Regulation of nitrate reductase activity in *Mycobacterium tuberculosis* by oxygen and nitric oxide

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Nitrate reduction by *Mycobacterium tuberculosis* is regulated by control of the transport of nitrate into the cell by NarK2. When oxygen was introduced into hypoxic cultures, nitrite production was quickly inhibited. The nitrate-reducing enzyme itself is relatively insensitive to oxygen, suggesting that the inhibition of nitrite production by oxygen was a result of interference with nitrate transport. This was not due to degradation of NarK2, as the inhibition was reversed by the removal of oxygen although chloramphenicol prevented new synthesis of NarK2. The oxidant potassium ferricyanide was added to anaerobic cultures to produce a positive redox potential in the absence of oxygen. Nitrite production decreased, signifying that oxidizing conditions, rather than oxygen itself, were responsible for the inhibition of nitrate transport. Nitric oxide added to cultures allowed NarK2 to be active even in the presence of oxygen. A similar result was obtained with hydroxylamine and ethanol, both of which interfere with oxygen utilization and the electron transport chain. It is proposed that NarK2 senses the redox state of the cell, possibly by monitoring the flow of electrons to cytochrome oxidase, and adjusts its activity so that nitrate is transported under reducing, but not under oxidizing, conditions.

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INTRODUCTION

Mycobacterium tuberculosis can persist in the human host even after the development of an immune response. Following initial infection, *M. tuberculosis* actively divides within naive macrophages. As cell-mediated immunity develops, the bacteria stop replicating, but they can remain viable for years and reactivate, resulting in active disease. It has been estimated that up to one-third of the world's population has latent tuberculosis (Dolin *et al.*, 1994). With so many people harbouring *M. tuberculosis*, control and possibly even eradication of the disease will require a more complete understanding of the factors that regulate non-replicating persistence.

In the human host, tubercle bacilli reside in granulomas, structures which may limit the availability of oxygen. Although *M. tuberculosis* is classified as an obligate aerobe,

in vitro it displays a complicated response to oxygen deprivation, with the induction of over 100 genes (Sherman *et al.*, 2001). When grown in sealed tubes with slow stirring, tubercle bacilli cease replicating when oxygen decreases to the microaerobic level and they enter a non-replicating persistence (NRP) state, NRP-1. With continued incubation, the anaerobic NRP-2 state follows (Wayne & Hayes, 1996). Many similarities in gene expression between chronic infection of mice and *in vitro* NRP cultures have been identified (Shi *et al.*, 2005).

One of the activities induced in *M. tuberculosis* by hypoxia is the dissimilatory reduction of nitrate to nitrite, which serves to provide energy as the bacteria adapt to anaerobiosis (Wayne & Hayes, 1999; Wayne & Sohaskey, 2001). Cultures in NRP-1 synthesize greater levels of nitrite than do actively growing (AG) aerobic cultures, but in both cases the nitrite is produced by the nitrate reductase enzyme encoded by *narGHJI* (Weber *et al.*, 2000; Sohaskey & Wayne, 2003). Expression of this operon is constitutive and not induced by hypoxia, although transcription of the *narK2* nitrate transporter is. This transporter is a main point of regulation, and is responsible for the upregulation of nitrate reductase activity seen in NRP cultures (Sohaskey & Wayne, 2003). Aerobic cultures are thought to rely on the slow diffusion of nitrate into the cells to provide substrate for the NarGHJI enzyme. Neither *narGHJI* nor *narK2* shows induction by either nitrite or nitrate.

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Abbreviations: AG, actively growing; c-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DETA/NO, *N*-(2-aminoethyl)-*N*-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylene diamine; GSNO, *S*-nitrosoglutathione; NRP, non-replicating persistence.

A graph showing the effects of DETA/NO and c-PTIO on nitrite synthesis is available as supplementary data with the online version of this paper.

One source of nitrate *in vivo* is the breakdown of nitric oxide (NO). Both hypoxia and NO can act as signals to *M. tuberculosis* that result in the initiation of the NRP state (Voskuil *et al.*, 2002). It has been suggested that oxygen limitation may be the important signal for NRP in humans, but in mice it may be NO (Boshoff & Barry, 2005). NO is essential in the control of *M. tuberculosis* in the murine model (Adams *et al.*, 1997; Chan *et al.*, 1995; MacMicking *et al.*, 1997) and humans (Nathan, 2002; Chan *et al.*, 2001). NO is synthesized by activated macrophages in mice when the cell-mediated immune system is activated, so there is an increase in available nitrate at the same time that the tubercle bacilli cease replicating.

A set of 48 genes, called the dormancy regulon, is induced by either hypoxia or low levels of NO (Voskuil *et al.*, 2002; Ohno *et al.*, 2003). The *narGHJI* operon is not induced by these stimuli, while *narK2* is induced by both (Sohaskey & Wayne, 2003; Ohno *et al.*, 2003; Voskuil *et al.*, 2002). We analysed the effect of oxygen and NO on nitrate reduction in both aerobic and NRP cultures of *M. tuberculosis*.

METHODS

Culture conditions. *M. tuberculosis* H37Rv and RVW3 were from the culture collection of this laboratory (Sohaskey & Wayne, 2003). *M. tuberculosis* was grown in Dubos Tween-albumin broth (DTA) (Difco). Aerobic cultures were incubated at 37 °C on a model G24 rotary shaker-incubator at a speed of 225 r.p.m. (New Brunswick Scientific Co., Inc.). For NRP cultures, conditions were as previously described, with a headspace ratio of 0.5 (Wayne & Hayes, 1996). Growth was monitored by measuring the OD₅₈₀ in a Coleman model 35 spectrophotometer (Coleman Instruments) with linear correction for particle interference at an OD₅₈₀ above 0.200 (Wayne & Hayes, 1996). Nitrate, when used, was added at a final concentration of 5 mM, unless otherwise indicated.

Chemicals. Kanamycin was used at 25 µg ml⁻¹ for *M. tuberculosis*. All chemicals were from Sigma. Potassium ferricyanide (III) [K₃Fe(CN)₆], Triton X-100 and hydroxylamine hydrochloride were dissolved in water and filter-sterilized. The sources of nitric oxide were *N*-(2-aminoethyl)-*N*-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylene diamine (DETA/NO) and *S*-nitrosoglutathione (GSNO), and the NO trap was 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), all of which were dissolved in sterile water.

Measurement of nitrite in culture. Cultures were inoculated to contain approximately 2.5 × 10⁶ cells ml⁻¹, and were grown either in 10 ml DTA with shaking, for aerobic (AG) cultures, or in 17 ml DTA in sealed tubes with slow magnetic stirring, for NRP cultures. After 112 h of incubation, aerobic cultures were in the mid-exponential phase of growth (OD₅₈₀ ~0.4), whereas the NRP cultures had by then been in microaerobic NRP-1 for approximately 45 h (Wayne & Hayes, 1996).

NRP-1 cultures, which contained approximately 10⁸ cells ml⁻¹, were opened, vortexed to introduce air and then pooled. Aerobic cultures were pooled and diluted with DTA to the same approximate cell concentration based on optical density (OD₅₈₀ 0.1 = 6.25 × 10⁷ c.f.u. ml⁻¹). Chloramphenicol to 20 µg ml⁻¹ and NaNO₃ to 5 mM were added where indicated. The cultures were then dispensed into tubes (in triplicate). Loosely capped tubes were incubated with shaking for aerobic conditions. For anaerobic incubation, Oxyrase (Oxyrase Inc., Mansfield, OH) was added to tightly capped tubes to

completely remove dissolved oxygen. Attainment of anaerobiosis was monitored by the decolourization of the redox indicator methylene blue present in independent control tubes. In every experiment, control tubes of AG and NRP cultures were incubated both aerobically and anaerobically.

At intervals, samples were removed to determine nitrite concentrations by the Griess reaction (Wayne & Doubek, 1965). In experiments with DETA/NO, this level had to be measured in control tubes containing no cells and subtracted as background. In experiments with GSNO or hydroxylamine, spontaneous conversion to nitrite was measured, and subtracted as background. *M. tuberculosis* did not produce nitrite from hydroxylamine under the conditions used here (data not shown).

Measurement of oxygen utilization. Mid-exponential-phase cultures containing approximately 2.5 × 10⁸ cells ml⁻¹ (OD₅₈₀ ~0.4) were opened, vortexed to introduce air and pooled. Methylene blue was added to 0.0003 % and cultures were aliquoted to 8 ml screw-capped culture tubes. DETA/NO, GSNO, hydroxylamine, ethanol or water was added to samples in triplicate, which were then sealed with Parafilm and incubated at 37 °C. Decolourization of methylene blue indicating utilization of oxygen was monitored by the measurement of absorbance at 665 nm.

RESULTS

Effect of oxygen on the reduction of nitrate

We measured nitrate reduction by cultures of *M. tuberculosis* that had been grown initially under aerobic or microaerobic non-replicating conditions as they were subsequently subjected to a variety of conditions. Assessment of the low rate of nitrite production is complicated by the fact that over the course of time needed to measure the rate of nitrite production, aerobic, but not NRP, cultures continue to replicate. In addition, both types of culture alter transcription and translation patterns to adapt to changes in the environment. Therefore, chloramphenicol was added at the start of these experiments to inhibit the synthesis of any new protein. This allowed the measurement of existing nitrate reductase activity in AG or NRP cultures under both aerobic and anaerobic conditions.

To investigate the effect of oxygen on nitrate reduction, cultures of *M. tuberculosis* were grown either aerobically (AG) to mid-exponential phase or to microaerobic phase (NRP). Subsequently, chloramphenicol and nitrate were added, the cultures were aerated by vigorous vortexing, and the cell densities adjusted so that all cultures contained similar numbers of cells in the tested volumes. Both cultures were split into identical samples which were then incubated either aerobically or anaerobically. Oxyrase was used to establish anaerobiosis rapidly, and it permitted multiple readings from a single tube. Samples were withdrawn from each tube at 3 h intervals and nitrite levels measured. An AG culture incubated aerobically had a low rate of increase of 1 µM NO₂⁻ h⁻¹ (Fig. 1), while one incubated anaerobically had greater activity, creating nitrite at the rate of 3 µM NO₂⁻ h⁻¹. An NRP culture incubated anaerobically with Oxyrase showed a linear increase in nitrite concentration for at least 12 h at a rate of 75 µM NO₂⁻ h⁻¹. In contrast, the NRP

Fig. 1. Nitrite synthesis during aerobic and anaerobic incubation. Cultures were grown either aerobically (AG) or to microaerobic conditions (NRP) and then divided for incubation with chloramphenicol and nitrate (5 mM), either aerobically or anaerobically. Nitrite concentrations were measured at intervals. Empty symbols indicate NRP cultures and filled symbols AG cultures. Circles, cultures incubated anaerobically; triangles, cultures incubated aerobically (barely visible at the bottom of the graph); squares, NRP cultures incubated initially anaerobically for 6 h and then aerobically; diamonds, NRP culture initially incubated aerobically for 6 h and then anaerobically; +, AG culture incubated anaerobically without chloramphenicol. Standard deviation is indicated.

culture incubated aerobically showed low activity of $1 \mu\text{M NO}_2^- \text{ h}^{-1}$. The rate was linear for at least 12 h, indicating no decrease in nitrate-reduction capacity during the course of the experiment. One set of NRP tubes was incubated anaerobically, and after 6 h the samples were opened and vortexed to aerate and inhibit Oxyrase activity. Incubation was then continued aerobically in loosely capped tubes. The rate of nitrite production decreased abruptly from anaerobic levels to aerobic levels (Fig. 1). Another set of NRP cultures was initially incubated aerobically, and after 6 h Oxyrase was added and the tubes sealed, resulting in anaerobiosis. This set showed an increase in the rate of nitrite synthesis approaching that of other NRP samples that had been incubated anaerobically. Results were similar when rifampicin was used in place of chloramphenicol (data not shown). An aerobic culture incubated anaerobically but without the chloramphenicol used in the other samples showed an increasing rate of nitrite production at each time point.

Effect of Triton X-100

The decrease in the rate of nitrite production of NRP cultures exposed to oxygen suggested a site of action present in NRP cells but missing from AG ones. One possible candidate is the nitrate transporter NarK2. If the decrease in nitrite synthesis in NRP cultures due to the presence of oxygen was a result of inhibition of the transport of nitrate into the cells, increasing the permeability of the membrane

to nitrate should lessen the effect. Triton X-100 incorporates into the membrane bilayer, thereby increasing the permeability to small ions. The rate of nitrite production was determined in chloramphenicol-treated cultures with 0.01 % Triton X-100 and 10 mM NaNO_3 . At this concentration, Triton X-100 did not inhibit respiration as measured by methylene blue decolourization (data not shown).

In aerobically grown cells incubated aerobically, the addition of Triton X-100 increased the rate of nitrite production approximately fourfold (Table 1). This supports previous work showing that diffusion of nitrate into the cell is the limiting factor in the reduction of nitrate in aerobic cultures (Sohaskey & Wayne, 2003). NRP cultures incubated aerobically showed very low activity that was also increased threefold by Triton X-100, suggesting that increasing nitrate transport across the membrane partially relieved the inhibition caused by oxygen. An NRP culture incubated anaerobically showed decreased nitrite synthesis from 63 to $40 \mu\text{M NO}_2^- \text{ h}^{-1}$ with the addition of Triton X-100, possibly due to increased diffusion of nitrate out of the cell.

Effect of a positive redox potential

The decrease in nitrite production by NRP cultures in the presence of oxygen could be due to molecular oxygen or alternatively the high redox potential (E_h) resulting from the presence of oxygen. To address this, the redox potential of anaerobic cultures was increased by the addition of the oxidant potassium ferricyanide (III) (Uden *et al.*, 1990; Kucera *et al.*, 1981; Alefounder *et al.*, 1981). At the concentrations used it did not interfere with Oxyrase activity or the Griess reaction used to measure nitrite levels. Potassium ferricyanide at 60 mM was added to chloramphenicol-treated AG and NRP cultures, which were then incubated both aerobically and anaerobically. Potassium ferricyanide had little effect on nitrite production by AG cultures, indicating no interference with the NarGHJI nitrate

Table 1. Effect of Triton X-100 on the rate of nitrite production

Cultures were grown aerobically (AG) or to microaerobic conditions (NRP) and then divided for further incubation with chloramphenicol and nitrate (10 mM), either aerobically or anaerobically. Triton X-100 (0.01 %, w/v) was added to half the samples. Nitrite was measured at intervals for 12 h. The rate of nitrite production ($\mu\text{M NO}_2^- \text{ h}^{-1}$) was determined for the linear part of the curve. Standard deviation is indicated.

Growth	Incubation	Triton X-100	Rate
AG	Aerobic	—	1.8 ± 0.2
		+	7.4 ± 0.5
NRP	Aerobic	—	0.9 ± 0.2
		+	2.6 ± 0.4
NRP	Anaerobic	—	62.9 ± 0.9
		+	40.4 ± 0.7

Fig. 2. Effect of a positive redox potential on the rate of nitrite production. Cultures were grown either aerobically (AG) or to microaerobic conditions (NRP) and then divided for further incubation with chloramphenicol and nitrate (5 mM), either aerobically or anaerobically. Potassium ferricyanide to 60 mM was added to half the samples. Nitrite concentrations were determined at intervals for 12 h. The rate of nitrite synthesis was determined for the linear part of the curve. Empty bars, untreated cultures; filled bars, cultures with potassium ferricyanide.

reductase enzyme (Fig. 2). Control NRP cultures incubated anaerobically showed strong nitrite synthesis, but with the addition of potassium ferricyanide, the rate of nitrite production of these cultures decreased threefold. Thus the inhibition of nitrate reduction could be mimicked in the absence of oxygen with a positive redox potential.

Effect of nitric oxide

NO from activated macrophages would be a major source of nitrate for tubercle bacilli *in vivo*. NO is highly reactive and inactivates many enzymes. To determine if NO inactivates

components of the nitrate reductase system, nitrite levels were measured in the presence of two NO donors.

When 100 μM DETA/NO was added to chloramphenicol-treated AG cultures incubated both aerobically and anaerobically, only a slight inhibition of nitrite production was seen (Fig. 3A). The addition of 100 μM DETA/NO had little effect on an NRP culture incubated anaerobically. However, when NRP cultures were incubated aerobically with DETA/NO, there was an 18-fold increase in nitrite synthesis.

An identical experiment was performed with GSNO as the NO donor, giving similar results. GSNO at 2 mM only slightly inhibited nitrite production by AG cultures incubated either aerobically or anaerobically (Fig. 3B). There was little effect on an NRP culture incubated anaerobically. However, NRP cultures incubated aerobically with GSNO showed a fivefold increase in nitrite production.

To show that this increase in nitrite synthesis was due to NO, the NO-specific trap c-PTIO was used (Goldstein *et al.*, 2004). Addition of c-PTIO at the same time as DETA/NO resulted in no change in the rate of nitrite production (Supplementary Fig. S1). When c-PTIO was added to an NRP culture previously treated with DETA/NO, the rate of nitrite production decreased immediately.

Effect of inhibition of the electron transport chain

Nitric oxide inhibits terminal oxidases (Clementi *et al.*, 1999; Brunori *et al.*, 2004). The effect of inhibiting these oxidases by other means was determined. Either cyanide or hydroxylamine was added to chloramphenicol-treated AG and NRP cultures. Cyanide at a low level inhibited nitrate reduction in AG cultures. *M. tuberculosis* under these conditions does not express *narK2*, so this effect is probably due to inhibition of the nitrate reductase enzyme itself.

Fig. 3. Effect of NO on the rate of nitrite production. Cultures were grown either aerobically (AG) or to microaerobic conditions (NRP) and then divided for further incubation with chloramphenicol and nitrate (5 mM), either aerobically or anaerobically. NO donors, either (A) DETA/NO to 100 μM or (B) GSNO to 2 mM, were added to half the samples and nitrite concentrations measured at intervals for 12 h. Empty bars, untreated cultures; filled bars, cultures with the NO donor.

Fig. 4. Effect of hydroxylamine and ethanol on the rate of nitrite production. Cultures were grown either aerobically (AG) or to microaerobic conditions (NRP) and then divided for further incubation with chloramphenicol and nitrate (5 mM), either aerobically or anaerobically. (A) Hydroxylamine hydrochloride to 5 mM or (B) ethanol to 5% was added to half the samples. Nitrite concentrations were determined at intervals for 12 h. Empty bars, untreated cultures; filled bars, cultures with hydroxylamine or ethanol.

Therefore no additional work was done with cyanide. Hydroxylamine at 5 mM inhibited growth of *M. tuberculosis* (data not shown), but did not interfere with nitrite production in AG cultures incubated either aerobically or anaerobically (Fig. 4A). NRP cultures incubated anaerobically showed some decrease in nitrite synthesis. However, when incubated aerobically with hydroxylamine, these NRP cultures showed a 15-fold increase in the rate of nitrate reduction.

Role of NarK2

The *narK2* knockout mutant RVW3 (Sohaskey & Wayne, 2003) was used to examine the role of NarK2 in the control of nitrate reduction by oxygen and NO. Both wild-type and RVW3 AG cultures showed similar low rates of activity when incubated aerobically (Fig. 5). Anaerobically incubated wild-type AG cultures showed a fourfold increase in nitrite production, and RVW3 cultures a 3–4-fold increase (Fig. 5). NRP cultures of both strains showed similar low activity when incubated aerobically. Incubated anaerobically, NRP cultures of RVW3 lacked the strong induction of nitrite synthesis, verifying the role of NarK2 in the hypoxic induction of nitrate reductase activity (Sohaskey & Wayne, 2003).

When exposed to 100 μM DETA/NO, NRP cultures of RVW3 did not show an increase in aerobic production of nitrite (Fig. 5). There was no increase in activity in the same cultures after exposure to 5 mM hydroxylamine. This demonstrates that the increase in the rate of nitrite synthesis seen in the wild-type NRP cultures incubated aerobically with NO is due to NarK2.

The effect of Triton X-100 on the RVW3 strain was also tested. NRP cultures were used and the rate of nitrite production was $0.7 \pm 0.2 \mu\text{M NO}_2^- \text{h}^{-1}$. This was boosted approximately 10-fold to $10.3 \pm 0.4 \mu\text{M NO}_2^- \text{h}^{-1}$ by the

addition of Triton X-100. When incubated anaerobically the increase was twofold from 4.0 ± 0.2 to $8.4 \pm 1.4 \mu\text{M NO}_2^- \text{h}^{-1}$. Therefore, the effect of Triton X-100 was independent of the presence of NarK2.

Effect of ethanol

Transcription of *narK2* is regulated by the hypoxic regulator DosR/DevR. Ethanol induced the transcription of *dosR/devR* under aerobic conditions (Kendall *et al.*, 2004).

Fig. 5. Nitrite production by RVW3. Cultures of *M. tuberculosis* RVW3 (*narK2::aph1*) and wild-type were grown either aerobically (AG) or to microaerobic conditions (NRP) and then divided for further incubation with chloramphenicol and nitrate (5 mM), either aerobically or anaerobically. DETA/NO (100 μM) or hydroxylamine (5 mM) was added to half the samples. Nitrite concentrations were determined at intervals for 12 h. Empty bars, wild-type cultures; filled bars, untreated RVW3; striped bars, RVW3 treated with DETA/NO; cross-hatched bars, RVW3 treated with hydroxylamine.

Therefore, the effect of ethanol on nitrite synthesis was determined. At 5% (800 mM), the level shown to cause a strong induction of *dosR/devR*, there was little effect on nitrite production by an AG culture (Fig. 4B). An NRP culture incubated anaerobically also showed only a slight inhibition by ethanol. The same NRP culture incubated aerobically produced an almost 40-fold increase in nitrite production (Fig. 4B).

Inhibition of respiration

Although NO, hydroxylamine and ethanol can have many effects, one they have in common is the ability to inhibit respiration. To determine if the concentrations used here resulted in inhibition of respiration in *M. tuberculosis*, oxygen consumption was followed with the redox indicator methylene blue. Methylene blue was added to mid-exponential phase AG cultures which were incubated in sealed tubes. The presence of oxygen was monitored by decolourization of the methylene blue (Fig. 6). The untreated control tubes faded in 7 h, while treated samples remained blue.

DISCUSSION

The system used here allowed the analysis of aerobic, actively growing (AG) and non-replicating persistent (NRP) cultures of *M. tuberculosis* under both aerobic and anaerobic conditions. AG cultures, which express *narGHJI* but not *narK2*, were included as controls to separate effects on the nitrate reductase enzyme from those on the nitrate transporter. Chloramphenicol blocked the synthesis of new proteins and enabled the measurement of changes in nitrate reductase activity independent of transcription and translation. Using

Fig. 6. Effect of NO, hydroxylamine and ethanol on respiration. Cultures were grown aerobically to mid-exponential phase and then divided for further incubation in full, sealed tubes with 0.0003% methylene blue. DETA/NO (500 mM, ▲), GSNO (2 mM, ▽), hydroxylamine (5 mM, ◆), ethanol (5%, ■) or water (●) was added. Decolourization of the methylene blue was monitored by A_{665} .

this technique, three different points of control of nitrate reductase by oxygen could be identified.

(1) Microaerobic NRP cultures had greater activity than aerobically growing ones. This difference has been previously described (Sohaskey & Wayne, 2003; Wayne & Hayes, 1999). In the paradigm system, *Escherichia coli*, the large difference in activity between aerobic and anaerobic cultures is mainly due to differences in the level of the nitrate reductase enzyme NarGHJI. In contrast, in *M. tuberculosis*, there is no change in transcription of *narGHJI* in response to hypoxia. The genome of *M. tuberculosis* encodes four genes with homology to nitrate transporters (Cole *et al.*, 1998). Transcription of *narK2* increases markedly in response to hypoxia and exposure to NO, while none of the other three are induced by these stimuli (Sohaskey & Wayne, 2003; Voskuil *et al.*, 2002; Ohno *et al.*, 2003). Their role, if any, in nitrate reduction is unknown. The presence of NarK2 in NRP cultures, not the level of nitrate reductase enzyme, was responsible for the approximately 75-fold difference in the rate of nitrite synthesis between AG and NRP cultures (Fig. 1) (Sohaskey & Wayne, 2003). The *narK2* mutant, RVW3, lacked the strong hypoxic induction of activity (Fig. 5). Without chloramphenicol, the rate of nitrite synthesis increased with each time point, probably due to induction and synthesis of *narK2* (Fig. 1).

(2) Oxygen inhibited nitrate reduction in aerobic, AG cultures. An aerobic culture of *M. tuberculosis* showed an approximately fourfold increase in nitrite production when incubated anaerobically. Aerobically grown cells do not produce NarK2, indicating that this increase is independent of NarK2. As further evidence, an aerobic culture of RVW3 also showed a threefold increase in nitrite production when incubated anaerobically (Fig. 5). The increase in nitrite production could be due to a diversion of electrons from cytochrome oxidase. In both *Paracoccus denitrificans* and *E. coli*, competition for electrons between the pathways to oxygen or nitrate has been proposed to be a main influence on nitrate reductase activity (Kucera *et al.*, 1996; Denis *et al.*, 1990). Partial inhibition of the nitrate reductase enzyme could also play a role, although it appears to be resistant to oxygen (Sohaskey & Wayne, 2003).

(3) Oxygen inhibited the activity of NarK2. An NRP culture that was exposed to air did not totally stop nitrate reduction, but continued at a level characteristic of aerobic cultures (Fig. 1). Increasing the diffusion of nitrate into the cell with Triton X-100, even in the *narK2* mutant, increased nitrite production. These experiments suggest that NarK2 was inhibited in the presence of oxygen. Inhibition was relatively rapid and was not due to degradation of NarK2, as the effect could be reversed by the removal of oxygen from cultures containing chloramphenicol.

The nitrate reductase system does not appear to sense oxygen levels directly. In the presence of potassium ferri-cyanide, nitrite production was inhibited in NRP cultures incubated anaerobically (Fig. 2), suggesting that the redox

state of some cell component, rather than molecular oxygen, is important.

Hypoxia results in an inactive cytochrome oxidase. NO and hydroxylamine can also inactivate cytochrome oxidase (Clementi *et al.*, 1999; Brunori *et al.*, 2004; Kucera & Skladal, 1990; Kucera *et al.*, 1981). Ethanol can inhibit the NADH dehydrogenase (complex I) (Kayser *et al.*, 2003). All of these treatments resulted in decreased respiration and an active NarK2 protein at oxygen concentrations that were previously inhibitory. Aerobic cultures did not show this increase in activity, suggesting the involvement of NarK2. This is supported by similar results with RVW3.

Inactivation of cytochrome oxidase either by lack of oxygen, or by the presence of NO or hydroxylamine, would increase the level of reduced components of the electron transport chain. A decreased electron flow may be part of the signal required for NarK2 activity. As a membrane protein, NarK2 is ideally located to sense components of the electron transport chain. A possible candidate is the quinone pool of *M. tuberculosis*. Quinone/quinol are non-protein components of electron transport and freely diffusible in the membrane. This pool could serve, not only as an electron source for the nitrate reductase enzyme, but also as an indirect indicator of oxygen levels. In *P. denitrificans*, it has been proposed that transport of nitrate is regulated by the redox level of the ubiquinone pool (Alefounder *et al.*, 1983).

Regulation of nitrate transport by oxygen has been documented in other bacteria (John, 1977; Noji & Taniguchi, 1987; Hernandez & Rowe, 1988). It is intriguing that *M. tuberculosis*, classified as an obligate aerobe, should have such intricate control of an anaerobic enzyme system. Transcription of *narK2* is controlled by DosR/DevR, which responds to hypoxia and NO (Ohno *et al.*, 2003; Voskuil *et al.*, 2002). Both the transcription and the activity of NarK2 are controlled by similar signals in what appears to be a redundant system, the purpose of which is not entirely clear.

The nitrate reductase system of *M. tuberculosis* may be important *in vivo*. The *narGHJI* operon is important for the survival of *Mycobacterium bovis* BCG in SCID mice (Weber *et al.*, 2000). A *narG* knockout mutant of *M. bovis* BCG shows decreased virulence in these mice in comparison to the wild-type strain. The mutant *M. bovis* BCG produces smaller granulomas with fewer bacteria. In immunocompetent mice, the mutant shows decreased ability to persist in the lungs, liver and kidneys, but no decrease in the spleen (Fritz *et al.*, 2002). A role for nitrate reductase in maintaining ATP levels in *M. tuberculosis* during hypoxia has been demonstrated from work with pyrazinamide (Wade & Zhang, 2004; Zhang *et al.*, 2003).

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