The cytochrome bd-type quinol oxidase is important for survival of Mycobacterium smegmatis under peroxide and antibiotic-induced stress

Ping Lu1,*, Marieke H. Heineke1,*, Anil Koul2, Koen Andries3, Gregory M. Cook3, Holger Lill1, Rob van Spanning1 & Dirk Bald1

Targeting respiration and ATP synthesis has received strong interest as a new strategy for combating drug-resistant Mycobacterium tuberculosis. Mycobacteria employ a respiratory chain terminating with two branches. One of the branches includes a cytochrome bc1 complex and an aa3-type cytochrome c oxidase while the other branch terminates with a cytochrome bd-type quinol oxidase. In this communication we show that genetic inactivation of cytochrome bd, but not of cytochrome bc1, enhances the susceptibility of Mycobacterium smegmatis to hydrogen peroxide and antibiotic-induced stress. The type-II NADH dehydrogenase effector clofazimine and the ATP synthase inhibitor bedaquiline were bacteriostatic against wild-type M. smegmatis, but strongly bactericidal against a cytochrome bd mutant. We also demonstrated that the quinone-analog aurachin D inhibited mycobacterial cytochrome bd at sub-micromolar concentrations. Our results identify cytochrome bd as a key survival factor in M. smegmatis during antibiotic stress. Targeting the cytochrome bd respiratory branch therefore appears to be a promising strategy that may enhance the bactericidal activity of existing tuberculosis drugs.

Mycobacterium tuberculosis is the causative agent of tuberculosis disease (TB). In 2013 there were 1.5 million TB-related deaths worldwide and 9 million people were newly infected with TB1. Despite the introduction of efficient antibiotics in the 1950s, TB treatment remains challenging, largely due to the emergence of drug-resistant strains2,3. Additionally, its metabolic flexibility allows the pathogen to exist in different states, ranging from actively replicating to dormant persisting4,5. The dormant population is difficult to eradicate and has the potential to cause active tuberculosis after resuscitation, which is especially threatening for immune-compromised patients suffering from HIV6. Therefore, drugs with novel mechanisms of action are urgently needed to adequately kill the heterogeneous population of bacteria and to counter multi-drug resistant (MDR) and extensively-drug resistant (XDR) tuberculosis strains. Since basal energy requirements and redox balance are essential for both replicating and persisting bacteria, components of the oxidative phosphorylation pathway are regarded as promising drug targets7–11.

1Department of Molecular Cell Biology, Amsterdam Institute for Molecules, Medicines and Systems, Faculty of Earth- and Life Sciences, VU University Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands. 2Infectious diseases and vaccines therapeutic area, Janssen Research & Development, Johnson & Johnson Pharmaceuticals, Turnhoutseweg 30, 2340-Beerse, Belgium. 3Department of Microbiology and Immunology, Otago School of Medical Sciences, University of Otago, Dunedin 9054, New Zealand. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to D.B. (email: d.bald@vu.nl)
The branched respiratory chain in mycobacteria. Cyd: cytochrome bd-type quinol oxidase subunits, Qcr: cytochrome bc complex subunits, Cta: subunits of aa₃-type cytochrome c oxidase. Note that M. smegmatis does not have a soluble cytochrome c. Instead QcrC is a di-heme cytochrome c, which transfers electrons between the cytochrome bc₁ complex and the aa₃-type cytochrome c oxidase.

The respiratory chain enzyme complexes that are part of the oxidative phosphorylation pathway establish a proton motive force across the bacterial cytoplasmic membrane and ATP synthase utilizes the energy of the proton motive force for synthesis of ATP. Mycobacterial ATP synthase has been validated as target of bedaquiline (BDQ), the lead compound of the diarylquinoline class of drugs, which selectively inhibits this enzyme in a variety of mycobacterial strains. BDQ has received accelerated approval by the US Food & Drug Administration (FDA) and the European Medicines Agency (EMA) for treatment of MDR-TB.17,18 Moreover, components of the respiratory chain such as the type-II NADH dehydrogenase (NDH-2) and the cytochrome bc₁ complex are targeted by small-molecule compounds that are currently in clinical development. Mycobacteria have a branched electron transport chain. Electrons from the menaquinone pool can be passed on either to the cytochrome bc₁ complex, which forms a supercomplex with the cytochrome aa₃ oxidase, or alternatively to the cytochrome bd-type quinol oxidase as an important contributor to stress resistance in (myco-) bacteria. 30–32. In Escherichia coli, exposure to exogenous hydrogen peroxide and nitric oxide induced expression of cytochrome bd and strains lacking cytochrome bd were found hyper-sensitive to peroxide and nitrosative stress as well as to low iron concentrations. In M. tuberculosis, cytochrome bd expression in the mouse lung is upregulated during chronic infection. During an inflammatory reaction, macrophages in the host can produce reactive oxygen species (ROS) to kill engulfed bacteria. Overexpression of cytochrome bd in M. tuberculosis is associated with increased peroxide resistance. Upregulation of cytochrome bd may represent a protection mechanism to survive the host's immune response. This data point towards the importance of each respiratory chain branch in protection against them.

In this study, the role of the two mycobacterial respiratory chain branches in response to stress elicited by peroxides and antimicrobials was investigated. For this aim we challenged strains of M. smegmatis lacking cytochrome bd or the cytochrome bc₁ complex in vitro with these stress factors to elucidate the importance of each respiratory chain branch in protection against them.

Results
Bioenergetic parameters of Mycobacterium smegmatis strains with inactivated respiratory chain branches. The role of two respiratory chain branches in mycobacteria was investigated using
mutant strains impaired in one of the two branches. These strains maintain either only the cytochrome \( \text{bd} \) branch (strain \( \Delta qcrCAB::\text{hyg} \)) or cytochrome \( \text{bc}_1 \) branch (strain \( \Delta cydA::\text{kan} \)) (Fig. 1). The growth rate of the \( \Delta cydA::\text{kan} \) strain was comparable to that of the wild-type, whereas growth of the \( \Delta qcrCAB::\text{hyg} \) strain was substantially lower (Fig. 2A), confirming previous data\textsuperscript{25,27}. We then extended the earlier reported microbiological characterization of the mutant strains and determined central bioenergetic parameters for the two mutants. Cellular ATP levels were unaltered in the \( \Delta cydA::\text{kan} \) mutant as compared with the wild-type, but were decreased by ~40% in the \( \Delta qcrCAB::\text{hyg} \) strain (Fig. 2B).

Similarly, oxygen consumption rates in inverted membrane vesicles isolated from aerobically grown cells were almost unchanged in the \( \Delta cydA::\text{kan} \) mutant versus wild-type, but lower in \( \Delta qcrCAB::\text{hyg} \) (Fig. 2C). These results reflect the higher respiratory efficiency of the cytochrome \( \text{bc}_1/\text{aa}_3 \) branch. Based on growth rate and bioenergetic characterization the cytochrome \( \text{bc}_1/\text{aa}_3 \) branch can be regarded as the more promising target pathway of the two branches.

**Sensitivity for hydrogen peroxide stress.** Next, we investigated the importance of the two respiratory chain branches in response to peroxide stress. Exponentially growing \( M. \text{smegmatis} \) cells were exposed to hydrogen peroxide (20 mM, final conc.) for various time intervals and colony-forming units were enumerated. Incubation with hydrogen peroxide had a bacteriostatic effect on wild-type \( M. \text{smegmatis} \) and for the \( \Delta qcrCAB::\text{hyg} \) mutant a minor decrease in viability was found (Fig. 3). For the \( \Delta cydA::\text{kan} \) mutant, a 99% decline in cell viability was observed after 60 min exposure (Fig. 3).
These results suggest that cytochrome \textit{bd} plays a protective role during oxidative stress in \textit{M. smegmatis}, whereas the cytochrome \textit{bc}1 complex is of minor importance for survival under these conditions.

**Sensitivity for the NDH-2 effector clofazimine.** We hypothesized that mycobacteria with impaired respiratory chain branches may also be more sensitive to antimicrobials that cause production of reactive oxygen species. Clofazimine (CFZ) is a front-line anti-leprosy drug that presently is repurposed for usage against tuberculosis. CFZ is an electron carrier that interferes with the type II NADH dehydrogenase (NDH-2) in mycobacteria\textsuperscript{19}. As such, it can transfer electrons from NDH-2 directly to oxygen, thereby producing ROS\textsuperscript{19}. First, we confirmed that CFZ caused time-dependent development of ROS by inverted membrane vesicles from the \textit{M. smegmatis} wild-type strain used in our laboratory (Supplementary Figure S1). Subsequently we investigated if either cytochrome \textit{bd} or the cytochrome \textit{bc}1 complex can protect \textit{M. smegmatis} against CFZ. For this purpose the bacteria were incubated for 72 hours in liquid culture with varying concentrations of the drug. CFZ was bacteriostatic against the wild-type strain, even at the highest concentration investigated (25x MIC, 7.5μg/mL) (Fig. 4). The \textit{ΔqcrCAB::hyg} mutant showed marginally higher sensitivity for CFZ as compared with the wild-type (Fig. 4). However, the viability
of the ΔcydA::kan mutant was strongly reduced in response to CFZ challenge. CFZ at concentrations >0.3 μg/mL was bacteriostatic for the ΔcydA::kan mutant and concentrations >1.5 μg/mL were bactericidal. With 7.5 μg/mL CFZ the limit of detection was reached after 72 hours of exposure (Fig. 4). These results indicate that cytochrome bd, but not the cytochrome bc1 complex, can protect the bacteria against the bactericidal effect of clofazimine. We hypothesized that the increased sensitivity of the ΔcydA::kan strain was due to ROS production by CFZ. To test this hypothesis we investigated the effect of chlorpromazine (CPZ), a phenothiazine-class drug that inhibits type-II NADH dehydrogenase20,23, but does not produce ROS19, on wild-type and the ΔcydA::kan mutant. As expected, CPZ did not discriminate between wild-type M. smegmatis and the ΔcydA::kan mutant (Supplementary Figure S1).

Sensitivity for the ATP synthase inhibitor bedaquiline. The results described above demonstrate that genetic inactivation of cytochrome bd, but not of the cytochrome bc1 complex, converts the bacteriostatic effect of hydrogen peroxide and of clofazimine into a bactericidal effect. Next, we expanded our experiments to the ATP synthase inhibitor bedaquiline (BDQ). Whereas BDQ is bactericidal against M. tuberculosis, it is bacteriostatic against M. smegmatis42. A transcriptional and proteomic analysis recently revealed that treatment of M. tuberculosis with BDQ triggers strong upregulation of cytochrome bd38 and deletion of cytochrome bd in M. tuberculosis enhanced the bactericidal activity of BDQ39. We therefore investigated if genetic inactivation of one of the respiratory chain branches would convert the bacteriostatic activity of BDQ on M. smegmatis into bactericidal activity.

BDQ was bacteriostatic against wild-type M. smegmatis, even at the highest concentration used (300x MIC, 5μg/mL) (Fig. 5). The ΔqcrCAB::hyg strain was less sensitive to BDQ as compared with the wild-type strain (Fig. 5). However, in case of the ΔcydA::kan mutant, challenge with BDQ (1μg/mL) led to a ~1 log10 reduction in colony forming units and 5μg/mL BDQ caused ~3 log10 kill, approaching the limit of detection after 3 days of treatment (Fig. 5). Cytochrome bd thus protects M. smegmatis against killing by bedaquiline, whereas the cytochrome bc1/aa3 branch does not. We attempted to link the protective function of cytochrome bd to production of ROS in the presence of BDQ, however, inverted membrane vesicles from M. smegmatis did not show increased ROS formation after treatment with BDQ (Supplementary Figure S1).

The results obtained for CFZ and BDQ demonstrate that inactivation of the cytochrome bd branch, but not of the cytochrome bc1/aa3 branch, can convert bacteriostatic activity of an antibacterial drug into bactericidal activity. Our findings identify cytochrome bd as an important survival factor in mycobacterial metabolism.

Inactivation of mycobacterial cytochrome bd by a small-molecule inhibitor. Genetic inactivation of cytochrome bd can considerably increase the potency of two prominent antibacterial drugs, CFZ and BDQ. Based on these findings we tested if small-molecule inhibitors can block the activity of cytochrome bd in M. smegmatis. The aurachin class of quinone analogs has been reported as inhibitors of a variety of quinone-modifying enzyme40-42. Within this class, aurachin D was previously shown to preferentially inhibit E. coli cytochrome bd as compared with other quinone-modifying enzymes42. We investigated the effect of aurachin D on the oxygen consumption activity of inverted membrane vesicles
from *M. smegmatis*. Aurachin D inhibited oxygen consumption in a dose-dependent manner with 50% maximal inhibition for wild-type strain (Fig. 6). Interestingly, this inhibitory effect was clearly stronger in membrane vesicles of the ΔqcrCAB::hyg strain, where ~90% maximal inhibition was reached (IC_{50} ~400 nM) (Fig. 6). This suggests that the main target in mycobacterial oxidative phosphorylation was cytochrome *bd*.

Subsequently, we evaluated the effect of aurachin D on mycobacterial growth. We found that for all three strains tested (wild-type, ΔcydA::kan, ΔqcrCAB::hyg) the minimal inhibitory concentrations (MICs) were >85 μM (data not shown). This result suggests that the inhibitor is not capable of effectively crossing the mycobacterial cell envelope.

**Discussion**

Previously it has been reported that genetic inactivation of cytochrome *bd* considerably decreased virulence or survival in the host of a variety of pathogenic bacterial strains. In *Shigella flexneri*, *Brucella abortus* and *Salmonella enterica Serovar Typhymurium*, the causative agents of bacterial dysentery, brucellosis and typhoid fever, inactivation of cytochrome *bd* considerably impaired intracellular survival and virulence. In *Klebsiella pneumonia* cytochrome *bd* was found crucial for free energy transduction under microaerobic conditions and for protection of anaerobic processes such as nitrogen fixation. In case of group B streptococci, inactivation of cytochrome *bd* led to decreased growth in human blood. Cytochrome *bd* may also allow strictly anaerobic bacteria such as *Bacteroides fragilis* to survive under nanomolar oxygen concentrations, potentially facilitating survival of opportunistic pathogens in the host.

In this study, we evaluated the function of the two mycobacterial respiratory chain branches in response to stress. The cytochrome *bc₁* complex is a validated drug target in *M. tuberculosis*, however, upregulation of cytochrome *bd* may partially compensate for inhibition of cytochrome *bc₁* function. Therefore, it has been postulated that simultaneously targeting both respiratory chain branches with inhibitors might be required to effectively disrupt mycobacterial respiration. Whereas the cytochrome *bd* branch may in part be able to compensate for inactivation of the cytochrome *bc₁* complex, our results indicate that the cytochrome *bc₁/aa₃* branch is not able to compensate for loss of cytochrome *bd* functionality. Inactivation of cytochrome *bd*, although not directly leading to a phenotype, exerts a strong impact on bacterial viability in the presence of antibiotic stress. This highlights the importance of the cytochrome *bd* branch as a survival factor in *M. smegmatis* and suggests that targeting this terminal oxidase may be a successful strategy for weakening the mycobacterial stress response.

The hypersensitivity of the *cydB* mutants to exogenous hydrogen peroxide is not due to impaired growth of the mutant strain, since growth rate and ATP levels are similar to the wild-type. Giuffre, Borisov and colleagues suggested two molecular mechanisms for peroxide protection by cytochrome *bd* in *E.coli*. First, cytochrome *bd* as oxygen scavenger may decrease the intracellular oxygen tension, thereby preventing the formation of reactive oxygen species. Second, cytochrome *bd* displays catalase activity and might thus directly metabolize peroxides. Both mechanisms may contribute to the
protective role of cytochrome bd against hydrogen peroxide stress in *M. smegmatis* and their respective importance in mycobacteria needs to be further elucidated.

Our experiments revealed that cytochrome bd plays an important role in protection against two prominent anti-tuberculosis drugs, both targeting oxidative phosphorylation. Protection against clofazimine, a ROS-producing drug, is most likely due to the ability of cytochrome bd to metabolize and/or prevent formation of peroxides. Our data do not allow for pinpointing the mechanism of protection against BDQ. Inhibition of ATP synthase may well result in reduction of the electron flow through the respiratory system. As a result, the reduction state of the respiratory complexes increases which in turn leads to increased production of ROS. Higher cellular NADH/NAD⁺ ratios and enhanced expression of bacteriostatic activity of bedaquiline is not restricted to *M. smegmatis*, but also found for *M. avium* ΔcydA deletion mutants in these pathogenic bacteria. It would be important to assess if cytochrome bd deletion mutants in these pathogenic bacteria display increased sensitivity to (ROS-producing) antibacterials as well.

Inactivation of cytochrome bd converts the bacteriostatic activity of clofazimine and bedaquiline against *M. smegmatis* into strong bactericidal activity. This finding may be of pharmaceutical and clinical relevance as the bacteriostatic activity of bedaquiline is not restricted to *M. smegmatis*, but also found for pathogenic non-tuberculous mycobacterial strains, such as the *M. avium* complex*. These pathogenic strains typically show only low susceptibility towards current antibacterial chemotherapy*. Inactivation of cytochrome bd may assist in improving treatment options for infections caused by these recalcitrant bacteria. Alternatively, protection by cytochrome bd may be due to its lack of proton pump functionality. Cytochrome bd in *E. coli* has been found electrogenic, but displays a low H⁺/e⁻ ratio*. In this way cytochrome bd may alleviate membrane hyperpolarization.

Inactivation of cytochrome bd converts the bacteriostatic activity of clofazimine and bedaquiline against *M. smegmatis* into strong bactericidal activity. This finding may be of pharmaceutical and clinical relevance as the bacteriostatic activity of bedaquiline is not restricted to *M. smegmatis*, but also found for pathogenic non-tuberculous mycobacterial strains, such as the *M. avium* complex*. These pathogenic strains typically show only low susceptibility towards current antibacterial chemotherapy*. Inactivation of cytochrome bd may assist in improving treatment options for infections caused by these recalcitrant bacteria. Alternatively, protection by cytochrome bd may be due to its lack of proton pump functionality. Cytochrome bd in *E. coli* has been found electrogenic, but displays a low H⁺/e⁻ ratio*. In this way cytochrome bd may alleviate membrane hyperpolarization.

Materials & Methods

**Chemicals.** Bedaquiline was obtained from Janssen, Pharmaceutical Companies of Johnson & Johnson. Aurachin D was a kind gift from Dr. Jennifer Herrmann (Helmholtz Centre for Infection Research and Pharmaceutical Biotechnology, Saarbrücken). All other chemicals were bought from Sigma unless indicated otherwise.

**Bacterial strains and growth conditions.** *M. smegmatis* mc² 155 was kindly provided by B.J. Appel melk, Department of Molecular Cell Biology & Immunology, VU University Medical Center Amsterdam, The Netherlands. *M. smegmatis* mc²155 mutants ΔgcrCAB::hyg and ΔcydA::kan were kindly provided by Dr. B. Kana, MRC/NHLS/WTTS Molecular Mycobacteriology Research Unit, National Health Laboratory Service, Johannesburg, South Africa. Replicating bacterial cultures were grown in Middlebrook 7H9 broth (Difco) supplied with 0.05% Tween-80 and 10% Middlebrook albumin dextrose catalase enrichment (BBL) at 37°C with shaking. If applicable, 50µg/ml kanamycin or 50µg/mL hygromycin was added to the medium to select for mutant strains.

**Growth curves.** Growth curves for wild-type and mutant *M. smegmatis* were determined using a 96-well plate system. Bacteria were diluted to an optical density at 600 nm of 0.01 and optical density was determined at 20 minute intervals for 60 hours. The optical density was measured with a UV-VIS spectrophotometer (Varian Cary50).

**Preparation of inverted membrane vesicles.** Inverted membrane vesicles (IMVs) of the bacterial strains were prepared as described previously*. Briefly, *M. smegmatis* was grown for three days in a pre-culture to late-exponential phase. Cells were sedimented by centrifugation at 6000 x g for 20 minutes. The pellet was washed with phosphate buffered saline (PBS, pH 7.4) and centrifuged at 6000 x g for 20 min. Each 5 g of cells (wet weight) was re-suspended in 10 mL of ice-cold lysis buffer (10 mM HEPES, 5 mM MgCl₂, and 10% glycerol at pH 7.5) including protease inhibitors (complete, EDTA-free; protease inhibitor cocktail tablets from Roche). Lysozyme (1.2 mg/mL), deoxyribonuclease I (1500 U, Invitrogen) and MgCl₂ (12 mM) were added and cells were incubated with shaking for one hour at 37°C. The lysates were passed three times through a One Shot Cell Disruptor (Thermo Electron, 40 K) at 0.83 kb to break
up the cells. Unbroken cells were removed by three centrifugation steps (6000 x g for 20 min at 4 °C). The membranes were pelleted by ultracentrifugation at 222,000 x g for one hour at 4°C. The pellet was resuspended in lysis buffer and snap-frozen until use. The protein concentration was measured using the BCA Protein Assay kit (Pierce) as described by the manufacturer.

**Oxygen respiration assays.** Oxygen respiration and the effect of inhibitors on oxygen respiration were measured by polarography using a Clark-type electrode. The electrode was fully aerated (212 μM O₂ at 37°C) and calibrated with sodium hydrosulfite. The inverted membrane vesicles were pre-incubated for three minutes with the inhibitors in a pre-warmed (37°C) buffer containing 50 mM MES and 2 mM MgCl₂ (pH 6.5). NADH was added as electron donor to a final concentration of 250 μM and oxygen respiration was measured for 90 seconds. Potassium cyanide was used as a control for inhibition. Two independent experiments were performed and average values plus standard errors were calculated.

**Cellular ATP levels** were determined using the luciferase bioluminescence method described previously55. Briefly, 1.0-mL samples taken from *M. smegmatis* cultures grown as described above were centrifuged at 8000 x g for 10 min. The pellets were re-suspended in 50 μL water and a 10-fold volume of boiling 100 mM TRIS-HCl, 4 mM EDTA (pH 7.75) was added. After incubation at 100°C for 2 min the samples were centrifuged (1000 x g, 60 s) and the supernatants transferred to fresh tubes. 100 μl luciferase reagent (ATP Bioluminescence assay, Roche) was added to 100 μl sample and luminescence was measured with a Luminometer (LKB).

**Hydrogen peroxide and antibiotic sensitivity assays.** Bacterial strains were grown to an optical density at 600 nm of 0.5. For hydrogen peroxide sensitivity assays, hydrogen peroxide (30% (w/v) stock) was added to an Eppendorf tube containing 0.49 mL of bacterial suspension to a final concentration of 20 mM. After the indicated time of incubation at 37°C with shaking, 15 μl of catalase (10 mg/mL) was added to degrade hydrogen peroxide and thereby stop the reaction. For antibiotic sensitivity assays, 10 μl of bacterial cultures were incubated with the antibiotic for three (clofazimine and chlorpromazine) or four days (bedaquiline) at 37°C with shaking. All samples were diluted in PBS and 0.1 mL was plated on 7H10 agar plates, containing oleic acid (0.05 g/l) and 10% Middlebrook albumin dextrose catalase enrichment (BBL). Cell viability was measured by counting colony-forming units per mL (CFU/mL) after 72 h (wild-type and ΔcydA::kan strain) or 96 h (ΔqcrCB::hyg strain) incubation at 37°C. The limit of detection was 100 CFU/mL. Survival was determined as percentage of surviving cells compared to untreated cells at day 0.

**ROS detection assays.** For detection of reactive oxygen species the Amplex Red® Hydrogen Peroxide/ Peroxidase Assay kit (Invitrogen) was used as described by the manufacturer with minor modifications. To measure ROS production in inverted membrane vesicles, 1 mL samples of 0.05 M sodium phosphate, pH 7.4 containing 20 μg M. smegmatis inverted membrane vesicles, 0.2 mM NADH, 50 μM Amplex Red®, 2 U horseradish peroxidase (HRP), 80 U superoxide dismutase (SOD) and the antibiotic diluted in DMSO in 1x reaction buffer (0.05 M sodium phosphate, pH 7.4 containing 20 mM) was added. After the indicated time of incubation at 37°C with shaking, 15 μl of catalase (10 mg/mL) was added to degrade hydrogen peroxide and thereby stop the reaction. For antibiotic sensitivity assays, 2 U horseradish peroxidase (HRP), 80 U superoxide dismutase (SOD) and the antibiotic diluted in DMSO in 1x reaction buffer (0.05 M sodium phosphate, pH 7.4) were prepared. Superoxide dismutase was added to allow for detection of superoxide. ROS production was determined by measuring absorbance at 563 nm for 30 minutes with a UV-VIS spectrophotometer (Varian Cary50).

**References**

1. The World Health Organization, *Global tuberculosis report 2014*. (2015) available at: http://www.who.int/tb/publications/global_report/en/ (date of access: 01/04/2015).
2. Dartois, V. The path of anti-tuberculous drugs: from blood to lesions to mycobacterial cells. *Nat. Rev. Microbiol.* 12, 159–167 (2014).
3. Lienhardt, C. et al. Global tuberculosis control: lessons learnt and future prospects. *Nat. Rev. Microbiol.* 10, 407–416 (2012).
4. Barry, C. E., III et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat. Rev. Microbiol.* 7, 843–855 (2009).
5. Humphries, C. Latency: A sleeping giant. *Nature* 502, S14–S15 (2013).
6. Koul, A., Arnoult, E., Lounis, N., Guillemont, J. & Andries, K. The challenge of new drug discovery for tuberculosis. *Nature* 469, 483–490 (2011).
7. Hurdle, J. G., O’Neill, A. J., Chopra, I. & Lee, R. E. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nat. Rev. Microbiol.* 9, 62–75 (2011).
8. Bald, D. & Koul, A. Respiratory ATP synthase: the new generation of mycobacterial drug targets? *FEMS Microbiol. Lett.* 308, 1–7 (2010).
9. Black, P. A. et al. Energy metabolism and drug efflux in Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 58, 2491–2503 (2014).
10. Lu, P., Lill, H. & Bald, D. ATP synthase in mycobacteria: special features and implications for a function as drug target. *Biochim. Biophys. Acta.* 1837, 1208–1218 (2014).
11. Cook, G. M., Hands, K., Vilchez, C., Hartman, T. & Berney, M. Energetics of Respiration and Oxidative Phosphorylation in Mycobacteria. *Microbiol. Spectr.* 2, (2014). doi: 10.1128/microbiolspec.MGM2.0015-2013.
12. Andries, K. et al. A diarylquinoline drug active on the ATP synthase of Mycobacterium tuberculosis. *Science* 307, 223–227 (2005).
13. Haagsma, A. C. et al. Selectivity of TMC207 towards mycobacterial ATP synthase compared with that towards the eukaryotic homologue. *Antimicrob. Agents Chemother.* 53, 1290–1292 (2009).
14. Haagsma, A. C. et al. Probing the interaction of the diarylquinoline TMC207 with its target mycobacterial ATP synthase. *PLoS. One* 6, e23375 (2011).
15. Koul, A. et al. Diarylquinolinolines target subunit c of mycobacterial ATP synthase. *Nat. Chem. Biol.* 3, 323–324 (2007).
24. Abrahams, K. A. et al. Characterization of novel imidazo[1,2-a]pyridine inhibitors targeting M. tuberculosis QcrB. PLoS. One. 7, e52951 (2012).
25. Matsoso, L. G. et al. Function of the cytochrome bc$_{2}$-aa$_{3}$ branch of the respiratory network in mycobacteria and network adaptation occurring in response to its disruption. J. Bacteriol. 187, 6300–6308 (2005).
26. Shi, L. et al. Changes in energy metabolism of Mycobacterium tuberculosis in mouse lung and under in vitro conditions affecting aerobic respiration. Proc. Natl. Acad. Sci. USA 102, 15629–15634 (2005).
27. Kana, B. D. Characterization of the cydAB-encoded cytochrome bd oxidase from Mycobacterium smegmatis. J. Bacteriol. 183, 7076–7086 (2001).
28. Arora, K. et al. Respiratory flexibility in response to inhibition of cytochrome C oxidase in Mycobacterium tuberculosis. Antimicrob. Agents Chemother. 58, 6962–6965 (2014).
29. Small, J. L. et al. Perturbation of cytochrome c maturation reveals adaptability of the respiratory chain in Mycobacterium tuberculosis. Mbio. 4, e00475–13 (2013).
30. Borisov, V. B., Gennis, R. B., Hemp, J. & Verkhovsky, M. I. The cytochrome bd respiratory oxygen reductases. Biochim. Biophys. Acta. 1807, 1398–1413 (2011).
31. Forte, E. et al. Cytochrome bd oxidase and hydrogen peroxide resistance in Mycobacterium tuberculosis. Mbio. 4, e01006–e01013 (2013).
32. Guiffre, A., Borisov, V. B., Ares, M., Sarti, P. & Forte, E. Cytochrome bd oxidase and bacterial tolerance to oxidative and nitrosoative stress. Biochim. Biophys. Acta. 1837, 1178–1187 (2014).
33. Borisov, V. B. et al. Interaction of the bacterial terminal oxidase cytochrome bd with nitric oxide. FEBS Lett. 576, 201–204 (2004).
34. Borisov, V. B. et al. Cytochrome bd oxidase from Escherichia coli displays high catalase activity: an additional defense against oxidative stress. FEBS Lett. 587, 2214–2218 (2013).
35. Lindqvist, A., Membrillo-Hernandez, J., Poole, R. K. & Cook, G. M. Roles of respiratory oxidases in protecting Escherichia coli K12 from oxidative stress. Antonie Van Leeuwenhoek 78, 23–31 (2000).
36. Mason, M. G. et al. Cytochrome bd confers nitric oxide resistance to Escherichia coli. Nat. Chem. Biol. 5, 94–96 (2009).
37. Cook, G. M. et al. A factor produced by Escherichia coli K-12 inhibits the growth of E. coli mutants defective in the cytochrome bd quinol oxidase complex: enterochelin rediscovered. Microbiology 144, 3297–3308 (1998).
38. Koul, A. et al. Delayed bactericidal response of Mycobacterium tuberculosis to bedaquiline involves remodelling of bacterial metabolism. Nat. Commun. 5, 3369 (2014).
39. Berney, M., Hartman, T. E. & Jacobs, W. R., Jr. A Mycobacterium tuberculosis cytochrome bd oxidase mutant is hypersensitive to bedaquiline. Mbio. 5, 01275–14 (2014).
40. Debnath, J. et al. Discovery of selective menaquinone biosynthesis inhibitors against Mycobacterium tuberculosis. J. Med. Chem. 55, 3739–3755 (2012).
41. Li, X. W. et al. Synthesis and biological activities of the respiratory chain inhibitor aurachin D and new ring versus circular analogues. Belsein. J. Org. Chem. 9, 1551–1558 (2013).
42. Meunier, B., Madgwick, S. A., Reil, E., Oettmeier, W. & Rich, P. R. New inhibitors of the quinol oxidation sites of bacterial cytochromes bo and bd. Biochemistry 34, 1076–1083 (1995).
43. Way, S. S., Sallustio, S., Magllozzo, R. S. & Goldberg, M. B. Impact of either elevated or decreased levels of cytochrome bd expression on Shigella flexneri virulence. J. Bacteriol. 181, 1229–1237 (1999).
44. Turner, A. K. et al. Contribution of proton-translocating proteins to the virulence of Salmonella enterica serovars Typhimurium, Gallinarum, and Dublin in chickens and mice. Infect. Immun. 71, 3392–3401 (2003).
45. Endley, S., Mchughay, D. & Fisher, I. A. Interruption of the cydB locus in Brucella abortus attenuates intracellular survival and virulence in the mouse model of infection. J. Bacteriol. 183, 2454–2462 (2001).
46. Juty, N. S., Moshiri, F., Merrick, M., Anthony, C. & Hill, S. The Klebsiella pneumoniae cytochrome bd$_{1}$ terminal oxidase complex and its role in microaerobic nitrogen fixation. Microbiology 143, 2673–2683 (1997).
47. Yamamoto, Y. et al. Respiration metabolism of Group B Streptococcus is activated by environmental haem and quinone and contributes to virulence. Mol. Microbiol. 56, 525–534 (2005).
48. Baughn, A. D. & Malamy, M. H. The strict anaerobe Bacteroides fragilis grows in and benefits from nanomolar concentrations of oxygen. Nature 427, 441–444 (2004).
49. Puustinen, A., Finel, M., Haltia, T., Gennis, R. B. & Wikstrom, M. Properties of the two terminal oxidases of Escherichia coli. Biochemistry 30, 3936–3942 (1991).
50. Kita, K., Konishi, K. & Anraku, Y. Terminal oxidases of Escherichia coli aerobic respiratory chain. II. Purification and properties of cytochrome b$_{558}$-d complex from cells grown with limited oxygen and evidence of branched electron-carrying systems. J. Biol. Chem. 259, 3375–3381 (1984).
51. Lounis, N., Gevers, T., Van den Berg, J., Vranckx, L. & Andries, K. ATP synthase inhibition of Mycobacterium avium is not bactericidal. Antimicrob. Agents Chemother. 53, 4927–4929 (2009).
52. van Ingen J. & Kuiper, E. J. Drug susceptibility testing of nontuberculous mycobacteria. Future Microbiol. 9, 1095–1110 (2014).
53. Bald, D. & Koul, A. Advances and strategies in discovery of new antibacterials for combating metabolically resting bacteria. Drug Discov. Today 18, 250–255 (2013).
54. Haagena, A. C., Driessen, N. N., Hahn, M. M., Lill, H. & Bald, D. ATP synthase in slow- and fast-growing mycobacteria is active in ATP synthesis and blocked in ATP hydrolysis direction. FEMS Microbiol. Lett. 313, 68–74 (2010).
55. Lu, P. et al. Pyrazinoin acid decreases the proton motive force, respiratory ATP synthesis activity, and cellular ATP levels. Antimicrob. Agents Chemother. 55, 5354–5357 (2011).
Acknowledgement

P.L. is indebted to the Chinese Scholarship Council for a fellowship. The authors wish to thank Henk Hakvoort and Marijke Wagner (VU Amsterdam) for technical assistance, Dr. Bavesh Kana (University of Witwatersrand) for providing the cytochrome bc1 and cytochrome bd mutants and Dr. Jennifer Herrmann (Helmholtz Centre for Infection Research and Pharmaceutical Biotechnology, Saarbrücken) and Dr. Thorsten Friedrich (University of Freiburg) for providing samples of aurachin D.

Author Contributions

P.L. and M.H. performed experiments; P.L., M.H., A.K., K.A., G.M.C., H.L., R.v.S. and D.B. designed experiments and analyzed data; D.B. and R.v.S. supervised and coordinated experiments; P.L., M.H. and D.B. wrote the manuscript with contributions from all co-authors, D.B. supervised the overall research.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

How to cite this article: Lu, P. et al. The cytochrome bd-type quinol oxidase is important for survival of Mycobacterium smegmatis under peroxide and antibiotic-induced stress. Sci. Rep. 5, 10333; doi: 10.1038/srep10333 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/