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Redox homeostasis in mycobacteria: the key to tuberculosis control?

Ashwani Kumar[1,*], Aisha Farhana[2,*], Loni Guidry[2], Vikram Saini[2], Mary Hondalus[3] and Adrie J.C. Steyn[2,4,†]

Mycobacterium tuberculosis (*Mtb*) is a metabolically flexible pathogen that has the extraordinary ability to sense and adapt to the continuously changing host environment experienced during decades of persistent infection. *Mtb* is continually exposed to endogenous reactive oxygen species (ROS) as part of normal aerobic respiration, as well as exogenous ROS and reactive nitrogen species (RNS) generated by the host immune system in response to infection. The magnitude of tuberculosis (TB) disease is further amplified by exposure to xenobiotics from the environment such as cigarette smoke and air pollution, causing disruption of the intracellular prooxidant–antioxidant balance. Both oxidative and reductive stresses induce redox cascades that alter *Mtb* signal transduction, DNA and RNA synthesis, protein synthesis and antimycobacterial drug resistance. As reviewed in this article, *Mtb* has evolved specific mechanisms to protect itself against endogenously produced oxidants, as well as defend against host and environmental oxidants and reductants found specifically within the microenvironments of the lung. Maintaining an appropriate redox balance is critical to the clinical outcome because several antimycobacterial prodrugs are only effective upon bioreductive activation. Proper homeostasis of oxido-reductive systems is essential for *Mtb* survival, persistence and subsequent reactivation. The progress and remaining deficiencies in understanding *Mtb* redox homeostasis are also discussed.

In 1890, Koch stated publicly that he had discovered the cure for tuberculosis (TB). In 1921, Calmette and Guerin introduced the vaccine against TB, and between 1944 and 1966,

[1]Institute of Microbial Technology, Chandigarh 160036, India

[2]Department of Microbiology and Centers for AIDS Research and Free Radical Biology, University of Alabama at Birmingham, AL, USA

[3]Department of Infectious Disease, University of Georgia, Athens, GA, USA

[4]KwaZulu-Natal Research Institute for Tuberculosis and HIV, Durban, South Africa

*These authors contributed equally.
†Corresponding author: Adrie J.C. Steyn, University of Alabama at Birmingham, AL 35294, USA and KwaZulu-Natal Research Institute for Tuberculosis and HIV, Durban, South Africa. Email: asteyn@uab.edu

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streptomycin, isoniazid (INH), ethambutol, rifampin and pyrazinamide were discovered as remedies for TB. Yet *Mycobacterium tuberculosis* (*Mtb*), the aetiological agent of TB, is still responsible for ~1.7 million deaths each year. In the majority of affected persons, *Mtb* enters a latent or persistent phase during infection (Fig. 1) associated with a state of drug unresponsiveness wherein the bacilli are not killed by currently available antimycobacterial agents (Refs 1, 2, 3). This situation, together with the emergence of multi-drug-resistant (MDR), extensively drug-resistant (XDR) and super XDR *Mtb* strains and the synergy with HIV infection, has created a frightening scenario. Studies show that malnutrition, tobacco smoking and indoor air pollution from solid fuel are the most important risk factors for TB worldwide, followed by HIV infection, diabetes and excessive alcohol intake (Fig. 1) (Ref. 4).This strongly suggests that improved nutrition and implementation of effective intervention strategies against tobacco smoke and indoor air pollution will have global socioeconomic and public health implications.

Dormancy refers to a physiological state of the bacillus generally typified by the absence of replication and the presence of metabolic shutdown. Latency is a clinical state characterised by purified protein deriviative (PPD) skin test responsiveness coincident with a lack of clinical representation of disease. For a more in-depth discussion of these terms, see Refs 5, 6, 7.

More than a hundred years of research has shown that *Mtb* is an obligate aerobe, but the phrase '*Mtb* anaerobic respiration' is frequently, albeit incorrectly, used in the TB literature. Nonetheless, it has been demonstrated that *Mtb* can survive in vitro for more than a decade under apparently anaerobic conditions.

Redox reactions have a key role in aerobic and anaerobic respiration. Within aerobic microbes, reactive species or oxidants are more or less balanced by the presence of antioxidants (Ref. 8). *Mtb*, similar to other bacterial species, has evolved pathways to monitor redox signals (such as O_2, NO and CO) and the alterations in intra- and extracellular redox states (Refs 2, 9, 10). We will begin this review by describing the basics of bacterial redox homeostasis and will then summarise the best-characterised redox mechanisms used by mycobacteria to sense and maintain redox homeostasis. A better understanding of these mechanisms should open new avenues for the development of improved diagnostic tools and effective vaccines, and lead to the identification of new drug targets.

Maintaining the balance: oxidative stress and oxidative damage

Oxidative stress can be defined as a disturbance in the prooxidant–antioxidant balance in favour of the former, leading to potential injury. Oxidative damage is characterised as the biomolecular impairment caused by the attack of reactive species upon the constituents of living organisms (Ref. 8). Oxidation can be described as a gain in oxygen ($C + O_2 \rightarrow CO_2$), a loss of hydrogen or a loss of electrons ($Na \rightarrow Na^+ + e^-$ or $O_2^{\bullet -} \rightarrow O_2 + e^-$), whereas reduction is defined as a loss of O_2 ($CO_2 + C \rightarrow 2CO$), a gain in hydrogen ($C + 2H_2 \rightarrow CH_4$) or a gain of electrons ($O_2 + e^- \rightarrow O_2^{\bullet -}$) (Ref. 8). Redox homeostasis can be defined as a 'relatively stable state of equilibrium or a tendency towards such a state between the different but interdependent elements or groups of elements of an organism, population, or group' (Merriam-Webster). Redox homeostasis is important to effectively harness reducing power produced through the catabolism of various substrates and to utilise this power in the anabolism of cellular components such as DNA, lipids and proteins.

Why is Mtb redox balance important?

During the course of infection, *Mtb* is exposed to a range of microenvironments that induce novel, as yet uncharacterised, compensatory metabolic pathways in an attempt by the bacillus to maintain balanced oxidation–reduction. It can be argued that redox imbalance can trigger mechanisms in the bacillus, which result in persistence and dormancy. Host-generated gases, carbon sources and pathological conditions such as hypoxic granulomas have a profound effect on bacterial metabolism and therefore redox balance, which through unknown mechanisms allow *Mtb* to successfully subvert the immune system and cause disease. These in vivo environmental conditions that cause intracellular redox imbalance might also affect antimycobacterial drug efficacy. For example, INH (Ref. 5), ethionamide (ETA) (Ref. 6) and PA-824 (a nitroamidazole

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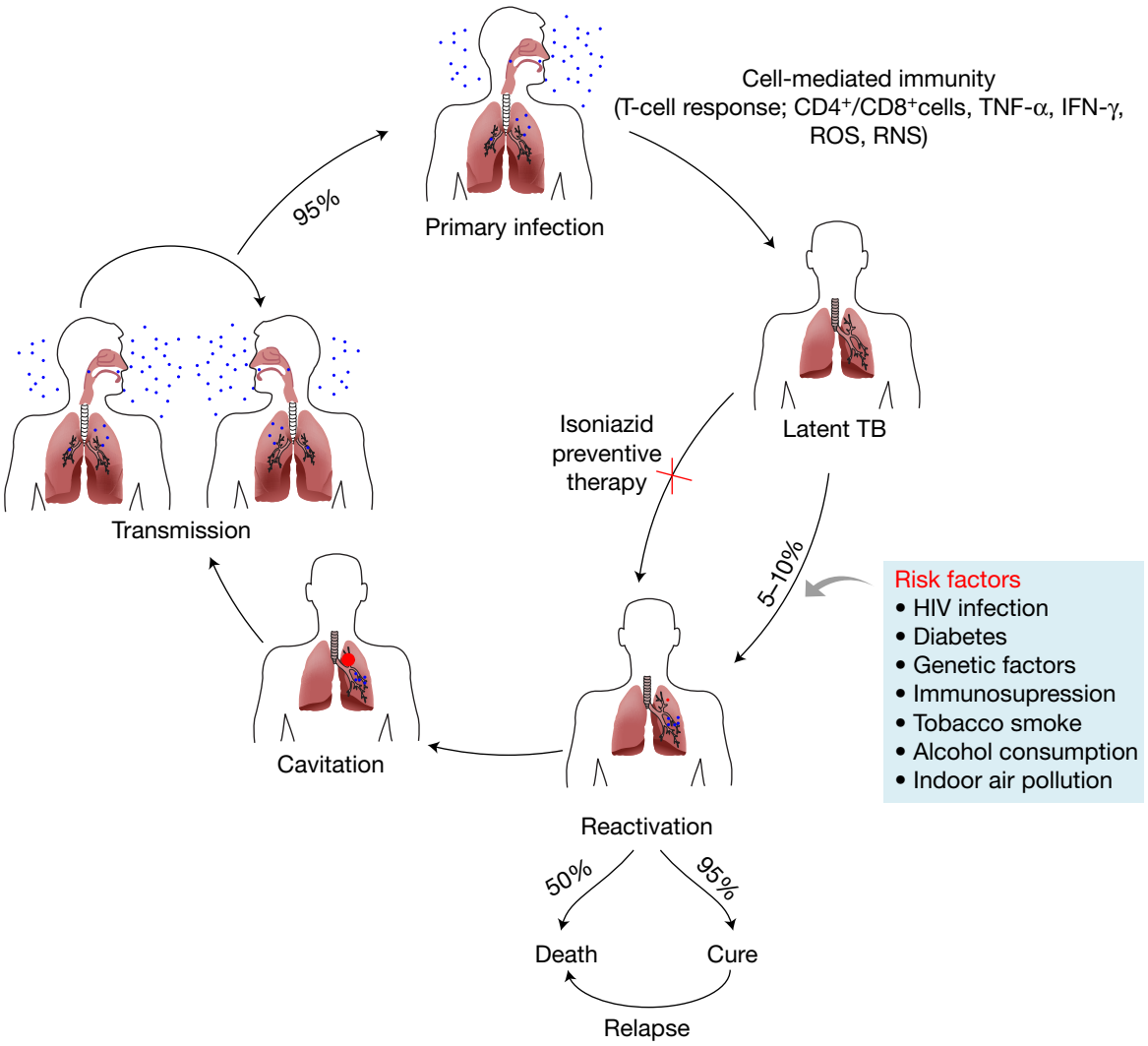

Virulence life cycle of *Mycobacterium tuberculosis* and progression of TB

Figure 1. Virulence life cycle of *Mycobacterium tuberculosis* and progression of TB. *Mtb* is transmitted by aerosol, and in 95% of cases, wherein the tubercle bacilli are inhaled, a primary infection is established. This is either cleared by the surge of the cell-mediated immunity or contained inside the granuloma in the form of latent TB, defined by no visible symptom of disease, but persistent, yet dormant, live bacilli within the host. The progress of TB can be stalled at this stage in some cases by isoniazid preventive therapy. This state might last for the lifespan of the infected individual, or progress to active TB by reactivation of the existing infection, with a lifetime risk of 5–10%. This risk of progression is exacerbated by immune-compromising factors such as HIV-AIDS, diabetes, indoor air pollution and tobacco smoke. Reactivation of TB is shown to occur at the upper and more oxygenated lobe of the lung, which can be cured by compliance with drug therapy. However, untreated or poorly treated TB might lead to the formation of tuberculous lesions in the lung. The development of cavities close to airway spaces allows shedding (e.g. coughing) of the bacilli through the airway, a stage of transmission. Subsequently, in a cyclic manner, the TB bacilli are transmitted to other individuals to establish primary infection.

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derivative) (Ref. 7) require bioreductive activation to exert an antimycobacterial effect. Further support is provided by studies demonstrating that an increased $NADH/NAD^+$ ratio leads to INH resistance (Ref. 11), and that mutations in the mycothiol biosynthetic pathway affect INH and ETA efficacy (Ref. 12). The identification of a link between INH resistance and mutations in *Mtb* type II NADH-menaquinone oxidoreductase (*Ndh-2*) is consistent with the present understanding that increased NADH reduces the frequency of INH-NAD (or ETA-NAD) adduct formation, which subsequently decreases its binding to InhA, the known target of INH (Refs 13, 11). Several studies have investigated the specific link between redox potential and nitroimidazole drug efficacy in other pathogens such as *Helicobacter pylori* (Ref. 14), *Bacteroides* spp. (Ref. 15) and *Trichomonas vaginalis* (Ref. 16). However, the mechanisms that *Mtb* uses to maintain redox homeostasis in vivo, and their role in drug susceptibility, remain unknown. A further understanding of how host environmental factors affect *Mtb* physiology, leading to perturbation of redox homeostasis might provide better insight into *Mtb* persistence and to the development of successful antimycobacterial intervention strategies.

What is a free radical?

A free radical is any species capable of independent existence that contains one or more unpaired electrons (Ref. 8), and is denoted by a superscript dot after the chemical formula. Free radicals can be beneficial (e.g. free radicals produced during phagocytosis) or detrimental (e.g. generating DNA damage or lipid peroxidation) to the free-radical-generating host. Reactive nitrogen species (RNS) refer to radicals such as $NO^\bullet$, $NO_2^\bullet$ and $NO_3^\bullet$, and nonradicals such as HNO_2, NO^+, NO^-, N_2O_4, N_2O_3, NO_2^+, ROONO and RO_2ONO. Reactive oxygen species (ROS) is a collective term that refers to O_2 radicals such as $O_2^{\bullet-}$, $HO_2^\bullet$, $HO^\bullet$, $RO_2^\bullet$, $RO^\bullet$ and $CO_2^\bullet$, and nonradical derivatives of O_2 such as H_2O_2, $ONOO^-$, ONOOH, $ONOOCO_2^-$, HOCl, HOBr and O_3 (Ref. 8).

Reduction potential is an important thermodynamic property that allows the prediction of the course of free radical reactions (Table 1). Important redox couples such as $NAD^+/NADH$ ($E^{0\prime} = -316$ mV), $NADP^+/NADPH$ ($E^{0\prime} = -315$ mV), $FAD/FADH_2$ ($E^{0\prime} = -219$ mV), ferredoxin (Fd_{ox}/Fd_{red}, $E^{0\prime} = -398$ mV) and GSSG/2GSH [$E_{hc} = -250$ mV (10 mM)] present in cells might function independently or be linked to other couples. Using linked sets of redox pairs, the redox environment can be defined as the summation of the products of the reduction potential and reducing capacity of the linked set of redox couples found in that cellular compartment (Ref. 17). In living systems, the reduction potential values predict what is feasible, but not what necessarily occurs (Ref. 8) (Table 1). Although a system of more negative reduction potential ($E^{0\prime}$) should reduce one with a less negative, zero or positive $E^{0\prime}$, there exists a hierarchy of oxidants. For example, the hydroxyl radical ($HO^\bullet$) will virtually always serve as an oxidant, whereas $NO^\bullet$ or H_2O_2 can function as oxidants or reductants depending on whether they react with molecules of lower or higher hierarchy (Ref. 18).

Measurement of all linked redox couples within bacterial cells is impractical and probably impossible, because some couples remain unidentified. Thus, quantification of a representative redox couple is used to infer changes in the redox environment. For example, in most bacteria (albeit not mycobacteria) the GSSG/2GSH couple represents the major intracellular redox buffer and can therefore be used to infer the status of the bacterial redox environment. Using this redox-couple-specific approach, the intracellular redox potential of *Escherichia coli* ($E^{0\prime} = -220$ to -245 mV) (Refs 19, 20) has been determined, which augurs well with that of a recent noninvasive fluorescent-based assessment ($E^{0\prime} = -259$ mV) (Ref. 19). The intracellular redox potential of mycobacteria has not yet been determined.

Free radicals and microbes

Endogenous oxidative stress arises from the univalent reduction of O_2 by various components of the electron transport chain (ETC) under normal aerobic conditions, resulting in the production of ROS such as superoxide radicals ($O_2^{\bullet-}$). The mechanism of O_2-mediated reoxidation of many reduced electron carriers such as reduced flavins, Fe^{2+} and NADH has been shown to occur by the formation of $O_2^{\bullet-}$. Although $O_2^{\bullet-}$ is less reactive than $HO^\bullet$ and does not react with most

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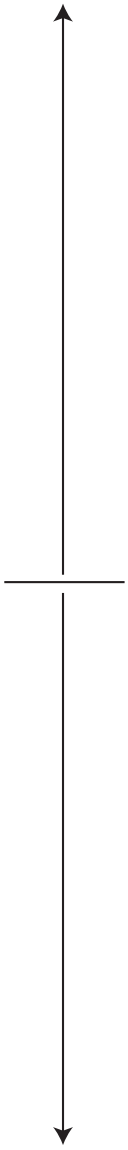
Table 1. Standard reduction potentials of biologically relevant redox couples

Redox couple	Redox potential (mV)
$CO_2/CO_2^{\bullet -}$	−1800
CO_2/CO	−520
Acetyl-Co/Apyruvate	−500
Succinyl-CoA/2-oxoglutarate	−491
$CO_2/HCOO^-$	−421
H^+/H_2	−414
$NAD^+/NADH$	−316
$NADP^+/NADPH$	−315
CO_2/acetate	−291
TrxC [TrxSS/Trx(SH_2)]	−269
TrxB [TrxSS/Trx(SH_2)]	−262
TrxA [TrxSS/Trx(SH_2)]	−248
$2H^+$/2Cys-SH (cystine)	−230
$FAD^+/FADH_2$	−219
$FMN^+/FMNH_2$	−219
Pyruvate, H^+/lactate	−183
Oxaloacetate, $2H^+$/malate	−166
Menaquinone	−74
ESSE/2ESH (ergothioneine)	−60
$CoQ/CoQ^{\bullet -}$	−36
Fumarate/succinate	+32
Ubiquinone/ubiquinol	+45
Fe^{3+}/Fe^{2+}(aq)	+110
$Ascorbate^{\bullet -}$/ascorbate$^-$	+282
O_2/H_2O_2	+295
Cytochrome a_3 (Fe^{3+})/cytochrome a_3 (Fe^{2+})	+350
NO_3^-/NO_2^-	+421
α-Tocopheroxyl$^{\bullet}$/α-tocopherol	+500
O_2/H_2O	+818
$RS^{\bullet}/RS^-$ (cysteine)	+920
$GS^{\bullet}/GS^-$ (glutathione)	+920
$NO_2^{\bullet}/NO_2^-$	+990
$ROO^{\bullet}$, H^+/ROOH (alkyl peroxyl radical)	+1000
$HO_2^{\bullet}$, H^+/H_2O_2	+1060
$ONOO^-/NO_2^{\bullet}$ (aq)	+1400
$RO^{\bullet}$, H^+/ROH (aliphatic alkoxyl radical)	+1600
$NO_2^+/NO_2^{\bullet}$	+1600
$CO_3^{\bullet -}$, H^+/HCO_3^-	+1780
$HO^{\bullet}$, H^+/H_2O	+2310

Highly reducing



Highly oxidizing

For a given couple, the reduction potential relative to the potential of the standard couple, hydrogen (H^+/H_2), is shown. Standard concentrations are 1.0 M for solutes and ions and 1 atm pressure for gases (e.g. H_2). The standard reduction potentials are symbolised by E^0. Note that the 'true' redox potentials within a cell can differ dramatically from standard values. The Nernst equation is used to correct E^0 values for the effect of temperature (T) and concentration: $= E^0 + \frac{-RT}{nF}\frac{\log_{10}[\text{oxidised}]}{[\text{reduced}]}$. Because protons are involved in many reactions, the values in the table are corrected to pH 7 ($E^{0\prime}$ rather than E^0). This is particularly important because the intracellular microbial and host pH probably vary widely during the course of infection. The bottom of the list (more positive) represents the highly oxidising couples, whereas the top of the list (more negative) represents the highly reducing couples. Therefore, the hydroxyl radical ($HO^{\bullet}$) at the bottom of the list is capable of oxidising everything else on the list. The data are largely from Refs 8, 17, 18.

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biological molecules, it readily reacts with $NO^{\bullet}$ to generate peroxynitrite ($ONOO^-$) (Ref. 8). $O_2^{\bullet-}$ also oxidises the 4Fe–4S clusters of dehydratases such as aconitase, leading to enzyme inactivation and release of Fe^{2+}. The released Fe^{2+} can then reduce H_2O_2 to intracellular $HO^{\bullet}$. Free Fe^{2+} is maintained in reduced form by intracellular reductants and will continue to reduce H_2O_2 to generate $HO^{\bullet}$. Further, Fe^{2+} can nonspecially bind to DNA, proteins and membranes, facilitating the localised production of $HO^{\bullet}$, which may result in oxidative damage to these molecules (Ref. 8).

Superoxide dismutase (SOD) catalyses the dismutation reaction between two superoxide radicals, resulting in the formation of molecular O_2 and H_2O_2 [equation (1)]:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

H_2O_2 can be detoxified by enzymes such as catalase and peroxidase as shown in equation (2), but its production in the presence of metal ions (such as Fe^{2+} and Cu^+) leads to the formation of extremely potent oxidant, $HO^{\bullet}$, through the Fenton reaction: [equation (3)]:

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^- \quad (3)$$

The superoxide anion generated as an unwanted byproduct of normal aerobic respiration can subsequently reduce the metal ion as shown in equation (4):

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2 \quad (4)$$

Reactions (3) and (4) combined are known as the Haber–Weiss reaction [reaction (5)], which was first described in 1934 (Ref. 21):

$$O_2^{\bullet-} + H_2O_2 \rightarrow HO^{\bullet} + HO^- + O_2 \quad (5)$$

The low reactivity of $O_2^{\bullet-}$ and H_2O_2 allows them to diffuse from their site of production, which, on reaction with free iron or copper ions in the cellular pool, leads to the generation of $HO^{\bullet}$. In *E. coli*, aerobic respiration leads to the generation of 0.1–0.2 μM H_2O_2 (Ref. 22). It is estimated that the intracellular stoichiometry of $O_2^{\bullet-}$ to $HO^{\bullet}$ is 2:1. The $HO^{\bullet}$ radical is particularly unstable and reacts rapidly with numerous bacterial components such as lipids, DNA and proteins (Ref. 22) to induce site-specific lesions. Studies have demonstrated that *Mtb* is susceptible to H_2O_2-induced damage in vitro (Ref. 23).

$O_2^{\bullet-}$ in aqueous solution can react as a reductant wherein it donates an electron to cytochrome *c*, and can also serve as an oxidant with ascorbic acid (AH_2) (Ref. 8):

$$\text{Cyt } c\,(Fe^{3+}) + O_2^{\bullet-} \rightarrow O_2 + \text{Cyt } c\,(Fe^{2+}) \quad (6)$$

$$AH_2 + O_2^{\bullet-} \rightarrow A^{\bullet-} + H_2O_2 \quad (7)$$

$O_2^{\bullet-}$ can also interact with NADH bound to the active site of lactate dehydrogenase and possibly other enzymes to generate a $NAD^{\bullet}$ radical; however, it does not oxidise free NADPH or NADH (Ref. 8).

Free radicals and the host

Mtb is a slow-growing bacillus transmitted by the respiratory route. The infection initiates on ingestion of the bacilli by alveolar macrophages. On phagocytosis of *Mtb*, lung macrophages and neutrophils produce large quantities of ROS and RNS. NADPH oxidase catalyses the one-electron reduction of O_2 using NADPH as electron donor, generating $O_2^{\bullet-}$, as depicted in the following (reviewed in Refs 24, 25, 26, 27):

$$2O_2 + NADPH \rightarrow O_2^{\bullet-} + NADP^+ + H^+ \quad (8)$$

Superoxide generated in the above reaction can be converted to H_2O_2 by SOD as described in equation (1).

A highly reactive hypochlorite ion (ClO^-) could be generated by myeloperoxidase, which catalyses the oxidation of chlorine, resulting in the formation of ClO^- according to the reaction below [equation (9)] (Ref. 28). Hypochlorite is an extremely reactive oxidant and can lead to oxidative damage of lipids, proteins and DNA:

$$Cl^- + H_2O_2 \rightarrow ClO^- + H_2O \quad (9)$$

$O_2^{\bullet-}$ also acts as a precursor of several other ROS (Refs 29, 30, 31) and RNS (Refs 32, 33, 34). In response to mycobacterial infection, another major antimicrobial pathway that acts through inducible NO synthase is activated, leading to increased production of NO (Refs 35, 36)

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(reaction (10)):

$$\text{L}-\text{arginine}+\text{NADPH}+\text{H}^{+}+\text{O}_2 \rightarrow \text{L-citrulline} + \text{NADP}^{+}+\text{H}_2\text{O}+\text{NO}^{\bullet} \quad (10)$$

NO is produced in parallel with $O_2^{\bullet -}$ and they both react with each other to produce highly reactive $OONO^-$. In addition to the generation of $OONO^-$, NO also leads to the generation of NO^-,$^{\bullet}NO_2$, NO_2^-, N_2O_3, N_2O_4, *S*-nitrosothiols and dinitrosyl-iron complexes (Refs 37, 38), which are all effective in killing bacteria (Ref. 39).

To dissect the role of ROS in TB, different murine knockout models lacking active NADPH oxidase components have been generated and compared with the wild-type strain for their capability to control the growth of *Mtb*. These experiments produced conflicting data among different laboratories. Two independent studies (Refs 40, 41) showed that *Mtb* growth was enhanced in the absence of active NOX (NADPH oxidase), whereas another study found no difference between $Phox^{-/-}$ and wild-type mice in their ability to control *Mtb* infection (Ref. 42).

Several murine studies (Refs 35, 43, 44, 45, 46) have shown that inducible NO synthase (iNOS or NOS2) produces NO, which is capable of killing mycobacteria. Furthermore, iNOS-deficient mice were demonstrated to be highly susceptible to TB infection (Refs 42, 47, 48), and NO was shown to be crucial in maintaining a latent TB infection in mice (Ref. 49). Clinical evidence in support of a role for NO in TB includes studies indicating increased iNOS protein and mRNA levels in bronchoalveolar lavage specimens from active pulmonary TB patients (Ref. 45), single-nucleotide polymorphism variations of NOS2A (Ref. 50), and increased exhaled NO and NO_2^- in patients with active pulmonary TB (Refs 43, 51).

Mtb physiology and the intracellular redox state

Mtb is a prototrophic, obligate aerobe that cannot replicate in the absence of O_2. However, the tubercle bacillus has an uncanny ability to survive extended periods of anaerobiosis even though classic manometric studies showed that several days of anaerobic exposure completely stalled bacterial respiration and the ability to grow on laboratory media (Ref. 52). In recent years, research into the mechanisms associated with the bacilli adaptive response to anaerobiosis has received much attention primarily because TB granulomas were shown to be hypoxic (Ref. 53), and because all current antimycobacterial drugs are ineffective against nonreplicating *Mtb* present in hypoxic granulomas. Thus, a more thorough understanding of *Mtb* redox physiology is critical to TB control.

Aerobic respiration is one of the most widespread bioenergetic pathways in microbial biology. Oxidation of a typical carbohydrate such as glucose can be divided into three separate phases: (1) a catabolic pathway (e.g. glycolysis) that breaks down glucose to pyruvate; (2) the TCA cycle, which oxidises organic molecules to CO_2 and H_2O, ATP and reduced coenzymes; and (3) oxidative phosphorylation, during which reduced coenzymes are oxidised and their electrons and protons establish a proton motive force across the membrane. Electrons are channelled (through NADH and $FADH_2$) to the ETC, which sequentially oxidises and reduces multiple redox centres before reducing O_2 to H_2O, and producing ATP. The respiratory metabolism is complex and regulated by many endogenous and exogenous (host) factors, including the carbon source, pH, O_2 (and ROS), NO (and RNS), CO, CO_2, etc. (Ref. 54).

The central role that redox reactions have in maintaining metabolic processes makes them essential to mycobacterial persistence. Unfortunately, the mechanisms used by *Mtb* to maintain redox homeostasis during active disease, persistence and reactivation are poorly understood and warrant further investigation. It is unknown how *Mtb* simultaneously regulates metabolic and signalling events in endogenous cellular compartments (e.g. the reducing environment of the cytoplasm and the oxidised periplasmic space and outer cell surface). Likewise, it is poorly understood how the bacterium senses and responds to the diverse environments encountered in vivo, for example in different organs or in different regions of the same organ (e.g. the natural O_2 gradients within the lung).

Important physiological players: gases and ATP

Mtb resides within a hypoxic microenvironment in the lungs (Ref. 55). However, aerobic and

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anaerobic microenvironments almost certainly exist, which in theory can explain the capacity of dormant bacilli to survive chemotherapy. Aerobic respiratory systems produce energy that comes from the movement of electrons from oxidisable organic substrates to O_2. Components of the ETC contain redox centres [redox-active prosthetic groups such as FMN, haem and iron–sulfur clusters (Fe–S)], with progressively greater affinities for electrons (from lower to higher standard reduction potentials). In general, these redox centres are very susceptible to host-generated ROS and RNS, which typically bind to or oxidise the prosthetic groups to affect protein activity, and therefore respiration. In agreement with the known mode of action of NO, which targets components of the respiratory chain, studies have shown that NO inhibits *Mtb* respiration. In fact, NO and lack of O_2 synergistically block respiration (Ref. 56). Lack of O_2 causes a loss of energy, which destroys the ordered state (life) of a cell, leading to its death. However, evidence suggests that *Mtb* has the extraordinary capacity to decrease respiration to a low, albeit not zero, level, and still remain viable (Ref. 52). Although nitrate (NO_3^-) prolongs the survival of *Mtb* under anaerobic growth conditions as demonstrated in vitro (Refs 57, 58), active replication was not promoted. By contrast, the *M. tuberculosis narGHJI* operon was capable of complementing a *nar E. coli* mutant, which acquired the ability to actively replicate anaerobically only in the presence of nitrate (Ref. 59). Therefore, because NO_3^- was unable to stimulate replication of *Mtb* under anaerobic conditions, this compound cannot be regarded as a terminal electron acceptor. Furthermore, it suggests that the reduction of NO_3^- could be redox balancing, or it might help provide energy under anaerobic conditions.

Consistent with the consequences of respiratory inhibition, ATP decreases to 25% of aerobic levels during hypoxic growth of *Mtb* (Ref. 60). A recent study has shown that *Mtb* maintains a fully energised cytoplasmic membrane to preserve ATP homeostasis during hypoxia without the use of alternate terminal electron acceptors (NO_3^-, fumarate, etc.) for respiration (Ref. 61). This suggests that *Mtb* retains a low level of metabolic activity to sustain an energised membrane even in the absence of respiration during hypoxic persistence.

Redox couples and electron transfer in *Mtb*

The NAD^+/NADH coenzyme system is required for catabolism, whereas the $NADP^+$/NADPH system is required for anabolism. NAD^+ is an efficient electron sink and hence is used as a cofactor in several oxidising reactions. A constant level of NADH is maintained during various phases of growth in vitro, whereas the concentration of NAD^+ is variable and is a major contributor to a change in NADH/NAD^+ ratio. In *Mtb*, the ratio of NADH/NAD^+ is typically ~1:3 to 1:10 (Refs 61, 62, 63), but a higher ratio of *Mtb* NADH/NAD^+ is generated during the transition from aerobic to anaerobic mycobacterial growth, owing to depletion of the NAD^+ pool, and is maintained by type II NADH dehydrogenase (Ref. 61). Although NAD^+ has an important role as an electron sink, NADPH acts as a major electron donor in many reductive reactions. Hence the NADPH/$NADP^+$ ratio is an indicator of reductive energy available to a cell. The concentration of the NADH/NAD^+ couple is submillimolar and is often higher than the phosphorylated form. In *Mtb*, the ratios of NAD^+/$NADP^+$, NADPH/NADH, $NADP^+$/NADPH and NAD^+/NADH are 1.95, 2.25, 2.39 and 10.5, respectively (Ref. 62).

Being an obligate aerobe, *Mtb* has to regenerate NAD^+ because the respiratory chain is downregulated in the absence of O_2 as terminal electron acceptor. An unexpected finding in the anaerobic model for in vitro dormancy was that the *Mtb* NAD^+ and NADH levels were only approximately 50% of the aerobic levels when O_2 was consumed, whereas the NAD^+/NADH ratio was similar to that in aerobic conditions (Ref. 60). These findings suggest that *Mtb* has evolved an as yet unidentified mechanism to survive severe hypoxia and regenerate NAD^+. Three plausible mechanisms that might allow regeneration of NAD^+ from NADH under hypoxic or nitrosative stress conditions exist: (1) nitrate reduction (*narGHJI*; Rv1161–1164) that catalyses the reduction of NO_3^- to NO_2^-; (2) triacylglycerol (TAG) anabolism (Ref. 64); or (3) electron bifurcation mechanisms by which two electrons bifurcate to a high and low potential pathway (Ref. 65). A recent in vivo study has shown that *Mtb* generates massive quantities of NAD(P)H in infected mouse lungs (Ref. 63) and therefore experiences significant reductive stress (see also Ref. 54 for a review). This finding again raises

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the issue of how reducing equivalents are regenerated to produce $NAD(P)^+$.

The carbon oxidation state

In vivo sources of energy include carbohydrates, organic acids, amino acids, nucleic acid precursors and fatty acids (Ref. 66). Several recent and historic studies have demonstrated that fatty acids are potential in vivo carbon sources for *Mtb* (Ref. 67). Isocitrate lyase (Icl), an enzyme from the glyoxylate cycle, has been shown to have an important role in fatty acid carbon utilisation in vivo (Ref. 68). Fatty acid utilisation has a profound effect on the amount of reducing equivalents produced [e.g. $NAD(P^+)H$]. For example, palmitate and oleate have highly reduced carbon oxidation states (COS values) of −28 and −30, respectively, compared with other fatty acid precursors such as propionate (COS = −1) and valerate (COS = −6) and carbohydrates such as glucose (COS = 0). Subsequent β-oxidation of palmitate generates 106 units of ATP, whereas oxidation of glucose produces only 38 ATP molecules. The β-oxidation of fatty acids yields one NADH and one $FADH_2$ molecule for every acetyl-CoA generated. Clearly, the 'type' of in vivo carbon source (most likely a mixture) has a profound effect on the energetics of the microbial cell, for example the amount of NAD(P)H to be recycled to maintain redox balance.

Redox balance and excretion

During aerobic respiration, the electron donor (e.g. organic substrates such as glucose) undergoes net oxidation whereas the external electron acceptor (e.g. O_2) is reduced to form a balanced oxidation–reduction process. Thus, the oxidation of the substrate is balanced by the reduction of the electron acceptor. *E. coli* regenerates NAD^+ under anaerobic conditions with the excretion of metabolic intermediates such as formate, ethanol and succinate (Ref. 69). By contrast, historical studies have shown that *Mtb* generates alkaline supernatants as opposed to acidic supernatants produced by other bacteria (Ref. 70). The lack of secreted acid intermediates suggests that carbohydrates are completely oxidised by *Mtb* and that unknown mechanisms are responsible for the generation of NAD^+ under hypoxic (dormant) conditions in which the TCA cycle is nonfunctional.

Mtb machinery that maintains intracellular redox homeostasis

Mycothiol

Although mycobacteria contain redox couples such as thioredoxin [$TrxSS/Trx(SH)_2$], $NADH/NAD^+$ and $NADPH/NADP^+$, the conventional redox couple glutathione (GSSG/2GSH) is absent in mycobacteria. Rather, mycobacteria contain oxidised–reduced mycothiol (MSSM/2MSH) in millimolar quantities as the major redox buffer.

Mycothiol is a low-molecular-weight thiol produced by many members of the actinomycetes, including mycobacteria. It functions like glutathione, the archetypal redox buffer, which is not produced by mycobacteria (Ref. 71). The reduction potential of MSSM–2MSH has not yet been determined, and studies are restricted to examining MSSM/2MSH ratios. Oxidised mycothiol is reduced by the FAD-binding mycothione reductase using NADPH as cofactor, which is indicated in equation (11) (Refs 72, 73):

$$NADPH + H^+ + MSSM \rightarrow 2MSH + NADP^+ \quad (11)$$

Another low-molecular-weight thiol produced by mycobacteria is ergothioneine (ERG_{ox}/ERG_{red}; $E^{0\prime} = -60$ mV) (Ref. 74). However, little is known about the role of this uncharacterised thiol in mycobacteria, but it has been shown to protect mammalian cells from oxidative stress (Refs 75, 76).

MSH consists of *myo*-inositol linked to glucosamine, which is in turn ligated to a cysteine residue with an acetylated amino group (Refs 77, 78, 79). There are five steps in MSH biosynthesis; the first is catalysed by a glycosyltransferase encoded by *mshA* (Ref. 80), which fuses 1L-*myo*-inositol-1-phosphate (derived from glucose-6-phosphate) and UDP-*N*-acetylglucosamine (Ref. 81). The resulting *N*-acetylglucosaminylinositol phosphate [1-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-D-*myo*-inositol 3-phosphate] is then dephosphorylated by MshA2 (its gene has not yet been identified) (Ref. 81) and deacetylated by MshB (Ref. 82). This glucosaminylinositol [1-*O*-(2-amino-1-deoxy-α-D-glucopyranosyl)-D-*myo*-inositol] is then ligated to the cysteine carboxyl group through MshC in an ATP-dependent reaction (Ref. 83). Finally, an acetyl group is added to the

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amino group of cysteine by an acetyltransferase encoded by *mshD* (Ref. 84). Published data strongly suggest that MSH has a pivotal role in maintaining the redox balance of mycobacterial cells. Evidence for this includes the sensitivity of various MSH mutants to oxidative stress caused by H_2O_2, cumene hydroperoxide and $O_2^{\bullet-}$ (Refs 85, 86, 87, 88). Inactivation of *mshA1* in *Mtb* and *Mycobacteruim smegmatis (Msm)* results in loss of the production of mycothiol and its intermediates (Ref. 89). *Msm* and *Mtb* mutants of *mshB* accumulated the MshB substrate *N*-acetylglucosaminylinositol and were capable of producing low levels of MSH, which is probably due to the presence of an unidentified enzyme with overlapping function (Refs 86, 90). By contrast, in *Msm* mutants, the loss of MshC activity completely blocks the production of MSH and causes increased levels of glucosaminylinositol (Ref. 87). *Mtb* mutants lacking *mshC* are not viable (Ref. 86), suggesting that mycothiol is necessary for *Mtb* survival; however, MSH-deficient *mshA1* mutants have been recovered in various *Mtb* strains (Ref. 89). *mshD* mutants of *Mtb* and *Msm* produce low levels of MSH and high levels of its immediate precursor, as well as two novel thiols (Refs 85, 91). As stated previously, increased sensitivity to oxidative stress is a common characteristic of the mycothiol mutants. *Msm* mutants independently disrupted in the four known mycothiol synthesis genes and the *Mtb mshD* mutant are more sensitive to H_2O_2 compared with the wild type (Refs 85, 88, 92). Additionally, increased sensitivity to cumene hydroperoxide was demonstrated for the *Mtb mshD* mutant (Ref. 86), whereas the *Msm* mycothiol mutants, compared with *Mtb*, are less resistant than the wild type to plumbagin, a superoxide generator (Refs 87, 88). Many of the mycothiol mutants are also more resistant to the prodrugs INH and ETA (Refs 80, 88, 90, 92).

Thioredoxin (Trx)

Trx is a small redox protein with two redox-active Cys residues in its active site. It is a superior thiol reductant that binds proteins and reduces disulfide bonds by a thiol-exchange reaction through the two Cys residues to generate a disulfide or dithiol. This results in oxidised Trx [equation (10)], which is then reduced by the FAD-containing enzyme Trx reductase (TrxR) that extracts electrons from NADPH (Ref. 93) [equation (13)]:

$$\text{Trx-(SH)}_2 + \text{protein} \rightarrow \text{Trx-S}_2 + \text{protein-(SH)}_2 \quad (12)$$

$$\text{NADPH} + \text{H}^+ + \text{Trx-S}_2 \xrightarrow{\text{TrxR}} \text{NADP}^+ + \text{Trx-(SH)}_2 \quad (13)$$

NADPH, TrxR and Trx comprise the thioredoxin system that is universally conserved. Trx is responsible for maintaining a reducing intracellular environment, regenerating the reduced forms of methionine sulfoxide reductase and peroxiredoxins, the redox regulation of enzymes and regulatory proteins by oxidoreduction and the detoxification of ROS (Refs 94, 95, 96). *Mtb* contains three types of Trx proteins, TrxA, TrxB and TrxC, with mid-point redox potentials of −248, −262 and −269 mV, respectively, along with one TrxR (Ref. 94). Trx and TrxR have also been shown to reduce H_2O_2 and dinitrobenzenes (Ref. 97).

A particularly interesting function of *E. coli* Trx, and probably also of *Mtb* Trx, is the reduction of a unique disulfide bond in ribonucleotide reductase (RNR), which allows RNR to reduce ribonucleotides to deoxyribonucleotides that feed into subsequent reactions (Ref. 98). Intriguingly, several *E. coli* thioredoxin and glutaredoxin double mutants were shown to be nonviable under aerobic conditions, but were rescued by DTT (a thiol-specific reductant) or anaerobiosis (Ref. 99).

The *Mtb sigH* (σ^H) mutant was found to be more susceptible to disulfide stress generated by diamide (a thiol-specific oxidant) and plumbagin (Refs 100, 101), suggesting that σ^H has a central role in protection against oxidative stress. The *Mtb* σ^H mutant was found to be attenuated for virulence in mice (Ref. 102), and microarray analysis has shown that σ^H regulates *trxB*, *trx1* and *thiX* (a hypothetical *trx*) expression. *Mtb sigH* exists in an operon with an antisigma factor *rshA* (Ref. 103). Dissociation of the σ^H and RshA interaction by oxidation allows expression of the *trx* and *trxR* genes and the σ^H operon (Ref. 103).

The Dsb disulfide oxidoreductases

Disulfide bond formation is a two-electron oxidation event in which two Cys residues (2RSH) are covalently bonded (RS–SR). Two protons and two electrons are released during

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this process, as shown below:

$$RSH + RSH \rightarrow RS\text{-}SR + 2H^+ + 2e^- \qquad (14)$$

Disulfide bond formation inside a cell is a rapid process, whereas in vitro conditions might require hours or days for the reaction to proceed. Dsb proteins are thioredoxin-like proteins that promote rapid disulfide formation and folding of periplasmic or secreted proteins. Although many Dsb proteins have been characterised, most in *E. coli*, only three Dsb homologues, DsbE (Ref. 104), DsbF (Ref. 105) and the transmembrane protein DsbD (Ref. 104), are present in *Mtb*.

Catalase peroxidase

Catalase peroxidases (Kat) are enzyme systems that efficiently protect the bacterium from ROS damage (Refs 106, 107, 108) and are used to detoxify H_2O_2. *Mtb* harbours one catalase, KatG (Ref. 109), that shows catalase, peroxidase and peroxinitritase activity. KatG has been demonstrated to be a virulence factor (Ref. 110) that mediates resistance against the prodrug INH. Additionally, clinical *Mtb* strains resistant or sensitive to INH that were exposed to the drug show higher levels of AhpC (alkyl hydroperoxide reductases) (Ref. 111), a member of the peroxiredoxin family.

Alkyl hydroperoxide reductases

Reaction of peroxides with cellular components such as lipids could lead to the generation of highly reactive alkyl hydroperoxides. Mycobacteria use a nonhaem peroxiredoxin called alkyl hydroperoxidase (AhpC) to detoxify by reduction such organic peroxides into less reactive alcohol derivatives (Ref. 112). Peroxiredoxins typically use two redox-active Cys residues to reduce their substrates; however, mycobacterial AhpC contains three Cys residues that are directly involved in this catalysis. AhpC was demonstrated to confer protection against both oxidative and nitrosative stress (Ref. 113). *Mtb* Trx and TrxR are not capable of reducing AhpC (Ref. 97); AhpD, which is reduced by dihydrolipoamide and dihydrolipoamide dehydrogenase (Lpd) (Ref. 114), is needed for the physiological reduction of AhpC. AhpC is linked to dihydrolipoamide dehydrogenase (Lpd) and dihydrolipoamide succinyltransferase (SucB) through AhpD, which acts as an adapter protein (Ref. 114). Lpd is a component of three major enzymatic complexes: the pyruvate dehydrogenase complex, the branched amino acid dehydrogenase complex and the peroxynitrate reductase complex. Thus, the peroxidase activity is uniquely linked to the metabolic state of *Mtb*. More recently, another peroxiredoxin system, thioredoxin reductase (TPx), was shown to be highly effective in protecting *Mtb* against oxidative and nitrosative stress (Refs 95, 115). The TPx system depends on TrxR, TrxB and TrxC for its activity and was recently shown to be involved in virulence (Ref. 116).

Superoxide dismutases

SODs are metalloproteins produced by prokaryotes and eukaryotes to detoxify superoxide radicals. They catalyse the dismutation of $O_2^{\bullet -}$ into H_2O_2 and molecular oxygen. *Mtb* contains two SODs, an iron-containing SOD called SodA or FeSOD (Ref. 117) and a Cu- and Zn-containing SOD called SodC or CuZnSOD (Ref. 118). SodA is constitutively expressed under normal conditions and is demonstrated to be a major secretory protein of *Mtb* that lacks a clearly defined signal peptide sequence (Refs 117, 118). Its expression is enhanced by H_2O_2 exposure and on nutrient starvation (Ref. 119). An antisense approach has successfully been used to show that SodA protects *Mtb* against superoxide in vitro (Ref. 120), whereas another study has demonstrated the role of *Mtb*SodC in protection against ROS (Ref. 121).

Methionine sulfoxide reductases

MSRs use NADPH, Trx and TrxR as the system to reduce methionine sulfoxide to methionine and to protect bacteria against ROS and RNS (Ref. 122). Usually bacteria contain two MSRs, one active on both free and peptidyl methionine-(*S*)-sulfoxide, and one or more MSRs active on peptidyl, but not free, methionine-(*R*)-sulfoxide (Ref. 123). Few studies on MSRs in *Mtb* have been reported. However, recent studies have shown that *Mtb* produces two MSRs, MsrA and MsrB, which are both required for protection against ROS and RNS (Ref. 124).

Truncated haemoglobins

Truncated haemoglobins (trHbs) are small haem-binding globin proteins related to, but smaller than, haemoglobin and myoglobin (Refs 125,

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126). trHbs are traditionally divided into three classes based on their sequence similarity: group I (trHbN), group II (trHbO) and group III (trHbP). trHbs differ significantly in their sequences and could be substantially different in function, ranging from transport or storage of oxygen to detoxification of ROS and RNS. *Mtb* has two trHbs: trHbN and trHbO. trHbO has high affinity for O_2 because of a high association constant and a low dissociation constant. trHbO can also react with H_2O_2 and NO, suggesting a role in detoxification of these two compounds (Ref. 127). trHbN was also shown to have potent NO oxidising activity (Ref. 128).

The environment of the lung

The human lung is the primary organ involved in uptake of atmospheric O_2 and is therefore naturally susceptible to oxidative damage because of its function. ROS production in the lung is further enhanced on exposure to exogenous oxidants such as tobacco smoke, diesel exhaust, ozone and nitrogen oxides. Antioxidants of the lungs include GSH, ascorbate, β-carotene, albumin-SH, mucus, uric acid, SODs, catalases and peroxidases (Ref. 129).The high concentration of GSH in the extracellular lining fluid (>400 μM) suggests that glutathione is a vital component of the defence mechanism against oxidant damage (Ref. 130). Not surprisingly, cigarette smoke was shown to significantly affect Cys/CySS and GSSG/2GSH ratios, suggesting an imbalance in thiol homeostasis (Ref. 130).

Hypoxia in the lung

It is widely accepted that oxygen depletion facilitates entry of *Mtb* into the nonreplicating persistent state. Within the lung, regional differences exist in ventilation and perfusion, and in the degree of blood oxygenation. In a seminal study using resected lung tissue, lesions classified as 'open' (oxygen rich) were found to contain actively growing, predominantly drug-resistant bacteria, whereas bacilli isolated from 'closed' (oxygen poor) lesions showed delayed growth and were drug sensitive (Ref. 131). This observation suggests that *Mtb* drug resistance could be due to the physiological heterogeneity of the bacilli caused by regional differences in O_2 levels. In agreement with this is the recent evidence suggesting that granulomas can be caseous, non-necrotising or fibrotic, sometimes within the same individual (Ref. 55), which is also supported by in vitro studies demonstrating that anaerobically exposed *Mtb* is a poor target for antimycobacterial drugs (Ref. 132).

High O_2 tension exists in the upper lung, whereas the ventral lung experiences low O_2 tension (Refs 133, 134). Consistent with anatomy and function, the partial O_2 pressure (pO_2) of atmospheric O_2 (~150–160 mmHg) drops in the lungs (60–150 mmHg), spleen (~16 mmHg demonstrated in rats) and thymus (10 mmHg) (Refs 53, 57, 135, 136). The diffusion distance of O_2 is ~100–200 μm, resulting in a pO_2 of zero within this distance from blood vessels. Using redox-active dyes that are reduced at pO_2 lower than 10 mmHg, studies in guinea pigs and monkeys have shown that the granulomas are indeed hypoxic (Ref. 53). Notably, *Mtb* is an obligate aerobe but has evolved as yet undefined mechanisms to survive within this hypoxic and perhaps anaerobic environment. Furthermore, O_2 is a highly diffusible gas, and despite the remarkable difference in pO_2 pressure between the granuloma (1.59 mmHg) and adjacent tissue (Refs 57, 53), it is not yet known how this pressure differential is maintained (Fig. 2).

The *Mtb* diet in the lung

The precise mechanism of nutrient acquisition by which *Mtb* senses nutrient availability and adjusts its metabolism in response to different carbon sources in vivo has not yet been elucidated. An additional level of complexity is added by the fact that nutrient availability and utilisation might change over the course of infection: for example, intracellular bacilli versus the late stages of infection where tissue has undergone caseation and liquefaction. Nonetheless, several studies suggest that host fatty acids might serve as the carbon and energy source in vivo (Refs 137, 138). The identification and current studies on Icl (Ref. 68), the fatty acid regulator KstR (Ref. 139) and an intracellular redox sensor, WhiB3, which controls the switchover from glucose to fatty acids (Refs 10, 140, 141), should give important information on how a persistent infection is established using fatty acids as a source of carbon.

The amount of fatty acids and lipids available for *Mtb* growth in vivo (g/l) considerably exceeds that of obtainable carbohydrates (Ref. 66). Lipids can be oxidised by β-oxidation to yield valuable ATP and

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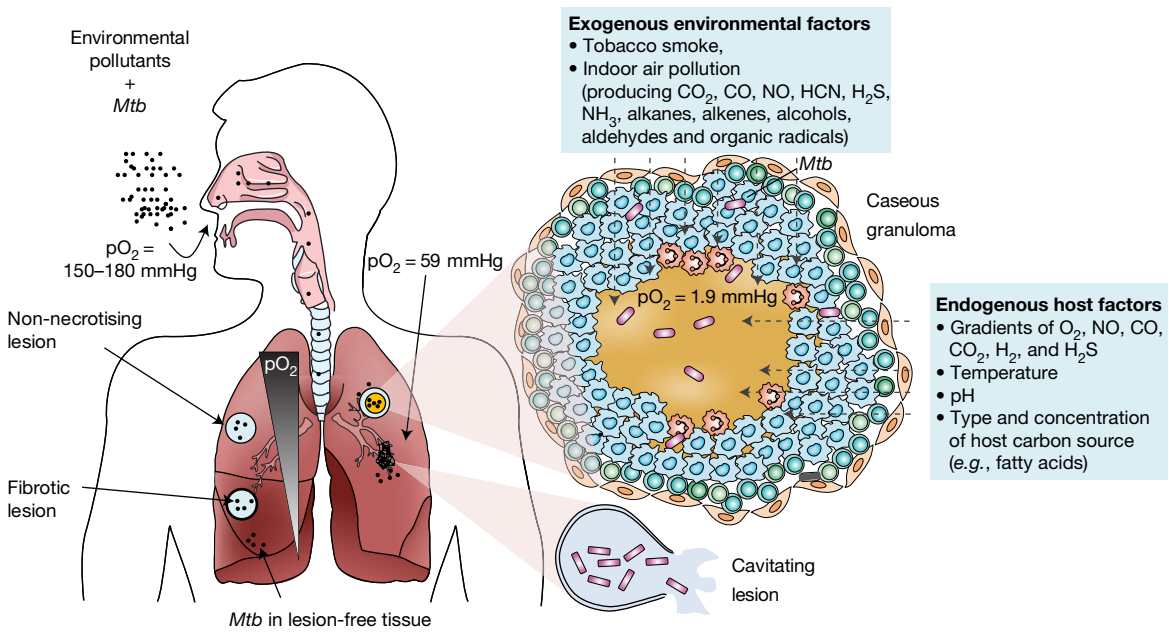

Effect of exogenous environmental and endogenous host redox factors on the pathogenesis of TB

Figure 2. Effect of exogenous environmental and endogenous host redox factors on the pathogenesis of TB. Infectious *Mycobacterium tuberculosis* (*Mtb*) bacilli are inhaled as aerosols from the atmosphere and phagocytosed by alveolar macrophages in the lung. A localised proinflammatory immune response causes the recruitment of mononuclear cells, leading to the establishment of a granuloma. However, *Mtb* cells are also present in lesion-free tissue. During the course of infection, caseous (typically hypoxic), fibrotic and non-necrotic granulomas can develop. The containment of *Mtb* by these granulomas never operates in isolation, and can fail as a consequence of malnutrition, diabetes, indoor air pollution, tobacco smoke and HIV infection, which are major risk factors for TB. Thus, any condition that weakens the immune status (in particular, a decrease in the function of $CD4^+$ T cells) of the host can lead to TB. Exogenous environmental pollutants, which consist largely of redox-active molecules, not only affect the host immune response, but also target the infecting bacilli. Exposure to these environmental agents, production of host redox molecules such as $O_2^{\bullet -}$, NO, $ONOO^-$, etc. that are generated during the oxidative burst, and the pathological and physiological host responses induced on infection (e.g. hypoxic granuloma, dysregulated host lipid production) can collectively cause an imbalance in *Mtb* redox homeostasis, leading to oxidative stress or damage. Conversely, exogenous factors and the dysregulation of endogenous host redox factors might lead to the establishment of *Mtb* infection, maintaining a persistent state or allowing the bacillus to emerge from persistence. Dormant *Mtb* cells residing inside hypoxic granulomas are resistant to current antimycobacterial drugs and therefore have substantial implications on therapeutic intervention strategies. Moreover, the dynamic physiology and structure of the lung further complicate the situation because no two regions inside the lungs are similar in terms of their architecture and oxygen tension. This also makes it extremely difficult to study the progression of TB using animal models. Inside the lung, *Mtb* cells are exposed during transmission to a range of oxygen levels that varies from 150 to 180 mmHg in the upper respiratory tract to 1.9 mmHg within the granuloma, compared with pO_2 levels of healthy lungs (~59 mmHg). In addition, host pH and the type of in vivo carbon source, along with its concentration, will also have an impact on *Mtb* redox homeostasis. Nonetheless, it is still not clear how exposure of *Mtb* to these exogenous and endogenous redox molecules affects *Mtb* physiology and redox homeostasis in vivo to favour disease.

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acetyl-CoA. Because the COS of fatty acids is highly reduced, substantial quantities of reducing equivalents [NAD(P)H] will be generated during β-oxidation, which must be dissipated to maintain intracellular equilibrium. This begs the question: does *Mtb* experience 'reductive stress' (Ref. 54) during its in vivo growth, and if so, how is that stress dissipated? The serendipitous discovery that *Mtb* $NAD(P)^+$/NAD(P)H present in infected lungs of mice predominantly exists in the reduced [NAD(P)H] state (Ref. 63) provides strong evidence that *Mtb* experiences substantial reductive stress during infection (for a review, see Ref. 54). Furthermore, recent findings have suggested a role for *Mtb* WhiB3 in the modulation and dissipation of reductive stress (Ref. 140), which implicates host fatty acids as a source of reductive stress, and perhaps a signal acting synergistically with hypoxia, NO and CO to modify the course of infection. Recently, *Mtb* TAG, which is under control of the Dos dormancy regulon and WhiB3, was suggested to be a source of carbon and energy when the bacilli emerge from a latent state (Ref. 64). Finally, studies have shown that cholesterol can also be used as a carbon source during infection (Ref. 142).

Mtb redox-sensing mechanisms: model paradigms

Although *Mtb* contains 11 paired two-component signalling systems and ~180 regulatory proteins, only a few proteins have been shown to directly react with NO, CO or O_2, and the downstream effects of these interactions are mostly unexplored. Nonetheless, in recent years the DosR–S/T two-component haem sensor system and the intracellular WhiB Fe–S cluster family of proteins, particularly WhiB3 (Refs 9, 10), have emerged as model signalling pathways that specifically respond to these gases.

The DosR/S/T dormancy regulon

The DosR/S/T (Dos) dormancy system [first reported as the DevR/S system (Ref. 143)] is a 'three-component' system capable of integrating two haem histidine kinase sensors (DosS and DosT) with a single response regulator, DosR. The Dos system has been implicated in virulence and is probably the most characterised system in *Mtb*. An identical overlap exists between the gene expression profiles of *Mtb* cells treated with NO or CO, and when cultured under low O_2 conditions (Wayne model for in vitro dormancy) (Refs 9, 144, 145, 146). The Dos regulon comprises ~47 genes thought to have crucial roles in the metabolic shift of *Mtb* to the persistent state (Refs 60, 147). Several of these genes are speculated to have a role in adaptation to hypoxic stress, such as *acr* (rv2031c; chaperone function), *narX* (rv1736c; unknown function), *nark2* (rv1737c; nitrate/nitrite transport), *fdxA* (rv2007c; ferredoxin), *nrdZ* (rv0570; ribonuclease reductase), *tgs1* (rv3130; triglyceride synthase) and *Mtb* orthologues of the universal stress protein family (rv1996, rv2005c, rv2028c, rv2623, rv2624c, rv3134c) (Ref. 148).

A key finding was the discovery that DosS and DosT are haem proteins that can be oxidised by O_2 or can directly bind NO and/or O_2 through their haem irons (Refs 9, 149, 150). The discovery that CO directly binds the haem irons of DosS and DosT, induces the Dos dormancy regulon (Refs 144, 146), and is produced at the site of *Mtb* infection has profound implications for the importance of CO generated by host haem oxygenase I (HO-1) in *Mtb* pathogenesis. A role for environmental CO in TB was described as early as 1923 (Ref. 151) and has been recently discussed in more detail (Ref. 152). The induction of the identical genetic expression profile in response to three diatomic gases (O_2, NO and CO) is an unparalleled finding in bacteriology, and suggests that *Mtb* has evolved an exquisite sensory system to allow the bacilli to continuously monitor and counter the effects of host NO, CO and O_2 levels during the course of infection (Fig. 3).

The survival of *Mtb* under hypoxic conditions depends on many factors in general and oxidative phosphorylation in particular. By contrast, it was shown that the NAD^+/NADH ratio in the hypoxic Wayne model remained comparable to aerobic cultured cells (Ref. 60). This is an unusual finding because this ratio in bacteria typically decreases with a diminished O_2 concentration. As expected, ATP levels decrease under hypoxia (Ref. 60), but are then maintained at a constant low level. Any further reduction in the ATP levels led to rapid death of *Mtb* (Ref. 61).

Although the clinical role and significance of the Dos regulon in human TB is yet to be established, an indication of its clinical relevance emerges by its ~50-fold overexpression in *Mtb* Beijing (W2) clinical strains (Ref. 153) and the

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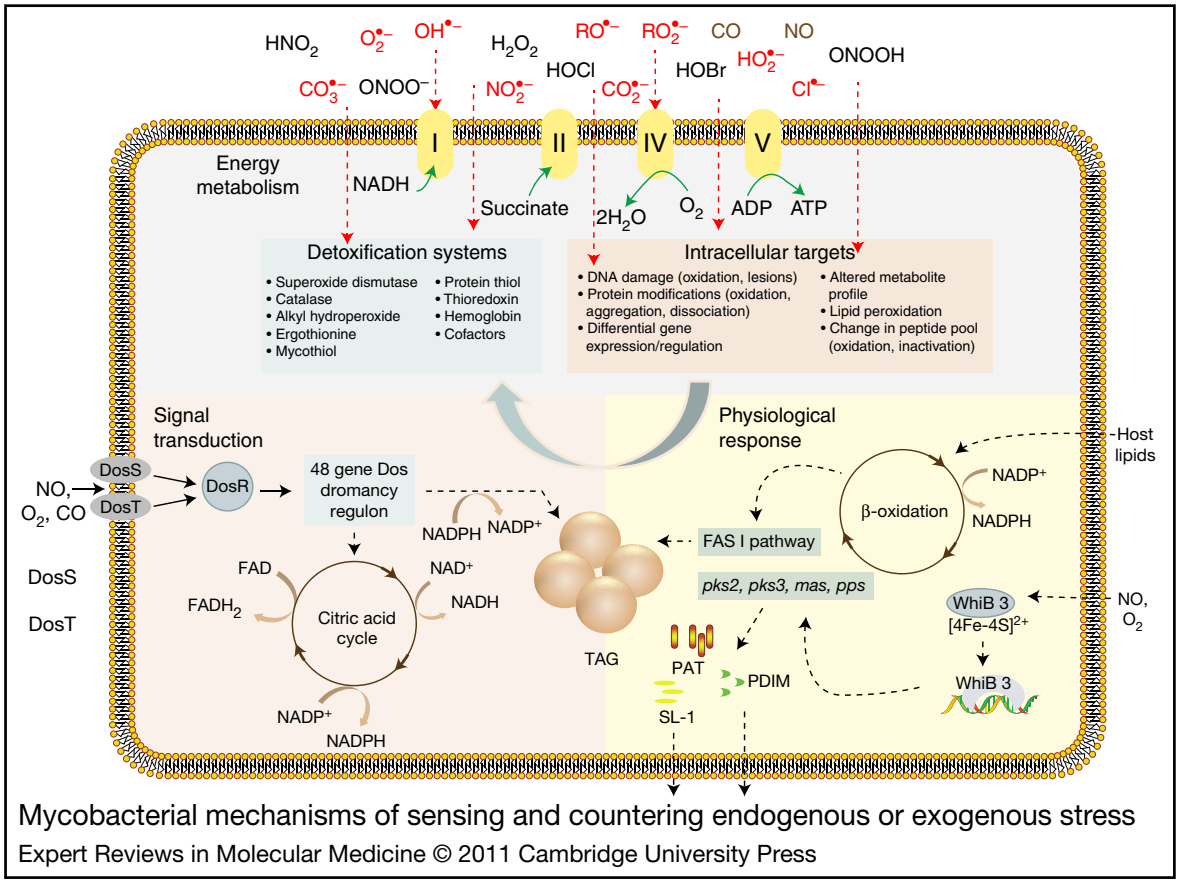

Mycobacterial mechanisms of sensing and countering endogenous or exogenous stress

Figure 3. Mycobacterial mechanisms of sensing and countering endogenous or exogenous stress. The host generates free radicals, non-free-radical molecules and numerous gases as a mechanism to counter *Mtb* infection. These molecules target mycobacterial DNA, proteins and lipids, may alter *Mtb* gene expression, and change the overall metabolic profile or peptide pool. Free radicals can react with prosthetic groups such as Fe–S clusters or haem groups of the respiratory complexes. *Mtb* responds to these free radical stresses by adjusting its energy metabolism, physiological response and signal transduction cascade. Most of the radical-mediated damage is countered by detoxification processes comprising (a) enzymes such as catalase, superoxide dismutase and alkyl hydroperoxide, (b) redox buffering systems (thioredoxins, mycothiol, ergothioneine and protein thiols) and (c) truncated haemoglobins and cofactors (NAD^+, FAD^+ and coenzyme A). *Mtb* also possesses sensing mechanisms to detect environmental gases such as gradients of O_2, NO and alterations in its intracellular redox state to allow its survival. Well-studied examples are the Dos dormancy regulon and the WhiB3 redox sensor. The Dos regulon senses O_2, NO and CO through the DosS and DosT haem proteins. The signal is relayed to DosR, which leads to the induction of the 48-member Dos dormancy regulon that includes genes involved in energy production, dissipating reducing equivalents and assimilation of storage lipids, which is thought to facilitate mycobacterial persistence. WhiB3 functions as a regulator of cellular metabolism, which responds to O_2 and NO through its Fe–S cluster and integrates it with intermediary metabolic pathways. WhiB3 is an intracellular redox regulator that dissipates reductive stress generated by utilisation of host fatty acids through β-oxidation. Through the transcriptional activation of genes involved in lipid anabolism, WhiB3 is thought to direct reducing equivalents into the production of cell wall components and virulence lipids such as sulfolipids, phthiocerol dimycocerosates, polyacyltrehaloses and DAT. Under certain conditions, WhiB3 regulates the production and accumulation of triacylglycerol, indicating a link with the Dos dormancy signalling pathway.

strong immune responsiveness of latently infected patients to the DosR regulon antigens (Refs 154, 155). In addition, the Dos regulon genes have been shown to be upregulated in sputum (Ref. 156) and in adipose tissue (Ref. 157) of *Mtb*-infected individuals.

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WhiB3 and redox homeostasis

It is known that *Mtb* survives a constant threat of redox stress either as a consequence of its aerobic metabolism or as infliction by the host to prevent the establishment of a successful infection. The nondividing persistent state of *Mtb* is attributable mainly to hypoxia, wherein mycobacteria adapt to low oxygen pressures by transcriptional regulatory networks that function to maintain redox homeostasis. Identification of such mechanisms allowing mycobacteria to counter oxidoreductive stress during infection, latency and reactivation is central to the development of effective intervention strategies.

The WhiB-like proteins are found in actinomycetes, and virtually all members of this family contain four conserved Cys residues that coordinate the Fe–S cluster. WhiB orthologues have been implicated in sporulation in *Streptomyces coelicolor* (Ref. 158), in pathogenesis and cell division in mycobacteria (Refs 140, 141, 159, 160), in oxidative stress in *Corynebacterium glutamicum* (Ref. 161), and in antibiotic resistance in mycobacteria and streptomyces (Ref. 162). However, the mechanistic basis for how these WhiB homologues sense and respond to endogenous and exogenous signals to exert their effect is not known. A comprehensive study examining the expression profiles of all seven *Mtb whiB* genes (*whiB1–whiB7*) after exposure to antibiotic and in vitro stress conditions provides insight into the biological function of the WhiB family (Ref. 163).

Mtb WhiB3, a homologue of a putative sporulation transcription factor in *Streptomyces*, has a role in virulence in mice and guinea pigs (Ref. 141), and was shown to contain a (4Fe–4S) cluster that directly associates with NO and is degraded by O_2 (Ref. 10). It was also proposed that *Mtb* WhiB3 senses changes in the intracellular redox environment associated with O_2 depletion and the metabolic switchover to the preferred in vivo carbon source, fatty acids (Ref. 10). Several lines of evidence (Refs 10, 140) suggest that WhiB3 is involved in maintaining redox homeostasis through its 4Fe–4S cluster by regulating catabolic metabolism and polyketide biosynthesis in *Mtb*. This has important implications for understanding how *Mtb* persists within the host, because it is widely accepted that fatty acids serve as a major source of carbon and energy in chronic infection. It was also shown that WhiB3 induces a metabolic shift that differentially modulates the assimilation of propionate into the complex virulence polyketides polyacyltrehaloses, sulfolipids, phthiocerol dimycocerosates and the storage lipid TAG in defined oxidising and reducing environments (Ref. 140) (Fig. 3). What seems to be emerging is a link between *Mtb* virulence lipid production and the response to oxidoreductive stress (Ref. 10). Because TAG production, which is under conditional WhiB3 control, is also induced on exposure to NO, CO and hypoxia through the Dos dormancy system (Refs 147, 164, 165), these data establish a novel link between an intracellular (WhiB3) and extracellular (Dos) signalling pathway.

Future challenges and conclusions

Redox reactions in the microbial cell have key roles in intracellular and extracellular signalling, DNA, RNA and protein synthesis, energy production and metabolic homeostasis. However, to date, we lack knowledge on the intracellular *Mtb* redox environment, the identity of all main redox couples and buffers, the behaviour of these redox couples under different environmental conditions, and the mechanisms of sustained redox homeostasis in *Mtb*. In particular, a fundamental challenge in the oxidative stress biology of *Mtb* is to understand how carbonyl, nitrosative and oxidative stress modulate *Mtb* pathogenesis. Using genome-wide tools, it is important to refine our understanding of the *Mtb* 'redoxome'. It should be possible to generate numerical indicators of the intracellular *Mtb* redox environment, the redox state of each redox pair, and determine how these indicators change on exposure to various environmental signals, particularly NO, O_2 and CO. Noninvasive technology such as the redox-sensitive green fluorescent protein (Ref. 166) can serve as a novel tool to explore global intracellular redox status and should be exploited to examine these changes in *Mtb* during infection. An important issue is the identification of the major redox couples and buffers in *Mtb*, and to ascertain their roles in pathogenesis and drug resistance. Is MSSM/2MSH the major redox buffer in *Mtb*? What is the function of ERG_{ox}/ERG_{red} in redox homeostasis? Presently, the ERG_{ox}/ERG_{red} redox couple is an understudied system, but it might have important implications for maintaining redox homeostasis and in disease progression.

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Another particularly interesting and unexplored area is the link between redox homeostasis and drug efficacy. For example, the identity of redox couples that participate in the bioreductive activation of antimycobacterial prodrugs (e.g. INH, ETH and PA-824) and an understanding of the underlying mechanisms involved will have a major impact on drug development strategies. Although some progress has been made in this field (Ref. 7), our knowledge remains sparse. Proper treatment of latent *Mtb* infection requires a more precise understanding of the true physiological status of *Mtb* within the microenvironment of the host, for example the granuloma. Lase-capture microdissection combined with mass spectroscopy and RNA amplification strategies could be exploited to quantitatively catalogue host and bacterial proteins, lipids and metabolites within granulomas. This would help to define what a true dormant bacillus is, and how we differentiate 'dead' from 'live' dormant bacilli. Recently, progress has been made in this regard, and stochastic *Mtb* phenotypes have been identified as a possible mycobacterial strategy to rapidly adjust to changing in vivo conditions (Ref. 167).

A fundamentally important subject to be addressed is the extent of *Mtb* respiration within a hypoxic granuloma. Do fully anaerobic granulomas exist? Identification of the terminal electron acceptors used, and determination of the mechanisms of $NAD(P)^+$ regeneration used under hypoxic (and perhaps anaerobic) conditions are crucial to understanding TB pathogenesis. An attractive hypothesis is that *Mtb* resides within a spectrum of aerobic, hypoxic and anaerobic microenvironments in the lungs (Ref. 55), which in theory can explain the capacity of dormant bacilli to survive chemotherapy. Other important areas to study include the mechanisms of how pO_2 levels are maintained in these microenvironments and the independent or combined roles of NO, CO and O_2 in *Mtb* persistence.

Although it is likely that the preferred in vivo carbon source for *Mtb* includes fatty acids or cholesterol, conclusive experimental evidence in vivo is still lacking. The use of labelled fatty acids in in vivo studies should allow us to identify metabolic pathways that are specifically geared towards in vivo growth and survival. Similar studies will also shed light on *Mtb* reductive stress (Ref. 54) in vivo, and whether it impacts the course of human TB. Furthermore, the role of H_2 as an energy source has been reported for other infectious agents (Ref. 168), but is an unexplored area for *Mtb* pathogenesis. Interestingly, because the oxidation of H_2 generates protons, some bacteria use it to dispose of excess reducing equivalents (Ref. 169). Study of the impact of complex host risk factors for TB such as tobacco smoke, indoor air pollution, malnutrition and diabetes on the bacilli by exploiting metabolomics, proteomics and microarray analyses will have broad public health and socioeconomic implications.

In conclusion, a fundamental challenge faced by investigators is the translation of their combined research findings into novel in vitro and in vivo experimental tools and ultimately into successful TB intervention and control strategies. This will dictate the success of ongoing and future efforts to combat the unrelenting threat of TB.

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Further reading

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Further reading *(continued)*

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Singh, A. et al. (2007) *Mycobacterium tuberculosis* WhiB3 responds to O_2 and nitric oxide via its [4Fe-4S] cluster and is essential for nutrient starvation survival. Proceedings of the National Academy of Sciences of the United States of America 104, 11562-11567

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Features associated with this article

Figures

Figure 1. Virulence life cycle of *Mycobacterium tuberculosis* and progression of TB.
Figure 2. Effect of exogenous environmental and endogenous host redox factors on the pathogenesis of TB.
Figure 3. Mycobacterial mechanisms of sensing and countering endogenous or exogenous stress.

Table

Table 1. Standard reduction potentials of biologically relevant redox couples.

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