THE ROLE OF CYTOCHROME BD IN
MYCOBACTERIUM SMEGMATIS IN
PROTECTION AGAINST REACTIVE
OXYGEN SPECIES AND
ANTIBACTERIALS

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Abstract

Targeting oxidative phosphorylation recently has received strong interest as new strategy for combating drug-resistant *Mycobacterium tuberculosis*. Several components of oxidative phosphorylation such as the type-II NADH dehydrogenase, the cytochrome bc₁ complex and ATP synthase have been exploited as target and drugs inhibiting these complexes presently are in clinical development. However, the terminal oxidases of the mycobacterial respiratory have attracted comparatively little attention. Mycobacteria display a branched respiratory chain with two terminal oxidases, the cytochrome bc₁/aa₃ branch and the cytochrome bd branch. In this report, we characterize mutants in *Mycobacterium smegmatis* in which one of the two respiratory chain branches is inactivated due to gene knockout. Basic microbiological characterization showed that inactivation of the cytochrome bc₁ significantly decreased bacterial growth, cellular ATP levels and oxygen consumption rates whereas inactivation of cytochrome bd did not. In contrast, we found that inactivation of cytochrome bd, but not of cytochrome bc₁, lead to enhanced susceptibility for hydrogen peroxide, for the ATP synthase inhibitor bedaquiline and for the type-II NADH dehydrogenase effector clofazimine. From these results, it can be concluded that cytochrome bd, although not required for aerobic growth, plays an important role in response to antibacterial stress. Targeting cytochrome bd therefore appears highly promising in order to weaken bacterial metabolism and for efficient drug combination regimen.
**Introduction**

*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), infected 8.6 million people in 2012\(^1\). Despite the introduction of efficient antibiotics in the 1950s, TB treatment remains challenging, partly due to the emergence of drug-resistant strains\(^2\). Additionally, its metabolic flexibility allows the pathogen to exist in different states, ranging from actively replicating to dormant persisting\(^3\)\(^-\)\(^5\). 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 HIV\(^6\). Therefore, drugs with novel mechanisms of action are urgently necessary 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 involved in energy metabolism are potential drug targets\(^5\),\(^7\)\(^-\)\(^9\).

ATP synthase (ATPase) has been validated as target of bedaquiline (BDQ), the lead compound of the diarylquinoline class of drugs, which potently inhibits both replicating and dormant mycobacteria\(^10\)\(^-\)\(^12\). BDQ has received accelerated approval by the FDA and the EMA for treatment of MDR-TB\(^13\),\(^14\). Moreover, components of the respiratory chain such as the type-II NADH dehydrogenase (NDH-2) and the cytochrome bc\(_1\) complex can also be targeted by small-molecule compounds, which are currently in clinical development\(^15\)\(^-\)\(^18\). To date, however, there has been only little attention given to the terminal oxidases of the respiratory chain. Mycobacteria employ a branched electron transport chain, from the menaquinone pool, electrons can be passed on either to the cytochrome bc\(_1\) complex which forms a
supercomplex with the cytochrome aa₃ oxidase, or alternatively to the cytochrome bd-type terminal oxidase⁹,¹⁹ (Figure 1). Cytochrome bd is of particularly interest as potential drug target, as it is only found in prokaryotes. The absence of a human homologue may facilitate selective targeting.

Figure 1. The branched respiratory chain in mycobacteria.

Cytochrome bc₁-aa₃ is the main respiratory branch utilized during aerobic conditions, however, it was found down regulated during hypoxia and chronic infection in a mouse model²²-²⁴. These conditions induced the expression of cytochrome bd, suggesting an important role for this enzyme in respiration during hypoxia²⁰-²². Additionally, cytochrome bd was induced when cytochrome c maturation was disturbed²³ or when the cytochrome bc₁ complex was impaired due to deletion mutations²⁰ suggesting that cytochrome bd may (partially) be able to compensate for lack of function in the bc₁/aa₃ branch of the respiratory chain²⁴.

Cytochrome bd accepts electrons from the electron carrier menaquinol to reduce O₂ to H₂O. During this reaction a
proton motive force is generated, which is subsequently used by ATP synthase to produce ATP from ADP and inorganic phosphate. Cytochrome bd is thought to contribute to the proton motive force by transferring electrons onto protons derived from the cytoplasmic side of the membrane whereas the protons produced by reduction of menaquinone are released on the periplasmic side\textsuperscript{25}. However, in contrast to the cytochrome aa\textsubscript{3}-type terminal oxidase, cytochrome bd apparently does not act as proton pump, making this branch of the respiratory chain less energy efficient\textsuperscript{26}.

Next to its role in oxidative phosphorylation, it is hypothesized that cytochrome bd protects mycobacteria from different types of stress\textsuperscript{23, 25, 27}. During an inflammatory reaction, macrophages in the host can produce Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) to kill engulfed bacteria\textsuperscript{28}. Induction of cytochrome bd expression in the mouse lung during chronic infection of \textit{M. tuberculosis} is exemplary of increased virulence associated with cytochrome bd expression\textsuperscript{21}, which is also observed for other pathogenic strains such as \textit{B. abortus}\textsuperscript{29, 30}, \textit{S. aureus}\textsuperscript{31} and \textit{S. flexneri}\textsuperscript{32}. Mutation of cytochrome bd in bacterial species such as \textit{B. abortus}, \textit{A. vinelandii} and \textit{E. coli} led to a hypersensitivity to hydrogen peroxide\textsuperscript{30, 33, 34}. Exposure to exogenous H\textsubscript{2}O\textsubscript{2} induced expression of cytochrome bd in \textit{E. coli}\textsuperscript{34} and similarly cytochrome bd gene expression is stimulated upon exposure to nitric oxide (NO) for \textit{E. coli}\textsuperscript{35}, \textit{M. tuberculosis}\textsuperscript{21}, \textit{B. subtilis}\textsuperscript{36} and \textit{S. aureus}\textsuperscript{37}. Overexpression of cytochrome bd in \textit{M. tuberculosis} leads to increased H\textsubscript{2}O\textsubscript{2} resistance\textsuperscript{23}. Upregulation of cytochrome bd may constitute a protection mechanism to survive the host’s immune response. Moreover, recently a transcriptional and proteomic analysis revealed that cytochrome bd is upregulated in response to bedaquiline\textsuperscript{38}. These data point toward cytochrome bd as an important factor for stress resistance in (myco-) bacteria.
In this study, the role of mycobacterial cytochrome bd in various stress situations is investigated. Strains of *M. smegmatis* lacking either cytochrome bd or the cytochrome bc₁ complex are exposed to oxidative and nitrosative stress as well as to antibacterials to elucidate the role of cytochrome bd (and of the cytochrome bc₁-aa₃ branch) in protection against these stress factors.

**Materials & Methods**

**Chemicals**

Bedaquiline was obtained from Johnson & Johnson. All chemicals were bought from Sigma unless indicated otherwise.

**Bacterial strains and growth conditions**

*M. smegmatis* mc²155 was kindly provided by B.J. Appelmelk, Department of Molecular Cell Biology & Immunology, VU University Medical Center Amsterdam, The Netherlands. *M. smegmatis* mc²155 cydA:kan was a generous gift by G.M. Cook, Department of Microbiology, Otago School of Medical Sciences, University of Otago, New Zealand. *M. smegmatis* mc²155 qcrCAB::hyg was kindly provided by B. Kana, MRC/NHLS/WITS 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 suitable, 50 µg/ml kanamycin or hygromycin was added to the medium to select for a mutant strain.
Preparation of inverted membrane vesicles

The inverted membrane vesicles (IMVs) of the bacterial strains were prepared as described previously\(^\text{39}\). Briefly, *M. smegmatis* was grown for 3 days in a preculture to late-exponential phase. Cells were sedimented by centrifugation at 6000 xg for 20 minutes (min). The supernatant was washed with Phosphate Buffered Saline (PBS, pH 7.4) and centrifuged at 6000 xg for 20 min. Each 5 g of cells (wet weight) was resuspended in 10 ml of ice-cold lysis buffer (PA\(_3\) buffer, containing 10 mM HEPES, 5 mM MgCl\(_2\) and 10% glycerol at pH 7.5) including protease inhibitors (complete, EDTA-free; protease inhibitor cocktail tablets from Roche). Lysozyme (1.2 mg/ml), deoxyribonucleaseI (1516.2 U, Invitrogen) and MgCl\(_2\)(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 (Thermo Electron, 40K) at 0.83 Kb to break the cells. The unbroken cells were removed by triple centrifugation at 6000 xg for 20 min at 4°C. The membranes were pelleted by ultracentrifugation at 55,000 rpm at 4°C for one hour. The pellet was resuspended in PA\(_3\) buffer and snap frozen until use. 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 using polarography with a clark-type electrode. The electrode was fully aerated (200 μM O\(_2\)) and calibrated with sodium hydrosulfitie. The inverted membrane vesicles were pre-incubated for three minutes with the inhibitors in a pre-warmed (37°C) buffer consisting of 50 mM MES and 2 mM MgCl\(_2\) (pH 6.5). The electron donor NADH was added in a final concentration of 250 μM and oxygen respiration was measured for 90 seconds. The
inhibitor KCN was used as a positive control. Two independent experiments in duplo were performed and averages plus standard errors was calculated.

**Growth curves**

Growth curves were determined in a 96-well plate using a spectrophotometer. Bacteria were diluted to an OD$_{600}$ of 0.01 and optical density was determined each 20 minutes for 60 hours. The samples in triplo were shaken in between and before each measurement.

**ROS, RNS and antibiotic sensitivity assays**

Bacterial strains were grown for 28 hours to an optical density at 600 nm of approximately 0.5. For hydrogen peroxide sensitivity assays, 0.01 ml of H$_2$O$_2$ was added to an Eppendorf tube containing 0.49 ml of cells. After the indicated time of incubation at 37°C with shaking, 15 µl of catalase (10 mg/ml) was added to degrade H$_2$O$_2$ and thereby stop the reaction. For DETA/NO sensitivity assays, 50 µl of DETA/NO was added to 450 µl bacteria and incubated for 24 h at 37°C with shaking. For antibiotic sensitivity assays, the antibiotic was incubated with 10 ml of bacterial cultures for three (clofazimine, 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. These plates contained 7H10 agar (Middlebrook), oleic acid (0.05 g/l), 10% Middlebrook albumin dextrose catalase enrichment (BBL) and kanamycin or hygromycin if suitable. Cell viability was measured by counting colony-forming units per ml (CFU/ml) after 72 h (wt, bd−) or 96 h (bc1−) incubation at 37°C. Detection limit was Log 2 CFU/ml. Survival was determined as percentage of surviving cells compared to untreated cells at day 0.
**ROS detection assays**

To measure ROS produced after antibiotic treatment, 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, samples of 1.0 ml containing 20 µg IMVs of M. smegmatis, 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) were prepared. Superoxide dismutase was added to include oxidative stress caused by superoxide. ROS production was determined by measuring absorbance for 30 minutes at 563 nm with a UV-VIS spectrophotometer (Varian Cary50).

**Results**

**Microbiological characterization of M. smegmatis strains with inactivated terminal oxidase branches**

The role of cytochrome bd in the respiratory chain of mycobacteria was investigated using mutant strains impaired in one of the two branches of the respiratory chain (Table 1). These strains maintain either only the cytochrome bd branch (bc\(_1^-\) (ΔQcrCAB::hyg)) or the cytochrome bc\(_1/\alpha\alpha_3\) branch (bd\(^-\) (ΔCydA::kan))(Figure 1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Source</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mC(^2)155</td>
<td>Fast-growing non-pathogenic <em>Mycobacterium</em> species</td>
<td>Dr. B. Appelmelk (VUmc Amsterdam)</td>
<td>WT</td>
</tr>
</tbody>
</table>
Table 1: *Mycobacterium smegmatis* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Supplier</th>
<th>Symbol</th>
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<tbody>
<tr>
<td>ΔCydA::kan</td>
<td>Cytochrome bd inactivation mutant of mc²155; kanamycin resistant</td>
<td>Dr. B. Kana (U.Witwatersr and South Africa)</td>
<td>bd⁻</td>
</tr>
<tr>
<td>ΔQcrCAB::hyg</td>
<td>Cytochrome bc₁₁ deletion mutant of mc²155; hygromycin resistant</td>
<td>Dr. B. Kana (U.Witwatersr and South Africa)</td>
<td>bc₁⁻</td>
</tr>
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The growth rate of the bd⁻ strain was comparable to that of the wild type, whereas growth of the bc₁⁻ strain was significantly lower (Fig 2A), confirming previous data⁰⁻²². We then extended the earlier reported microbiological characterization of the deletion strains and determined key bioenergetic parameters of the two mutants. Cellular ATP levels were unaltered in the bd⁻ mutant as compared with the wild type, but were strongly decreased in the bc₁⁻ strain (Figure 2B, C). These findings reflect the role of the cytochrome bc₁ complex as energetically more efficient primary terminal branch the respiratory chain in replicating *M. smegmatis*²⁰,⁴⁰. Similarly,
also oxygen consumption rates were lower in the bc1⁻ strain.

**Figure 2: Microbiological characterization of *M. smegmatis* strains lacking the cytochrome bd or the cytochrome bc1 complex.** (A) Wild-type (wt) and respective mutant strains were grown overnight, subcultured in fresh medium and incubated at 37°C for 60 h. The optical density at 600 nm was measured in 20 min intervals. Data are representative of two independent experiments, each done in triplicate. (B) Cellular ATP levels in WT and mutant *M. smegmatis* as determined by the Luciferase method. (C) Oxygen consumption rates of inverted membrane vesicles (IMVs) from wild type and mutant *M. Smegmatis* strains using NADH as substrate. Data represent average plus standard error (SEM) for one experiment done threefold. One-way ANOVA was used for statistical analysis, ** represent P value < 0.01.

**Cytochrome bd protects against oxidative stress**

Next, we investigated if cytochrome bd in *M. smegmatis* protects against peroxide stress, as reported for *E. coli*. Exponentially growing bacteria were exposed to hydrogen peroxide (20 mM, final conc.) for various time intervals and colony-forming units were counted. Whereas incubation with hydrogen peroxide had a bacteriostatic effect of WT *M. smegmatis*, for the d⁻ strain a 99% decline in cell viability was observed after 60 min of exposure (Figure 3). In contrast, only a minor decrease in viability was found for the bc1⁻ strain. These results suggest that cytochrome bd plays a protective role during oxidative stress in *M. smegmatis*. 
Figure 3: Cytochrome bd protects against oxidative stress. The effect of hydrogen peroxide (20 mM) on the survival of exponentially growing *M. smegmatis* is shown: wild type (filled squares), bd⁻ (filled triangles), and bc₁⁻ (open circle). Results represent means of two independent experiments with standard error of the mean (SEM).

Role of cytochrome bd during nitrosative stress

In addition to protecting against oxidative stress, cytochrome bd has been plays a role during nitrosative stress in *E. coli*\(^{28}\), and a similar role in pathogenic bacteria has been postulated\(^{25}\). To test this hypothesis, exponentially growing bacteria were exposed to DETA/NO as nitric oxide donor. This compound slowly releases NO and thereby induces nitrosative stress. For concentrations up to 5 mM no difference in survival was observed (Figure 4), only at the very highest concentration (25 mM) the bd⁻ strain displayed a minor increase in susceptibility. These results indicate that cytochrome bd does not play a major role in protection against nitrosative stress under the conditions investigated here.
Figure 4: Role in protection of cytochrome bd against nitric oxide stress. Strains of *M. smegmatis* were treated with indicated amounts of the nitric oxide donor DETA/NO for 24 hours and CFU/ml were counted on agar plates after three days of incubation at 37°C. Black bars: WT; white bars: bd−. Results represent means of at least two experiments with standard error of the mean (SEM).

Cytochrome bd protects against the ATP synthase inhibitor bedaquiline

Then, we investigated the role of cytochrome bd in the response to drugs targeting oxidative phosphorylation. We first investigated the ATP synthase inhibitor bedaquiline (BDQ), since cytochrome bd is unregulated in the presence of this drug\(^\text{38}\). BDQ acts bacteriostatic against WT *M. smegmatis*, even at the highest concentration used (5 microg/ml) (Figure 5). For the bd− strain ~1 log unit kill was observed with 1 microg/ml BDQ and ~ 3 log unit kill with 5 microg/ml BDQ, reaching the LOD (detection limit) after 3 days of treatment (Figure 5). In contrast, the bc\(_1\)− strain was even slightly less
sensitive as the WT strain (Figure 5). Cytochrome bd thus efficiently protects \textit{M. smegmatis} against bedaquiline stress, whereas the cytochrome bc\textsubscript{1}/aa\textsubscript{3} branch does not.

![Figure 5: Cytochrome bd protects against the ATP synthase inhibitor bedaquiline.](image)

**Figure 5: Cytochrome bd protects against the ATP synthase inhibitor bedaquiline.** Strains of \textit{M. smegmatis} were treated with indicated amounts of bedaquiline for 96 hours and CFU/ml were counted on agar plates after three (WT, bd\textsuperscript{-}) or four days (bc\textsubscript{1}\textsuperscript{-}) of incubation at 37°C. Black bars: WT; grey bars: bc\textsubscript{1}; white bars: bd\textsuperscript{-}. Results represent the means of two independent experiments with standard error of the mean (SEM).

**Cytochrome bd protects against the NDH-2 effector clofazimine**

We hypothesized that mycobacteria with impaired cytochrome bd may also be more sensitive to other inhibitors of oxidative phosphorylation. Clofazimine (CFZ) is a front-line anti-leprosy drug that presently is repurposed for usage against tuberculosis\textsuperscript{41}. CFZ interferes with the type II NADH
dehydrogenase in *M. smegmatis* (and likely *M. tuberculosis*)(NDH-2), accepts and transfers electrons onto oxygen, thereby producing ROS\(^42\). To investigate if cytochrome bd protects against CFZ, bacterial cells were incubated for 72 hours with varying concentrations of the drug. CFZ at up to 25x MIC (7.5 µg/ml) was not bactericidal for the WT strain, while growth of the bd\(^-\) strain was significantly reduced at CFZ concentrations > 0.3 microg/ml and with 7.5 µg/ml the LOD was reached (Figure 6). The bc\(_1\)\(^-\) strain showed the same or only marginally higher sensitivity for CFZ as the WT strain. These results indicate that cytochrome bd protects the bacteria against clofazimine.

**Figure 6: Cytochrome bd protects against clofazimine.** Strains of *M. smegmatis* were treated with the indicated amounts of clofazimine for 72 hours and CFU/ml were counted on agar plates after three (WT, bd\(^-\)) or four days (bc\(_1\)\(^-\)) of incubation. Black bars, WT; grey bars: bc\(_1\)\(^-\); white bars: bd\(^-\).
Bars represent means of at least two independent experiments with standard error of the mean (SEM).

The protective mechanism by cytochrome bd may be based on scavenging of ROS or alternatively, in case of BDQ, due to alleviating membrane hyperpolarization triggered upon inhibition of ATP synthase. Therefore, we used chlorpromazine (CPZ) as control, which is a phenothiazine-class drug that inhibits NDH-2\(^{15}\), but does not produce ROS nor cause membrane hyperpolarisation. Consistent with our hypothesis, chlorpromazine did not discriminate between WT and bd- \textit{M. smegmatis} (Figure 7).

\textbf{Figure 7: Cytochrome bd does not protects against the NDH-2 inhibitor chlorpromazine} Strains of \textit{M. smegmatis} were treated with indicated amounts of chlorpromazine (CPZ) for 72 hours and CFU/ml was counted on agar plates after three days of incubation at 37°C. Black bars: WT; white bars:bd. Results represent means of at least two independent experiments with standard error of the mean (SEM).
Next, we determined if incubation with BDQ and CFZ leads to ROS production in our experimental system. CFZ caused time-dependent development of ROS by inverted membrane vesicles from \textit{M. smegmatis} WT, confirming earlier results from Yano \textit{et al}^{42} (Figure 8). In contrast, we were not able to detect ROS production by BDQ in our experimental system (Figure 8).

Figure 8: ROS production by clofazimine and BDQ. ROS production was measured for 30 min after adding the indicated drugs to inverted membrane vesicles of wild-type \textit{M. smegmatis}. The production of resofurin, a product of the 1:1 reaction of H$_2$O$_2$ with Amplex Red®, was measured at 562 nm. Representative results from two independent experiments are shown.

Inactivation of mycobacterial cytochrome bd by a small-molecule inhibitor

Our results reveal that genetic inactivation of cytochrome bd can significantly increase the potency of two prominent antibacterial drugs. Based on these findings we tested if small-molecule inhibitors can block the activity of cytochrome bd in \textit{M. smegmatis}. The aurachin class of quinone analogs has been
reported as inhibitors of quinone-modifying enzymes in *E. coli*. Within this class, aurachin D was previously shown to preferentially inhibit *E. coli* cytochrome bd as compared with other quinone-modifying enzymes. We investigated the effect of aurachin D on the oxygen consumption activity of IMVs from *M. smegmatis* and found dose-dependent inhibition, with a 50% maximum inhibition for wild type strain (Figure 9). Interestingly, the inhibitory effect was strongly enhanced in the bc\(_1^-\) strain (IC\(_{50}\) ~400 nM), indicating that the preferential target in mycobacterial oxidative phosphorylation is indeed the cytochrome bd (Figure 9).

Subsequently, we evaluated the effect of aurachin D on mycobacterial growth. We found that for all three strains tested (WT, bd\(^-\), bc\(_1^-\)) the minimal inhibitory concentrations (MICs) were > 50 microg/ml (data not shown). These results suggest that the inhibitor is not capable of effectively crossing the mycobacterial cell envelope.
Figure 9: Aurachin D inhibits cytochrome bd activity of *M. smegmatis* membrane vesicles. Oxygen consumption activity of inverted membrane vesicles (IMVs) from *M. smegmatis* was measured with a Clark-type electrode. The reaction was started by addition of NADH (250 µM final conc.) as electron donor and followed for 90 sec. Black bar: WT; gray bar: bc₁⁻. Results represent the means of two independent experiments with standard error of the mean (SEM).

**Discussion**

**Role of Cytochrome bd in protection against ROS and RNS**

In this study, we found that the respiratory enzyme cytochrome bd plays a protective role during oxidative stress in *M. smegmatis*. The hypersensitivity of the bd⁻ strain to exogenous H₂O₂ is not due to the instability of the mutant strain, since growth rate and ATP levels are similar for wild type and bd⁻ strains. Cytochrome bd, although not essential for aerobic growth and respiration, interestingly plays an important role in protecting the bacterium from ROS. Surprisingly, the mutant strain that only has cytochrome bd as terminal oxidase (bc₁⁻) does not show increased survival during H₂O₂ treatment. It is possible that lower cellular ATP levels in this strain impair the maintenance capability and therefore may decrease survival chances.

The molecular mechanism of protection is not identified in this study, but Giuffrè et al. present two non-mutually exclusive hypotheses. First, since cytochrome bd acts as an oxygen scavenger, it might reduce the intracellular oxygen level and thereby prevent the formation of ROS. This suggests that cytochrome bd oxidase acts as an electron sink, thereby avoiding the production of oxidative stress. Second, it is possible that cytochrome bd directly metabolizes ROS. In *E. coli*, it was found that cytochrome bd endows catalase activity, and it would therefore be interesting to measure
catalase activity of cytochrome bd in *M. smegmatis*. Our study does not provide evidence to distinguish between these two hypotheses, the mechanism of protection against ROS needs to be further elucidated.

Previous studies showed that nitric oxide (NO) can inhibit mitochondrial cytochrome c oxidase\(^4\) and cytochrome bd in *E. coli\(^4\)*, which led us to the hypothesis that mycobacterial cytochrome bd may also protect against nitrosative stress. Nitric oxide can be produced in human alveolar macrophages and can control growth of mycobacteria. In our experiments we did not find significant differences in survival between wild type *M. smegmatis* and the cytochrome bd deficient strain, except for at extremely high concentrations. Cytochrome bd may thus not play a major protective role in *M. smegmatis*. Instead, the truncated hemoglobin (trHbn) or other factors may defend this bacterium from inhibition by NO during aerobic conditions\(^4\). In addition, the function of cytochrome bd in NO detoxification may be restricted to conditions of hypoxia. Furthermore, It was found that protection against nitric oxide in *E. coli* is associated with the fast \(k_{\text{off}}\) rate from cytochrome bd\(^2\). This aspect is not represented in our experimental system with constant nitric oxide levels, but may be more relevant in a dynamic environment with fluctuating nitric oxide concentrations.

**Cytochrome bd protects against two promising antibacterials**

Our experiments revealed that cytochrome bd plays an important role in protection against two prominent antibacterials, both targeting oxidative phosphorylation. Protection against clofazimine can be explained by production of ROS by this drug. Dwyer *et al* proposed that several major
antibiotic classes share a common mechanism of killing by generating ROS\textsuperscript{48}. Cytochrome bd may thus protect against a variety of antibacterials. Our data do not allow for pinpointing the mechanism of protection against BDQ. Increased production of ROS is expected from an ATP synthase inhibitor, which creates backpressure on the respiratory chain and thereby increases the likelihood of electron transfer from respiratory chain complexes upon oxygen. A signature of BDQ-induced backpressure, such as increased cellular NADH\textsuperscript{+} levels and increased expression of bacterioferritin in \textit{M. tuberculosis}, has previously been reported\textsuperscript{38}. However, it is possible that levels of ROS produced by BDQ are not sufficiently high for detection in case the membrane vesicles are too leaky, which maybe the case for most IMVs. An attempt to measure ROS production in whole bacteria failed (data not shown). Alternatively, cytochrome bd may protect against BDQ by preventing membrane hyperpolarization due to its comparatively low electrogenic capacities. The mechanism of protection needs to be further investigated.

**Targeting cytochrome bd as new antibacterial strategy?**

Our results highlight the importance of cytochrome bd in mycobacterial metabolism and strongly suggest that targeting this terminal oxidase may be a successful strategy to weaken bacterial metabolism. Based on standard microbiological characterization such as growth rate and cellular ATP levels the cytochrome bc\textsubscript{1}/aa\textsubscript{3} branch may appear to be the most promising target of the two branches in the mycobacterial respiratory chain. However, our results demonstrate that targeting cytochrome bd, whose inactivation does not directly lead to a phenotype, leads to a stronger impact on bacterial viability in drug combinations. Our results thus provide a striking example for a counter-intuitive choice of a drug target.
Inhibition of cytochrome bd in mycobacterial membranes by aurachin D serves as proof-of-principle for small-molecule inhibition of this important new drug target. Improved aurachin derivates with better ability to penetrate the mycobacterial cell envelope may constitute a new class of anti-tuberculosis drugs. Whole-cell or target-based screenings with compounds from chemical libraries might lead to the discovery of other potent cytochrome bd inhibitors. Inhibitors of cytochrome bd likely will synergize with BDQ and CFZ (and possibly with other drugs as well) and may improve the potency of anti-tuberculosis drugs, potentially turning a bacteriostatic drug into a bactericidal one. Combining cytochrome bd inhibitors with existing anti-mycobacterial drugs may enhance and improve current treatment regimen and contribute to solving the problem of drug-resistant M. tuberculosis strains.

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Reference List


