1 **Mycobacteria tolerate carbon monoxide by remodelling**

2 **their respiratory chain**

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18 **Abstract**

19 Carbon monoxide (CO) is a gas infamous for its acute toxicity. The toxicity of CO
 20 predominantly stems from its tendency to form carbonyl complexes with transition
 21 metals, thus inhibiting the heme-prosthetic groups of proteins, including the terminal
 22 oxidases of the respiratory chain. While CO has been proposed as an antibacterial
 23 agent, the evidence supporting its toxicity towards bacteria is equivocal, and its
 24 cellular targets remain poorly defined. In this work, we investigate the physiological
 25 response of mycobacteria to CO. We show that *Mycobacterium smegmatis* is highly
 26 resistant to the toxic effects of CO, exhibiting normal growth parameters when
 27 cultured in its presence. We profiled the proteome of *M. smegmatis* during growth in
 28 CO, identifying strong induction of cytochrome *bd* oxidase and members of the *dos*
 29 regulon, but relatively few other changes. We show that the activity of cytochrome *bd*
 30 oxidase is resistant to CO, whereas cytochrome *bcc-aa₃* oxidase is strongly inhibited
 31 by this gas. Consistent with these findings, growth analysis shows that *M. smegmatis*
 32 lacking cytochrome *bd* oxidase displays a significant growth defect in the presence
 33 of CO, while induction of the *dos* regulon appears to be unimportant for adaption to
 34 CO. Altogether, our findings suggest that *M. smegmatis* has considerable resistance
 35 to CO and benefits from respiratory flexibility to withstand its inhibitory effects.

36

37 **Importance**

38 Carbon monoxide has an infamous reputation as a toxic gas and it has been
 39 suggested that it has potential as an antibacterial agent. Despite this, the means by
 40 which bacteria resist its toxic effects are not well understood. In this study we
 41 determine the physiological response of *Mycobacterium smegmatis* to growth in CO.

42 We show for the first time that the cytochrome *bd* oxidase is inherently resistant to
43 CO and is deployed by *M. smegmatis* to tolerate the presence of this gas. Further,
44 we show that aside from this remodelling of its respiratory chain, *M. smegmatis*
45 makes few other functional changes to its proteome, suggesting it has a high level of
46 inherent resistance to CO.

47

48 **Introduction**

49 Carbon monoxide (CO) is notorious as a noxious gas, largely due to the acute
 50 toxicity observed in humans and higher vertebrates upon inhalation (1). However,
 51 while CO is an energy-rich molecule that can be oxidized to yield low-potential
 52 electrons, it is largely chemically inert under physiological conditions (2). The toxicity
 53 of CO stems from its tendency to form carbonyl complexes with transition metals (3),
 54 specifically the Fe ion of heme groups, which are indispensable for many
 55 biochemical processes (4). Despite considerable knowledge of the chemistry of CO-
 56 metal interactions, the specific cellular targets of CO toxicity are still only partially
 57 defined (5). Toxicity at a cellular level is thought to arise primarily from competitive
 58 inhibition of heme-containing respiratory enzymes, i.e. heme-copper terminal
 59 oxidases; however, given the abundance of heme-containing proteins in most cells,
 60 this is unlikely to be the sole target (6).

61 While CO is acutely toxic to mammals, the evidence demonstrating this gas is also
 62 potentially toxic towards bacteria is equivocal. A number of studies working with
 63 diverse bacterial species have demonstrated that, while gaseous CO is inhibitory to
 64 bacteria, this inhibition is only observed at high CO partial pressures (2-30%), is
 65 transient, and acts to slow rather than halt bacterial growth (7-10). Studies treating
 66 bacteria with CO-releasing molecules (CORMs) have reported more acute toxicity
 67 and a bactericidal mode of action, which was attributed to CO (10, 11). However,
 68 subsequent analysis of the effect of CORMs strongly suggests that this toxicity is
 69 largely due to the transition metal complex that constitutes many CORMs, rather
 70 than toxicity of CO (12, 13). Further investigation is required to determine the extent
 71 to which CO is toxic to bacteria, the molecular targets of toxicity, and how bacteria
 72 are able to grow at high partial pressures of CO.

73 The role of CO in the physiology of *Mycobacterium* has remained controversial. This
 74 diverse actinobacterial genus includes saprophytic species such as *Mycobacterium*
 75 *smegmatis*, which are prevalent in soils (14). The genus also includes numerous
 76 human and animal pathogens, including *Mycobacterium tuberculosis*, the causative
 77 agent of tuberculosis, which is currently the world's deadliest infectious disease (15).
 78 Mycobacterial species, including *M. smegmatis* and *M. tuberculosis*, seem to be
 79 relatively resistant to CO and exhibit robust growth in the presence of a 20-30% CO
 80 atmosphere (8, 16). Furthermore, both *M. smegmatis* and *M. tuberculosis* possess
 81 the enzyme CO dehydrogenase to use CO as an energy source; it was recently
 82 demonstrated that *M. smegmatis* enhances its long-term survival by scavenging
 83 atmospheric CO when exhausted for preferred organic energy sources (9).

84 Mycobacteria are obligate aerobes, meaning they require a functioning aerobic
 85 respiratory chain to grow (17). As a result, the inhibitory effect of CO on the oxygen-
 86 binding heme groups of the terminal oxidases must be resisted for these bacteria to
 87 grow in the presence of CO. The mycobacterial respiratory chain is branched, with
 88 the final reduction of molecular oxygen mediated by one of two terminal oxidases:
 89 the cytochrome *bcc-aa₃* complex or cytochrome *bd* oxidase (18). The cytochrome
 90 *bcc-aa₃* complex is a supercomplex composed of components analogous to
 91 mitochondrial complex III and IV; it is primarily synthesized under optimal growth
 92 conditions (19) and is the more efficient of the two oxidases given it acts as a proton
 93 pump (18). Alternatively, cytochrome *bd* oxidase is non-proton pumping and is
 94 therefore less efficient, but is thought to have a higher O₂ affinity and is induced
 95 during hypoxia (20, 21). Cytochrome *aa₃* oxidases are members of the heme-copper
 96 oxidase superfamily with binuclear heme-copper active sites that are highly
 97 susceptible to inhibition by ligands like CO, nitric oxide (NO), cyanide (CN⁻) and

hydrogen sulphide (H₂S) (22). Cytochrome *bd* oxidases are unrelated to heme-copper oxidases and utilise dual *b* and *d* hemes in their active site for O₂ reduction (21). In a number of bacteria, cytochrome *bd* oxidase is important for resistance to oxidative and nitrosative stress, as well as to NO, CN and H₂S, suggesting its active site is less susceptible to inhibition by non-O₂ ligands (23-25). However, it remains to be determined whether it plays a similar role in bacterial resistance to CO.

In addition to acting as an alternative energy source at low concentrations and respiratory toxin at high concentrations, CO has been shown to influence gene expression in mycobacteria, via the two-component DosS/DosR system (8, 26). The sensor histidine kinase DosS is a hemoprotein that activates the transcriptional regulator DosR via phosphorylation in response to low oxygen, low redox state or via binding of ligands to its heme functional group (27). The DosS/DosR regulator has been most thoroughly characterized in *M. tuberculosis*, which possesses an additional sensor kinase designated DosT that acts synergistically with DosS to modulate DosR function (28). In *M. tuberculosis*, the *dos* regulon contains 48 genes and contributes to survival during hypoxia-induced dormancy (27, 29, 30). While the Dos response plays a role in adaption to hypoxia and for resistance to respiratory inhibition by NO, the physiological role of its activation in response to CO in *M. tuberculosis* has not been determined (8, 26, 30). Moreover, in *M. tuberculosis*, *dos* activation in response to CO is much less pronounced than to NO and may potentially be a non-specific effect relating to the inherent affinity of CO for heme functional groups (8). In *M. smegmatis*, the *dos* regulon plays a similar role to *M. tuberculosis* in preparing cells for hypoxia and is largely analogous, with the notable addition of the hydrogenases Hhy and Hyh, which are responsible respectively for the oxidation hydrogen as an energy source and for fermentative hydrogen

production (17, 31-33). The activation of the *dos* response by CO and a potential role in CO resistance in *M. smegmatis* has not previously been investigated.

In this study, we sought to determine the effect of CO on *M. smegmatis* throughout its growth cycle. To achieve this goal, we combined systematic analysis of *M. smegmatis* growth in the presence of CO with whole-cell shotgun proteomics to determine the response of *M. smegmatis* to growth in CO at the protein level. To place these data in a functional context, we utilised terminal oxidase mutants to directly determine the ability of the *M. smegmatis* terminal oxidases to support respiration and growth in the presence of CO. Our results show that *M. smegmatis* utilises cytochrome *bd* oxidase as a primary means of resisting inhibition of its respiratory chain by CO. The mutant strain lacking cytochrome *bd* oxidase is impaired in its ability to grow in the presence of CO. Cytochrome *bd* oxidase is induced during growth in CO, and its oxidase activity is resistant to the presence of CO. These data provide the first direct evidence of the role of this terminal oxidase in resistance to CO in mycobacteria.

Results

***M. smegmatis* induces cytochrome *bd* oxidase and the *dos* regulon during growth in the presence of CO**

In order to determine the effect of CO on *M. smegmatis*, we systematically compared the growth of wild-type *M. smegmatis* in glycerol-containing minimal media in sealed vials, under an atmosphere with either 20% N₂ or 20% CO. Growth of *M. smegmatis* was slightly slower in the presence of 20% CO (Figure 1A), though exponential-

phase growth rate and final growth yield were near-identical under both conditions (Figure 1B, D). The lag phase during growth in 20% CO was 1.3-fold longer than in 20% N₂, accounting for the slight difference in growth. This suggests that *M. smegmatis* is initially inhibited by CO, but grows normally after adapting to the gas, likely through gene expression changes (Figure 1C). In order to identify changes in the *M. smegmatis* proteome that may account for this adaption to CO, we performed proteomic analysis on *M. smegmatis* cultures in the presence and absence of 20% CO during mid-exponential phase.

Proteomic analysis showed that 37 proteins are differentially abundant in response to growth in the presence of 20% CO, with 27 proteins more abundant and 10 less abundant (Figure 2A, Table S1). The cytochrome *bd* oxidase subunits CydA and CydB were highly induced (24- and 4.8-fold) in response to growth in 20% CO, while levels of cytochrome *bcc-aa₃* (QcrCAB) were unaffected (Figure 2A, Table S1). This suggests that in mycobacteria, cytochrome *bd* oxidase is induced to adapt to growth in CO and may be inherently resistant to inhibition by this gas. This is consistent with previous observations that other bacteria deploy cytochrome *bd* oxidase to cope with inhibition of cytochrome *aa₃*-type heme-copper oxidases by gaseous molecules like NO, CN and H₂S (23-25). Fifteen of the most induced proteins belong to the *dos* regulon, representing a subset the regulon, which includes the regulator of the pathway DosR (5.0-fold), universal stress protein family proteins (98 to 5.7-fold), and Acg (1007-fold) and Fsq (72-fold), two proteins that protect against oxidative stress through the sequestration of flavins (34, 35). The full *dos* regulon in *M. smegmatis* contains 49 proteins (17). Notable *dos*-regulated proteins not induced by CO include the hydrogenases Hhy or Hyh, which are important for the response to starvation and hypoxia respectively (17, 36).

There were fewer proteins with decreased abundance in response to CO exposure. The ESX-3 type-VII secretion system component EccC3, known to be involved in the export of proteins important for iron acquisition (37), exhibited the largest decrease in abundance (15-fold). Correlating with this, the acyl-dehydrogenase MbtN involved in synthesis of the iron binding siderophore mycobactin (38) was also less abundant (10-fold). The decreased abundance of proteins involved in iron acquisition suggests that *M. smegmatis* does not experience iron-limitation during growth in CO, due to iron sequestration in Fe-carbonyl complexes. Iron limitation induced by this mechanism was proposed for *E. coli*, which was shown to transcriptionally upregulate iron-acquisition systems in response to CO (39).

The 37 proteins with differential abundance in response to CO comprise only 0.56% of the total 6,625 proteins predicted to be synthesized by *M. smegmatis* mc²155 (40). This is in contrast to *E. coli*, where 20% of genes were shown to be differentially regulated at a transcriptional level 80 minutes after exposure CO gas (39). This suggests that *M. smegmatis* has a high level of inherent resistance to CO toxicity, requiring relatively few changes to its proteome to cope with this gas. Among proteins that were not differentially abundant during growth in CO were Cor and CO dehydrogenase. Cor was reported to be the most important gene for CO resistance in *M. tuberculosis* (41), and is highly conserved in *M. smegmatis* (MSMEG_3645, 94% amino acid identity); while the exact function of this protein remains unresolved, this results suggests Cor-mediated CO resistance is independent of induction by CO. The lack of induction of CO dehydrogenase in response to CO is consistent with previous findings that this enzyme is deployed to utilise trace quantities of CO as an energy source during persistence (9).

195

196 **Cytochrome *bd* oxidase, but not DosR, contributes to CO resistance**

197 Our proteomic analysis demonstrates that *M. smegmatis* induces cytochrome *bd*
 198 oxidase and members of the *dos* regulon in response to CO. To determine the role
 199 of the terminal oxidases and the *dos* regulon in resistance to CO, we systematically
 200 assessed the growth of *M. smegmatis* strains with genetic deletions to the
 201 cytochrome *bcc-aa₃* oxidase ($\Delta qcrCAB$), cytochrome *bd* oxidase ($\Delta cydAB$), and the
 202 regulator DosR ($\Delta dosR$), as above, in the presence of 20% CO or 20% N₂. Growth of
 203 the $\Delta dosR$ strain was identical to wild-type in both the N₂- and CO-supplemented
 204 conditions (Figure 1A). This shows that the inability of the $\Delta dosR$ strain to induce
 205 the *dos* regulon has no effect on growth in CO and suggests that the partial induction
 206 of the *dos* regulon by CO is a non-specific rather than adaptive response.

207 There were significant differences in the growth characteristics of the terminal
 208 oxidase mutants in the presence and absence of CO. In line with previous
 209 observations (42), the $\Delta qcrCAB$ mutant exhibited a longer lag phase, slower specific
 210 growth rate, and lower final growth yield compared to the wild-type, whereas the
 211 $\Delta cydAB$ mutant exhibited only minor growth defects (Figure 1A-C). The growth of the
 212 $\Delta qcrCAB$ strain did not differ in the presence and absence of CO, suggesting that
 213 cytochrome *bd* oxidase is inherently resistant to inhibition by CO (Figure 1A-D). In
 214 contrast, the $\Delta cydAB$ strain grew markedly slower in the presence of CO compared
 215 to growth in the 20% N₂-amended control. This slower growth is largely attributable
 216 to an increase in lag phase, as the specific growth rate of the $\Delta cydAB$ strain during
 217 exponential phase in CO was only slightly lower than wild-type (Figure 1B). The
 218 increase in lag phase of the $\Delta cydAB$ strain in CO was 2.5-fold longer than for wild-

type *M. smegmatis* (Figure 1C). This is consistent with our proteomic analysis that shows cytochrome *bd* oxidase is induced in response to CO, and demonstrates that this terminal oxidase is important for adaptation to growth in CO. Interestingly, final growth yield was significantly higher in the wild-type and $\Delta cydAB$ strains when grown in CO (Figure 1D), suggesting that oxidation of CO by CO dehydrogenase may provide an alternative energy source that enhances biomass generation under these conditions.

In order to determine if additional proteome changes occur during the adaptation of *M. smegmatis* terminal oxidase mutants to CO, we performed proteomic analysis of these strains during exponential growth in the presence and absence of 20% CO. In the absence of CO, the $\Delta qcrCAB$ mutant significantly increased synthesis of the cytochrome *bd* oxidase subunits CydA (52-fold) and CydB (9.6-fold) compared to the wild-type (Table S1), likely in order to compensate for the loss of the main terminal oxidase. In the $\Delta qcrCAB$ strain, only 28 proteins significantly differed in abundance in the presence of CO (23 higher, 5 lower) (Figure 2C-E, Table S1), including the same 15 proteins of the *dos* regulon and multiple hypothetical proteins (Figure 2C, D; Table S1). The lack of additional significant changes to the proteome of this strain suggests that it is inherently resistant to CO. In the $\Delta cydAB$ strain, the abundance of a larger subset of 77 proteins changed in response to CO (47 higher, 30 lower) (Table S1). Most of these differentially regulated proteins are poorly characterised, making it difficult to assess their role in adaptation to CO. Other than the *dos* regulon, the induced proteins include enzymes from the thiamine biosynthetic pathway (ThiC, 8.2-fold; ThiD, 3.7-fold), histidine biosynthetic pathway (HisD, 4.9-fold) and a NAD(P)⁺ transhydrogenase (6.8-fold) (Table S1), suggesting considerable metabolic remodelling. Overall, the additional proteome changes

observed in the $\Delta cydAB$ mutant suggest a larger-scale response to cope with increased respiratory inhibition due to the loss of cytochrome *bd* oxidase.

Cytochrome *bd* oxidase is resistant to CO inhibition in actively growing *M. smegmatis* cells

Our finding that in *M. smegmatis* cytochrome *bd* oxidase is important for optimal growth in the presence of CO and is induced under these conditions led us to hypothesise that this enzyme may be inherently resistant to inhibition by CO. To test this hypothesis, O₂ consumption was monitored amperometrically in *M. smegmatis* wild-type, $\Delta qcrCAB$, and $\Delta cydAB$ strains during mid-log phase; cells were spiked with glycerol to simulate respiration, followed by treatment with CO-saturated buffer. Spiking of glycerol stimulated O₂ consumption in all strains (Figure S1). Consistent with a high sensitivity of the cytochrome *bcc-aa*₃ complex to inhibition by CO, complete inhibition of O₂ consumption was observed in the $\Delta cydAB$ mutant upon addition of CO. Inhibition of the wild-type strain was significant but less pronounced than for the $\Delta cydAB$ mutant, while the $\Delta qcrCAB$ mutant was not significantly inhibited by CO (Figure 3A). These data are consistent with our hypothesis and indicate that the *M. smegmatis* terminal oxidases are differentiated in their sensitivities to CO poisoning.

Recently, we demonstrated that *M. smegmatis* is able to utilise the trace quantities of CO present in the atmosphere as an energy source during starvation, via the CO dehydrogenase (9). This study demonstrated that the addition of CO leads to enhanced O₂ consumption in *M. smegmatis*, suggesting that electrons derived from CO oxidation enter the respiratory chain, though the specific terminal oxidases

involved in this process were not determined (9). To test this, we spiked carbon-limited (3 days post-OD_{max}) *M. smegmatis* wild-type, $\Delta qcrCAB$, and $\Delta cydAB$ cultures with CO-saturated buffer and amperometrically monitored O₂ consumption. Upon addition of CO, all cultures consumed O₂, with consumption of the $\Delta cydAB$ culture 1.7-fold less than wild-type (Figure 3B). Conversely, O₂ consumption in the $\Delta qcrCAB$ culture was 1.5-fold higher than wild-type (Figure 3B). Inhibition of cytochrome *bcc-aa₃* through the addition of zinc azide in the $\Delta cydAB$ mutant completely abolished CO-dependent O₂ consumption (Figure 3B).

These data demonstrate that electrons generated by CO oxidation by CO dehydrogenase can be donated to either terminal oxidase. Furthermore, the complete inhibition of O₂ consumption by zinc azide in the $\Delta cydAB$ mutant shows that O₂-dependent CO oxidation is obligately coupled to the terminal oxidases of the respiratory chain. The higher rate of O₂ consumption in the $\Delta qcrCAB$ mutant may result from the insensitivity of cytochrome *bd* to inhibition by CO. In the wild-type and $\Delta cydAB$ strains, it is likely that the addition of CO concurrently stimulates O₂ consumption by providing electrons to the electron transport chain and inhibits respiration through inhibition of cytochrome *bcc-aa₃* oxidase. As cytochrome *bd* oxidase is insensitive to CO, in the $\Delta qcrCAB$ mutant only a stimulatory effect is observed. The respiration observed upon addition of CO to $\Delta cydAB$ strain during carbon-limitation contrasts with the complete inhibition observed when CO is added to actively growing, glycerol-stimulated cells (Figure 3A, B). This suggests that, as reported for mitochondria (22), differences in the respiratory chain redox state or a higher intracellular O₂ concentration of carbon-starved cells make cytochrome *bcc-aa₃* oxidase less susceptible to inhibition by CO under these conditions.

292

293 Discussion

294 Previous investigations showed that *M. smegmatis*, and mycobacteria more
 295 generally, exhibit considerable resistance to CO (8, 9, 16). However, prior to our
 296 current work, no underlying mechanism for CO resistance in mycobacteria had been
 297 determined. Here we show that induction of cytochrome *bd* oxidase is the key
 298 adaptive response for CO resistance in *M. smegmatis*, as the function of this oxidase
 299 is largely unaffected by high concentrations of CO. This finding adds to the growing
 300 body of evidence that cytochrome *bd* oxidases play a general role in the resistance of
 301 the bacterial respiratory chain to gaseous inhibitors (43). The resistance of
 302 cytochrome *bd* oxidase to CO in *M. smegmatis* is consistent with a previous report
 303 showing that, in *E. coli*, a mutant possessing only cytochrome *bd-I* oxidase is less
 304 sensitive to growth inhibition by the CO producing molecule CORM-3 than mutants
 305 possessing only the other terminal oxidases (cytochrome *bd-II* and cytochrome *bo'*)
 306 of *E. coli* (44). However, a recent study demonstrated that purified cytochrome *bd-I*
 307 and *bd-II* oxidases from *E. coli* are more sensitive to inhibition by gaseous CO than
 308 cytochrome *bo'* oxidase (45). The difference between these findings may result from
 309 the use of CORM-3 for CO delivery in the former study. CORM-3 is known to exert
 310 cytotoxic effects independent of CO, making the role of cytochrome *bd-I* oxidase in
 311 CO resistance in *E. coli* uncertain (12). As such, our current work represents the first
 312 definitive evidence that bacterial cytochrome *bd* oxidases display inherent resistance
 313 to CO.

314 The proteomic analyses showed surprisingly few proteins are differentially abundant
 315 in *M. smegmatis* during growth in a 20% CO atmosphere. Fifteen of the most

induced proteins, and the only proteins consistently induced in both the wild-type and terminal oxidase mutant backgrounds belong to the *dos* regulon of *M. smegmatis* (17). Considering that our growth data shows that DosR is not required for adaptation to growth in CO, the induction of these proteins is unlikely to be an adaptive response to CO. This further reduces the number of adaptive proteomic changes observed in response to CO. Excluding DosR regulated proteins and cytochrome *bd* oxidase, only 21 proteins are differentially abundant during growth of wild-type *M. smegmatis* in CO, with the majority of these changes less than 5-fold compared to growth in air. This is in contrast to the observation that in *E. coli* approximately 20% of the genome is differentially regulated at a transcriptional level in response to growth in CO (39). This together suggests that, aside from the resistance of its electron transport chain to CO inhibition mediated by cytochrome *bd* oxidase, *M. smegmatis* is otherwise highly resistant to CO.

Previously we showed that, in *M. smegmatis*, CO dehydrogenase is active during non-replicative persistence (9). This provides *M. smegmatis* with the ability to utilise CO at atmospheric concentrations (100 ppbv) as an alternative energy source in the absence of organic substrates (9). Our current work shows that electrons derived from CO are donated to O₂ via either of the two terminal oxidases, obligately coupling CO dehydrogenase to the aerobic respiratory chain. The fact that cytochrome *bcc-aa₃* oxidase is able to accept electrons from CO oxidation, despite the fact that it is inhibited by the gas, is seemingly paradoxical. However, CO is a competitive inhibitor of O₂ binding by heme-copper oxidases and CO dehydrogenase in *M. smegmatis* is a high-affinity enzyme that operates at very low CO partial pressures (9, 22). This means that, at the low concentrations of CO that are

physiologically relevant for CO dehydrogenase activity in *M. smegmatis*, no significant inhibition of cytochrome *bcc-aa₃* oxidase would be observed.

Despite the suggestion that CO has antibacterial potential against pathogenic mycobacteria and numerous other bacterial species (5, 13, 46), surprisingly little is known about physiological and biochemical effects of gaseous CO on the bacterial cell. The uncertainty regarding the effects of CO on bacteria is confounded by the fact that much of the work testing the effects of CO has been performed using CORMs, which have antibiotic activity in addition to the effects of CO release (12). In this work we have shown that *M. smegmatis* has a high level of resistance to CO that requires relatively few changes to its proteome. If these findings extend to pathogenic mycobacteria, then it is unlikely that CO, either produced by the host via heme oxygenase 1 (8) or delivered exogenously, will exert a significant antibacterial effect on pathogenic members of the genus.

Methods

Growth experiments

Mycobacterium smegmatis mc²155 (47), $\Delta qcrCAB$, and $\Delta cydAB$ mutant strains were maintained on lysogeny broth (LB) agar plates supplemented with 0.05% (w/v) Tween80. For broth culture, *M. smegmatis* was grown on Hartmans de Bont minimal medium (48) supplemented with 0.05% (w/v) tyloxapol and 2.9 mM glycerol. Liquid cultures were incubated at 37°C on a rotary incubator at ~180 rpm. For growth assays, triplicate cultures of *M. smegmatis* was grown in 30 mL media in 120 mL sealed serum vials. After inoculation, culture headspace was amended to either 20% CO (via 100% v/v CO gas cylinder, 99.999% pure) or 20% N₂ (via 100% v/v N₂ cylinder, 99.999% pure). N₂ was added to account for removed O₂. Growth was monitored by measuring optical density (OD) at 600 nm (1 cm cuvettes, Eppendorf BioSpectrometer Basic). When OD₆₀₀ was above 0.5, culture was diluted ten-fold before reading.

Proteomic analysis

For shotgun proteomic analysis, 30 mL cultures of *M. smegmatis* were grown on Hartmans de Bont minimal medium (48) supplemented with 0.05% (w/v) tyloxapol and 5.8 mM glycerol in triplicate in 120 mL serum vials sealed with rubber butyl stoppers. Immediately after inoculation, one triplicate set was amended with 20% CO (via 100% v/v CO gas cylinder, 99.999% pure). Cells were harvested in mid-exponential phase (OD₆₀₀ ~0.3) by centrifugation (10,000 × g, 10 min, 4 °C). They were subsequently washed in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄, pH 7.4), recentrifuged, and resuspended in 100 mM Tris + 4% SDS at a weight:volume ratio of 1:4. The resultant

suspension was then lysed by beat-beating with 0.1 mm zircon beads for five 30 second cycles. To denature proteins, lysates were boiled at 95 °C for 10 min, then sonicated in a Bioruptor (Diagenode) using 20 cycles of ‘30 seconds on’ followed by ‘30 seconds off’. The lysates were clarified by centrifugation (14,000 × *g*, 10 mins). Protein concentration was confirmed using the bicinchoninic acid assay kit (Thermo Fisher Scientific) and normalized for downstream analyses. After removal of SDS by chloroform/methanol precipitation, the proteins were proteolytically digested with trypsin (Promega) and purified using OMIX C18 Mini-Bed tips (Agilent Technologies) prior to LC-MS/MS analysis. Using a Dionex UltiMate 3000 RSL Cnano system equipped with a Dionex UltiMate 3000 RS autosampler, the samples were loaded via an Acclaim PepMap 100 trap column (100 μm × 2 cm, nanoViper, C18, 5 μm, 100 Å; Thermo Scientific) onto an Acclaim PepMap RSLC analytical column (75 μm × 50 cm, nanoViper, C18, 2 μm, 100 Å; Thermo Scientific). The peptides were separated by increasing concentrations of buffer B (80% acetonitrile / 0.1% formic acid) for 158 min and analyzed with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) operated in data-dependent acquisition mode using in-house, LFQ-optimized parameters. Acquired .raw files were analyzed with MaxQuant 1.6.5.0 (ref: PMID: 19029910) to globally identify and quantify proteins pairwise across the different conditions. Data visualization and statistical analyses were performed in R studio (49) with the ggplot2 package (50).

Respirometry measurements

For respirometry measurements, 30 mL cultures of wild-type, $\Delta qcrCAB$, and $\Delta cydAB$ *M. smegmatis* were grown on Hartmans de Bont minimal medium (48) supplemented

with 0.05% (w/v) tyloxapol and 5.8 mM glycerol to mid-exponential ($OD_{600} = 0.3$) or mid-stationary phase (72 hours post $OD_{max} \sim 0.9$) in 125 mL aerated conical flasks. Rates of O_2 consumption were measured using a Unisense O_2 microsensor in 1.1 mL microrespiration assay chambers that were stirred at 250 rpm at room temperature. Prior to measurement, the electrode was polarized at -800 mV for 1 hour with a Unisense multimeter and calibrated with O_2 standards of known concentration. Gas-saturated phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4) was prepared by bubbling PBS with 100% (v/v) of either O_2 or CO for 5 min. To determine how CO affected respiration in growing cells, 0.9 mL mid-exponential phase cultures of either *M. smegmatis* wild-type, $\Delta qcrCAB$, or $\Delta cydAB$ strains and 0.1 mL O_2 -saturated PBS were loaded onto respiration chambers and baseline rate of O_2 consumption was measured. Following this, glycerol (3.5 mM final concentration) and 0.1 mL CO-saturated PBS were sequentially amended into the chamber and O_2 consumption was measured before and after addition of CO. To determine the effect of CO in carbon-starved cells, initial oxygen consumption was measured in assay chambers sequentially amended with mid-stationary phase *M. smegmatis* cell suspensions (0.9 mL) and O_2 -saturated PBS (0.1 mL). After initial measurements, 0.1 mL of CO-saturated PBS was added into the assay mixture. Additionally, O_2 consumption was measured in $\Delta cydAB$ strain treated with 250 μ M zinc azide. Changes in O_2 concentrations were recorded using Unisense Logger Software (Unisense, Denmark). Upon observing a linear change in O_2 concentration, rates of consumption were calculated over a period of 20 s and normalized against total protein concentration.

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Author contributions

C.G. and R.G. conceived and supervised the study. C.G., K.B., P.R.F.C., and R.G. designed experiments. K.B., P.R.F.C., and C.H. performed experiments. K.B., P.R.F.C., C.G., R.G., and C. H. analysed data. K.B., R.G., P.R.F.C., and C.G. wrote the paper with input from all authors.

Data Availability

All raw proteomic data have been deposited at PRIDE with the dataset identifier: PXD018382.

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Figures

Figure 1. Growth of *M. smegmatis* wild-type and terminal oxidase mutants in air supplemented with either 20% CO or 20% N₂. (A) Growth curves of *M. smegmatis* wild-type, terminal oxidase, and *dosR* mutants grown in sealed culture vials in the presence of air supplemented with 20% CO or 20% N₂. The specific growth rate (B), length of lag phase (C) and maximum culture density (D) for each strain is also shown. Different letters above data bars (A, B, C, D) designate significantly different values (*p*-value < 0.05, two-way ANOVA).

Figure 2. Shotgun proteomic analysis of *M. smegmatis* wild-type and terminal oxidase mutants at mid-log phase grown in air in the presence and absence of 20% CO. Volcano plots showing differential detection of proteins in (A) wild-type, (B) $\Delta cydAB$, and (C) $\Delta qcrCAB$ strains in air in with and without 20% CO. Each protein is represented by a single point. DosS/DosR regulon proteins are represented by black dots, and proteins of interest are highlighted as per the legend. Strains were harvested at mid-exponential phase ($OD_{600} \sim 0.3$). Venn diagrams showing overlap of proteins identified by the shotgun proteomics with higher (D) and lower (E) abundances in wild-type, $\Delta qcrCAB$, $\Delta cydAB$ mutant strains grown in air + 20% CO.

Figure 3. Oxygen consumption of *M. smegmatis* terminal oxidases in the presence of CO (A) Rate of O₂ consumption by *M. smegmatis* wild-type, $\Delta qcrCAB$, and $\Delta cydAB$ mid-exponential, glycerol-spiked cultures in the presence and absence of CO. ns = non-significant, * = $p < 0.05$, *** = $p < 0.0005$ (B) Rates of O₂ consumption of carbon-starved (3-days post OD_{max}) *M. smegmatis* wild-type, $\Delta qcrCAB$, and $\Delta cydAB$ cultures before and after spiking with CO. O₂ consumption was measured with an oxygen electrode. Azide is a compound that targets the *bcc-aa₃* cytochrome oxidase and therefore acts as a negative control. Error bars

642 represent standard deviation of three biological replicates. *Different letters above*
643 *data bars (A , B, C) designate to significantly different values (p-value <0.05, two-*
644 *way ANOVA).*

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Supplemental Data

Table S1. Summary of shotgun proteomic data (xlsx file).

Figure S1. Stimulation of O₂ in *M. smegmatis* upon addition of glycerol. The specific O₂ consumption rate of mid-exponential phase *M. smegmatis* wild-type and terminal oxidase mutants, before and after the addition of 3.5 mM glycerol.