of the genes that comprise this NO/dormancy-regulated gene set were found to coincide with those reported

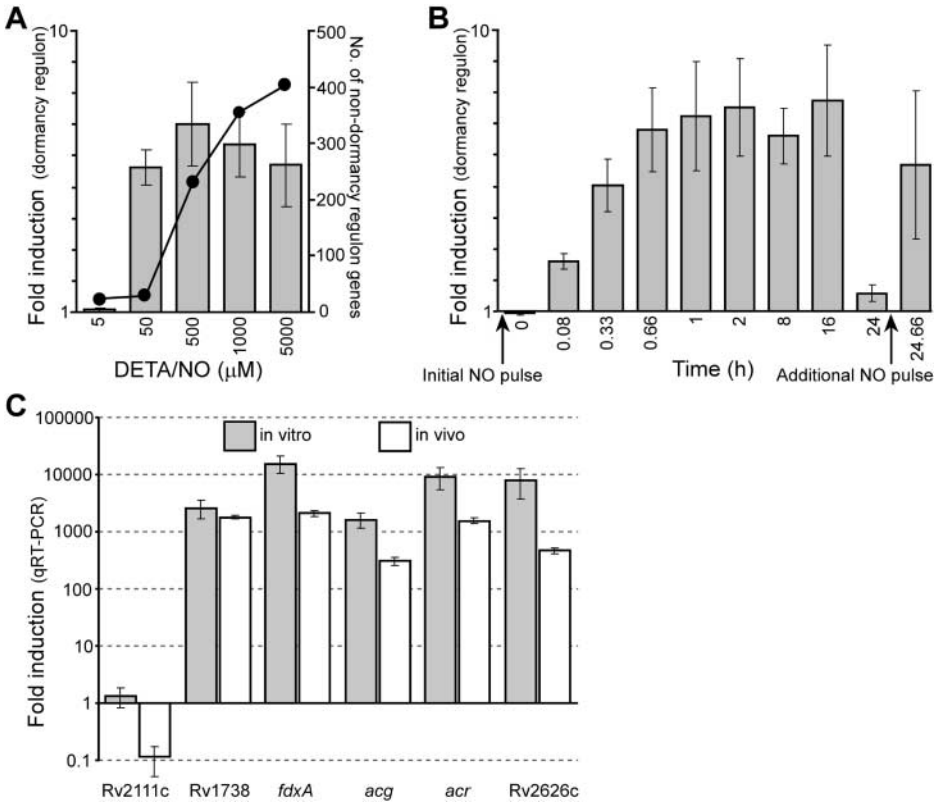


Figure 2. Dormancy regulon gene expression as measured by DNA microarray and qRT-PCR. (A) Average fold induction of all 48 genes as measured by DNA microarray after a 40 min DETA/NO exposure (left y axis); number of nondormancy regulon genes regulated at least twofold (curve; right y axis). (B) Temporal pattern of regulon induction after addition of 500 μM DETA/NO. (C) Quantitative in vitro and in vivo (infected mouse lung) induction ratios of the five regulon genes were obtained by qRT-PCR (35). Rv2111c was used as an NO-independent control gene. Gene induction ratios were calculated by first determining the copy number of each transcript normalized to the copy number of the major housekeeping sigma factor gene of *M. tuberculosis*, *sigA*. *sigA* mRNA remained constant during exposure to 50 μM DETA/NO (ratio 1:1 as measured by DNA microarray). The normalized copy numbers were compared with mRNA from midlogarithmic phase in vitro grown bacteria. Induction ratios are indicated by gray bars for in vitro exposure to 50 μM DETA/NO for 40 min, and white bars indicate in vivo infection, 21 d after challenge.

previously to be induced in hypoxic cultures provided with a continuous supply of 0.2% O_2 in the head space gas (12). Thus, low concentrations of NO, entry into the low O_2–induced in vitro dormant state or cultivation in hypoxic cultures induce a common set of 48 genes. The 48 genes induced by NO, dormancy, and hypoxia are not distributed randomly across the chromosome, but are clustered in nine discrete modules (Fig. 1). Seven of these modules contain contiguous genes, whose orientations indicate the presence of multiple transcription units, an arrangement suggestive of a supra-operonic organization that might facilitate a rapid, pronounced, and coordinated transcriptional response.

In Vivo Gene Expression of the NO/Dormancy/Hypoxia-induced Response. To determine if this gene set is also expressed within infected tissue, C57BL/6 mice were killed 21 d after aerosol infection with *M. tuberculosis*, and total RNA from lung tissue was assayed by qRT-PCR. During infection, *M. tuberculosis* highly expressed each of five sentinel genes from the group of 48 genes (Fig. 2 C). In a separate paper focused on the transcriptional response of *M. tuberculosis* in the macrophage phagosome, Schnappinger et al. confirmed that the five dormancy genes depicted in Fig. 2 C were highly expressed in mouse lung 21 d after infection and showed, in addition, that they remained expressed at high levels 56 d after infection (13). These in vivo results and the selective induction of these genes by NO and hypoxia provide compelling evidence that *M. tuberculosis* encounters one or both of these conditions during the course of infection in the murine lung. This idea is further supported by the studies of Schnappinger et al. with primary bone marrow–derived murine macrophages showing that most genes in the NO/dormancy/hypoxia gene set are strongly induced by *M. tuberculosis* within INFγ-activated macrophages from wild-type mice, but not by *M. tuberculosis* in activated macrophages from inducible nitric oxide synthase knockout ($NOS2^{-/-}$) mice (13). Thus, in the intraphagosomal environment of the IFNγ-activated macrophage, the induction of these genes is NO dependent. This interpretation of these data is also supported by a recent work by Shi et al. showing that expression of three dormancy genes (*acr*, Rv2623, and Rv2626c) within *M. tuberculosis*–infected murine lungs is delayed in $IFN\gamma^{-/-}$ mice compared with wild-type mice (14). Because NOS2 was not detected in the $IFN\gamma^{-/-}$ mice, in contrast to wild-type mice, the delayed induction of the three genes in the $IFN\gamma^{-/-}$ mice might be due to NO generated by endothelial NO synthase (15) and/or to localized areas of hypoxia resulting from inflammation. This paper also demonstrates that these genes are not expressed early in infection (days 10–12) and that expression commences by days 15–18 after infection, coincident with activation of the immune system and slowing of bacterial growth.

Two observations suggest that these conditions might also prevail in *M. tuberculosis*–infected human lungs. First, analysis of human tuberculosis granulomas demonstrates elevated levels of inducible and endothelial NO synthases as well as nitrotyrosine, an indicator of the presence of NO (15). Second, 85% of tuberculosis patients have antibodies to the protein encoded by *acr*, a NO/dormancy/hypoxia-induced gene (Fig. 1 B; reference 16).

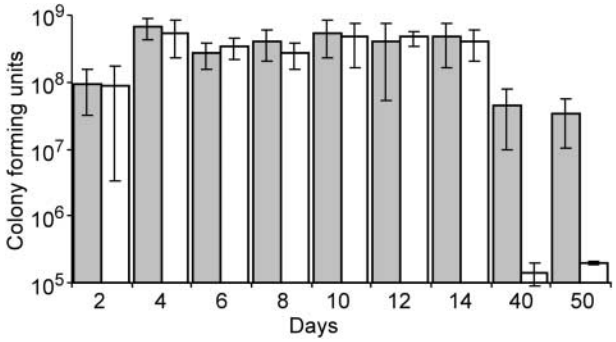


Figure 3. Survival of a dormancy regulon mutant during a low O_2–induced dormant state. The wild-type H37Rv parent (gray bars) and the Rv3134c mutant (white bars) were grown in the low O_2–induced dormancy model as described by Wayne and Hayes (11). Bacilli cultures were stirred in sealed test tubes to generate a slow depletion of O_2 via culture respiration. Test tubes were opened to remove samples for colony forming unit determination at each time point and discarded.

The 48-gene NO/Dormancy/Hypoxia Response Defines the Dormancy Regulon. The two-component response regulator, dormancy survival regulator (DosR) encoded by Rv3133c, but not a sensor histidine kinase encoded by the adjacent gene Rv3132c, is required for induction of *acr* by hypoxia (12, 17). Microarray expression profiling was used to determine if DosR also regulates the NO/dormancy/hypoxia-induced gene set. Inactivation of Rv3134c, the first gene of a presumed three-gene operon (composed of Rv3134c, *dosR*, and *R*/Rv3132c; reference 12), fully or partially reduced induction of each of the 48 genes in response to NO and during adaptation to the low O_2–induced dormant state. Complementation of the mutant with Rv3134c/*dosR* largely restored this effect (Table S1, available at http://www.jem.org/cgi/content/full/jem.20030205/DC1). We have demonstrated recently that hypoxia fails to induce any of the 48 genes when *dosR* is selectively disrupted (18). Thus, the capacity of NO or low O_2 to partially induce some of the 48 genes in the Rv3134c mutant was likely due to a low level of *dosR* expression by this strain. Together, these results show that NO, hypoxia, and adaptation to an in vitro dormant state induce a common set of 48 genes and do so via DosR. In view of these results and evidence that a DosR binding site is present in the up-stream promoter regions of several NO/dormancy/hypoxia-induced genes (18), we designate the gene set listed in Fig. 1 B the "dormancy regulon".

To determine if the dormancy regulon contributes to the survival of *M. tuberculosis* during the in vitro dormant state, we compared the viability of the wild-type parent with the Rv3134c mutant during the adaptive phase and after 40 and 50 d in the low O_2–induced dormant state (11). The dormancy regulon mutant grew to the same level as the wild-type strain during the initial adaptive phase after O_2 limitation (Fig. 3). After 40–50 d of oxygen limitation, 200-fold more wild-type bacteria were recovered compared with the dormancy regulon mutant (Fig. 3). The experiment demonstrating the survival phenotype of the Rv3134c mutant (Fig. 3) did not include the use of the complemented strain. Thus, in principle, this phenotype could have resulted from an unintended effect of this mutation. However, disruption of *dosR* in the nonpathogenic bacillus Calmette-Guerin vaccine strain of *M. bovis* was recently shown to yield a mutant with a 1,500-fold reduced survival phenotype during the low O_2–induced dormant state. Complementation of this mutant with a wild-type copy of *dosR* restored the survival phenotype of the parent strain (17). Together, these data show that the dormancy regulon increases the fitness of *M. tuberculosis* in an in vitro model of clinical latency.

Inhibition of Respiration and Growth by NO. Aerobic respiration declines and stops as O_2 levels fall during adap-

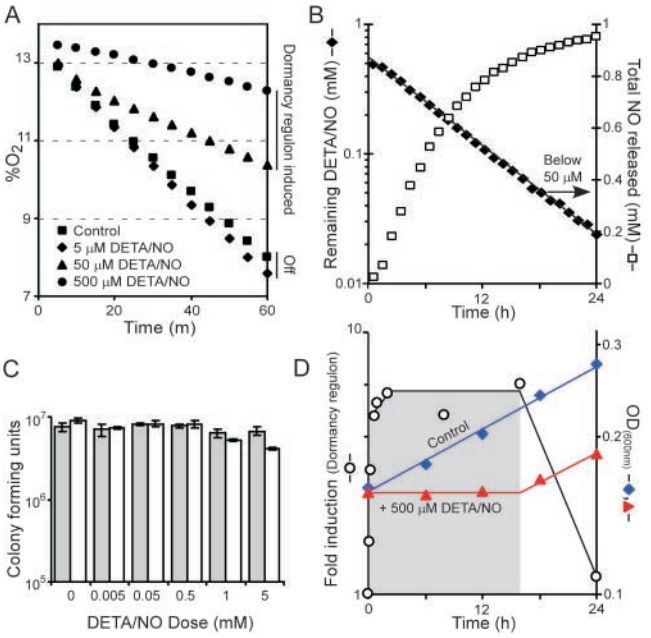


Figure 4. NO-mediated respiratory inhibition and reversible bacteriostasis. (A) The effect of 5, 50, and 500 μM DETA/NO on *M. tuberculosis* respiration and dormancy regulon induction. DETA/NO was added to a well-aerated 250 ml H37Rv stirring culture and the flask was sealed. A probe measured the depletion of O_2 by bacterial respiration. (B) Rate of DETA/NO decomposition and NO release. DETA/NO release of NO was monitored by absorbance at 252 nm in a 0.1 M phosphate buffer at pH 6.6 and 37°C. A standard curve was generated with known concentrations of DETA/NO to determine the molar absorptivity and used to calculate the DETA/NO concentration (closed diamonds) over time using Beer's law. One mole of DETA/NO decomposes to release 2 mol NO; therefore, the total NO released (open squares) was calculated by doubling the number of moles of DETA/NO decayed. (C) Survival of *M. tuberculosis* 1254 monitored by colony-forming units after exposure to various doses of DETA/NO for 4 (gray bars) and 24 h (white bars). (D) Growth inhibition by NO overlaid with the induction of the dormancy regulon. Average induction of dormancy regulon over time after addition of 500 μM DETA/NO (left y axis); optical density of control culture and culture exposed to 500 μM DETA/NO (right y axis). Refer to Fig. 4 B for the kinetics of NO production and DETA/NO consumption over the same time course after the addition of 500 μM DETA/NO. NO caused a state of bacteriostasis over ~16 h. Resumption of growth coincided with the disappearance of NO as indicated by reduction of regulon expression to basal levels (gray area) and decomposition of the NO donor (B).

tation of *M. tuberculosis* to a low O_2–induced dormant state. The drop in aerobic respiration is associated with reduced replication rates of *M. tuberculosis*, an obligate aerobe (5). To determine if NO also inhibits respiration and slows bacterial growth, we measured O_2 consumption and bacterial growth rate during NO exposure. NO was found to inhibit respiration in a dose-dependent manner and to cause growth arrest (Fig. 4, A and D). Fig. 4 illustrates that three effects (dormancy regulon induction, inhibition of respiration, and growth rate arrest), occurred contemporaneously and at the same concentrations of NO. Growth of the culture exposed to 500 μM DETA/NO resumed and dormancy regulon induction abated ~18 h after addition of the DETA/NO (Fig. 4 D). Fig. 4 B illustrates that 16–17 h after addition of 0.5 mM DETA/NO, its concentration decomposed below the 50-μM threshold concentration required for dormancy regulon induction and for inhibition of respiration and growth. Thus, it is possible that the dormancy regulon may encode a mechanism to actively modulate growth. However, no evidence currently exists to support this role. Indeed, the Rv3134c mutant and its parent strain are equally impaired in growth immediately after being exposed to NO. This indicates that the initial growth arrest by NO does not require full induction of the dormancy genes, but instead is most likely due to respiratory inhibition after NO exposure (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20030205/DC1).

To determine if concentrations of NO that cause growth arrest, dormancy regulon induction, and respiratory inhibition are bacteriostatic or bacteriocidal, viable plate counts were obtained after exposure of *M. tuberculosis* to low, intermediate, and high concentrations of NO. Fig. 4 C demonstrates that the viability of *M. tuberculosis* was largely unaffected by NO exposure, explaining why the effects of low to moderate concentrations of NO on growth were reversible. Only after *M. tuberculosis* was exposed to high concentrations of NO (1 and 5 mM DETA/NO) was a slight effect on the number of colony forming units observed. Together, these results show that exposure of *M. tuberculosis* to NO, like entry into the low O_2–induced dormant state, is associated with reduced respiration and replication rates and that both these effects are reversed by the removal of NO or provision of O_2, respectively.

Characterization of the NO and O_2 Sensor. Because both NO and low O_2 induce dormancy regulon expression, we investigated whether the presence of NO and the absence of O_2 are sensed by the same molecular sensor. First, we determined if O_2 could competitively inhibit NO-mediated induction of the dormancy regulon. Gene induction was assayed by microarray expression profiling after exposing *M. tuberculosis*, growing in high or low aerated cultures, to different concentrations of NO. In this experiment, aeration was controlled by culture stirring speed; therefore, in addition to the degree of aeration, it is possible that other factors affected by stirring could have influenced the results. At low aeration, only 1–5 μM DETA/NO was necessary to initiate dormancy regulon induction. By contrast, at least 5 times more NO was required to induce the dormancy regulon under conditions of high aeration (Fig. 5 B). This finding (the capacity of O_2 to inhibit NO-mediated regulon induction) is consistent with the idea that the same molecular sensor monitors the levels of O_2 and NO. One such sensor could be a heme-containing protein that binds both NO and O_2. To test this possibility, microarray expression profiling was used to determine if low levels of cyanide (CN^-), a heme-protein inhibitor, would prevent induction of the dormancy regulon by NO and hypoxia. Cyanide was found to block the expression of dormancy regulon genes by NO and low O_2, without significantly affecting the expression of genes outside the dormancy regulon (Fig. 5 A). CN^- did not prevent overall transcription or gene induction even when 10-fold more CN^- than necessary to block NO or low O_2 signaling of the dormancy regulon was used, as illustrated by the large number of genes induced after 2-h hypoxia in the presence of 0.5 mM KCN^- (Fig. 5 A). The interaction of NO and O_2 in dormancy regulon signaling and inhibition of signaling by cyanide strongly implicate a heme-containing protein as a component of the NO/low O_2 signal transduction system.

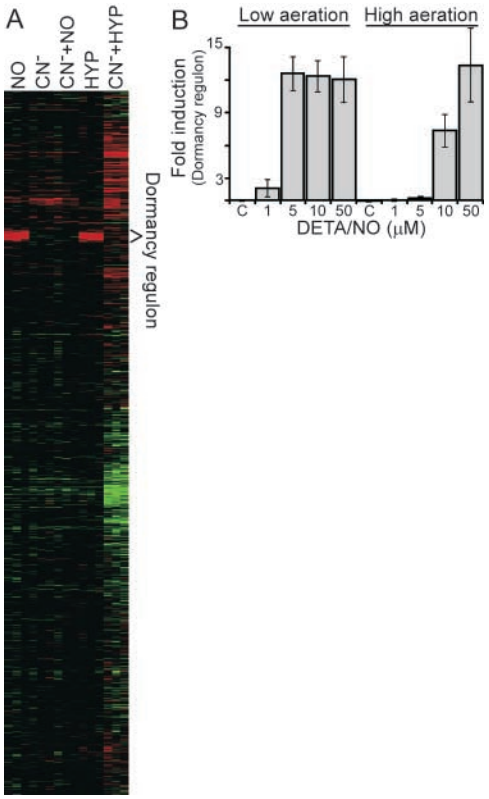


Figure 5. Competition between O_2 and NO for heme-containing protein. (A) Average linkage clustering comparison of expression data from all *M. tuberculosis* genes after a 40-min exposure to 50 μM DETA/NO, 50 μM KCN, KCN plus DETA/NO, 2-h hypoxia, and 500 μM KCN plus 2-h hypoxia. (B) Competition between NO and O_2 for control of dormancy regulon expression. DETA/NO was added to a 300-ml culture stirring at 100 rpm for low aeration and maximum rpm for high aeration. Induction of the regulon was assayed by DNA microarray after 40 min NO exposure.

Discussion

The results presented here show that low, nontoxic concentrations of NO induce a 48-gene regulon via the response regulator DosR, inhibit aerobic respiration, and reversibly slow replication. The correspondence of these effects by NO and the previously reported effects of hypoxia indicate that these apparently quite different treatments share a common mechanism of action. Differences in the survival of the wild-type and DosR mutant in a low-O_2 model of clinical latency were also demonstrated, indicating that this regulon adapts the organism for survival during prolonged periods of in vitro dormancy.

The individual contributions of the 48 genes that comprise this regulon to the enhanced survival of the wild-type strain during low O_2–induced dormancy are unknown. However, the predicted roles of some of the dormancy regulon genes appear to explain many of the previously reported biochemical or physiological properties of the low O_2–induced dormant state (5). Thus, a shift from aerobic to anaerobic respiration during adaptation to the dormant state, where nitrate rather than O_2 would serve as the terminal electron acceptor (19), is signaled by the induction of *narX*, which encodes nitrate reductase, *nark2*, which specifies a nitrite/nitrate transport function, and *fdxA*, which encodes feredoxin A, a protein involved with alternative electron transport. Induction of *nrdZ*, which encodes ribonucleotide reductase class II, an enzyme that converts nucloeside triphosphates to deoxynucleoside triphosphates to dNTPs under anaerobic conditions, could enable the chromosomal duplication that reportedly occurs during adaptation to low O_2–induced dormancy (20). Six of the dormancy regulon genes contain an *Escherichia coli* UspA motif. UspA is believed to be a nucleoid-associated DNA-binding protein that confers DNA resistance to UV radiation and mitomycin C (21). Recent findings support this correlation as mycobacteria in a low O_2–induced dormant state were found to be highly resistant to mitomycin C (22). Thus, protection of a diploid chromosome by these UspA-like proteins may enhance preservation of genome integrity. The induction of *otsB1*, which encodes trehalose phosphatase, indicates that trehalose may accumulate during adaptation to dormancy. Trehalose stabilizes living cells during anhydrobiosis (23). Previous analyses of *M. tuberculosis* show that the small chaperone protein α-crystallin (Acr) accumulates under hypoxic conditions of growth (24) and after NO exposure (25). Functional studies of the α-crystallin protein family (26) and the induction of *acr* during adaptation to the dormant state suggest that Acr might protect partially unfolded proteins during long periods of bacteriostasis. During the course of this work, we demonstrated high levels of transcript corresponding to each of five tested dormancy regulon genes in the lungs of *M. tuberculosis*–infected mice, thus, demonstrating that the entire regulon is likely to be induced in vivo in a murine model of tuberculosis. Though dormancy regulon genes are required for optimal survival of *M. tuberculosis* in an in vitro model of latency (reference 17 and this paper), we do not know if the postulated functions herein contribute to the persistence of the organism during in vivo latency in man.

Unlike the effect of hypoxia on *M. tuberculosis* in the oxygen depletion latency model, NO has typically been portrayed as a microbicidal molecule that, at the high concentrations generated by the inducible NO synthase of activated macrophages, can damage cellular components, resulting in cell death. Indeed, this is evident in the microarray expression profiles of *M. tuberculosis* treated with concentrations of NO that are higher than those needed to induce the dormancy regulon, where DETA/NO concentrations ≥1 mM induce the expression of stress response genes indicative of the organism's response to cell injury. However, NO is also a potent intercellular signaling molecule, regulating a variety of essential functions in eukaryotes (8). The effects of low, nontoxic concentrations of NO on *M. tuberculosis* respiration, growth, and dormancy regulon induction reported here may be one example of this in bacteria, where NO production by the activated immune system would signal *M. tuberculosis* to adopt a quiescent physiological state that, for most infected hosts, is lifelong and clinically innocuous. The postulated effect of NO on pathogen physiology and adaptation may also be true for *Toxoplasma gondii*, an intracellular protozoan parasite. *T. gondii* exists in a latent bradyzoite stage and a reactivated tachyzoite stage. NO has been shown to initiate a physiological stage conversion, whereby it inhibits parasite replication and promotes the conversion from the tachyzoite to the bradyzoite stage (27).

These hypothesized host-to-pathogen NO signaling functions differ significantly from most previously reported effects of NO on bacterial physiology and gene regulation. One such difference is the reported capacity of NO to modulate gene regulation in a manner that reproduces the effect of reactive O_2 species or molecular O_2. For example, in *E. coli*, NO is known to affect gene expression by modifying the oxidative stress-responsive transcriptional regulators, OxyR (28) and SoxR (29). Similarly, the anaerobic regulator, FNR, is inactivated by both O_2 and NO (30). We see effects of this kind with *M. tuberculosis* only at high concentrations of NO, which like H_2O_2, initiate an oxidative stress response. By contrast, O_2 and low concentrations of NO were found in this paper to mediate opposing, rather than analogous, effects on gene expression, as NO induces and O_2 represses dormancy regulon expression. Consequently, it seems unlikely that an OxyR, SoxR, or an FNR-like mechanism is used to monitor NO and O_2 levels for dormancy regulon regulation.

Alternatively, the truncated hemoglobin HbN (trHbN) and cytochrome *c* oxidase (CcO) are two heme-containing *M. tuberculosis* proteins that interact with NO and O_2 and, thus, could perform a NO/low O_2–sensing function. trHbN efficiently detoxifies NO and protects stationary phase *Mycobacterium bovis* from NO respiratory inhibition (31). CcO, the terminal oxidase in the aerobic respiratory pathway, catalyzes the reduction of O_2 to H_2O, and in the

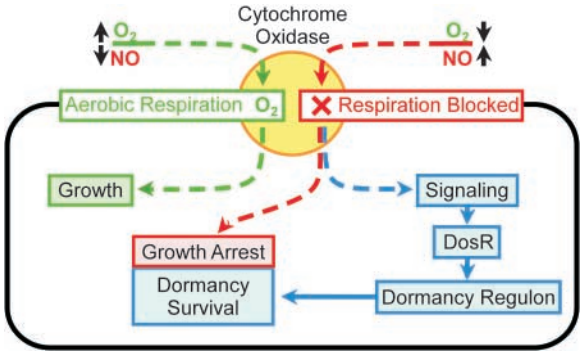


Figure 6. Model of O_2 and NO control of respiration, growth, and gene regulation. Cytochrome oxidase is posited as the sensor/integrator of O_2/NO levels in this model.

process, pumps protons across the cell membrane, which contribute to the proton gradient and ATP generation. Comprehensive studies of human mitochondrial aa3-type cytochrome oxidase (7) (homologous to the aa3-type cytochrome oxidase from *M. tuberculosis*) show that CcO is inhibited reversibly by very low (nM) concentrations of NO. By contrast, irreversible damage to the iron-sulfur centers of mitochondrial proteins requires ~1 mM NO (7). Cytochrome oxidase can also double as an O_2 sensor for the induction of genes responding to low O_2 in yeast (32) and for the repression of genes responding to high O_2 levels in the bacterium *Rhodobacter sphaeroides* (33). In view of these functional attributes and the effects of NO and O_2 on *M. tuberculosis* respiration, growth, and dormancy regulon signaling, we propose CcO as the most likely candidate for a NO/low O_2 sensor. However, proof of this conjecture will require O_2 and NO binding studies with purified *M. tuberculosis* CcO and functional studies with CcO *M. tuberculosis* mutants.

The findings that NO competes with O_2 to inhibit respiration, slow growth, and induce the dormancy regulon provide the basis for a model that describes the relationship between the tissue concentrations of NO and O_2 and the in vivo growth state of *M. tuberculosis* (Fig. 6). The main postulate of this model is the following: an important function of NO production and granuloma formation by the immune system is to limit aerobic respiration and impair growth of *M. tuberculosis*, an obligate aerobe. Granulomas in the middle and lower lung lobes of an immunocompetent person, particularly if surrounded by an avascular fibro-calcific capsule, exemplify this circumstance. The bacilli respond to the decrease in respiration by initiating a transcriptional response that transforms the pathogen. This transformation stabilizes vital components of the bacterial cell and enables survival during extended periods of latency. The fact that this response is exquisitely regulated and occurs at nontoxic concentrations of NO, points to the possibility that NO serves as an environmental signal from the host to the pathogen that discloses the level of immune activation.

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