Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in *Mycobacterium tuberculosis*

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*Mycobacterium tuberculosis* is a chronic, facultative intracellular pathogen that spends the majority of its decades-long life cycle in a non- or slowly replicating state. However, the bacterium remains poised to resume replicating so that it can transmit itself to a new host. Knowledge of the metabolic adaptations used to facilitate entry into and exit from nonreplicative states remains incomplete. Here, we apply 13C-based metabolomic profiling to characterize the activity of *M. tuberculosis* tricarboxylic acid cycle during adaptation to and recovery from hypoxia, a physiologically relevant condition associated with nonreplication. We show that, as *M. tuberculosis* adapts to hypoxia, it slows and remodels its tricarboxylic acid cycle to increase production of succinate, which is used to flexibly sustain membrane potential, ATP synthesis, and anaplerosis, in response to varying degrees of O2 limitation and the presence or absence of the alternate electron acceptor nitrate. This remodeling is mediated by the bifunctional enzyme isocitrate lyase acting in a noncanonical role distinct from fatty acid catabolism. Isocitrate lyase-dependent production of succinate affords *M. tuberculosis* with a unique and bioenergetically efficient metabolic means of entry into and exit from hypoxia-induced quiescence.

Q uiescence, or exit from cell cycle, is a physiologic prerogative of all cells, executed irreversibly by some upon terminal differentiation and reversibly by others as they adapt to changing conditions (1). For *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), quiescence has emerged as a hallmark of its pathogenicity. *M. tuberculosis* infects approximately one in every three people worldwide and is the leading bacterial cause of death. Following infection, *M. tuberculosis* enters a clinically asymptomatic state of non- or slowly replicating physiology that often lasts decades, if not the lifetime, of the infected host, and exhibits a form of nonheritable resistance to nearly all TB drugs that has hindered mass eradication strategies (2). Clinical TB is nonreplicating. This is thought to arise when *M. tuberculosis* reenters cell cycle and provokes an inflammatory response that inflicts host tissue damage and enables it to transmit itself to a new host. However, some of the *M. tuberculosis* in active TB is nonreplicating. This is thought to impose the need for chemotherapies that are longer and more sustained production of ATP, regeneration of NAD, and maintenance of proton motive force are required to preserve viability. Previous work identified a genetic regulon (DosR) essential for mediating adaptation to O2 limited environments and during reversible shifts between aerobic and anaerobic respiration (13, 20–23). However, the metabolic changes accompanying *M. tuberculosis* exit and entry into cell cycle remain incompletely defined.

Here, we applied liquid chromatography–time-of-flight mass spectrometry to monitor the pool size and turnover of metabolites in *M. tuberculosis* during its transitions through hypoxia-induced quiescence, focusing on intermediates of its putative tricarboxylic acid (TCA) cycle. The TCA cycle consists in a highly conserved set of biochemical reactions that serve to generate ATP, biosynthetic precursors and reducing equivalents. Recent work, using a chemostat model, showed that hypoxic *M. tuberculosis* metabolize glucose through a reverse TCA cycle to generate succinate as an obligatorily secreted fermentation product (24). However, prevailing evidence has implicated fatty acids and lipids as key carbon sources encountered by *M. tuberculosis* in the host, whose metabolism could not be readily supported by the foregoing mechanism (10, 25–28). We therefore sought to expand our understanding of the scope and nature of metabolic adaptations used by *M. tuberculosis* to enter into, reside in, and exit from hypoxia-induced quiescence.

**Results**

**Replicative Quiescence of *M. tuberculosis* at 1% O2.** We adapted our previously described filter culture method to model hypoxia-regulated entry and exit of *M. tuberculosis* into and out of cell cycle by combining a chemically defined agar medium with a disposable environmental chamber that depletes oxygen gradually and generates CO2 via a palladium catalyst (29, 30). This system achieves a final atmosphere of ~1% O2 (as reported by a resazurin-based indicator strip) and ~5% CO2, levels similar to those encountered in the tuberculous lungs of infected animals, within 4 h (31–33). We chose acetate as a carbon source based on genetic evidence implicating fatty acids as a potential carbon source encountered by *M. tuberculosis*.

Long been considered a feature faced by *M. tuberculosis* in humans and in some experimental animal models (11–15), *M. tuberculosis* exposed to hypoxia in vitro has been shown to cease replication but some proportion remain viable and virulent for decades, tolerant to nearly all first and second line TB drugs (16, 17).

Hypoxic *M. tuberculosis* down-regulates transcription of key complexes of the electron transport chain (ETC) and maintains ATP levels approximately five times lower than those of replicating counterparts (18, 19). However, even under hypoxic conditions, sustained production of ATP, regeneration of NAD, and maintenance of proton motive force are required to preserve viability. Previous work identified a genetic regulon (DosR) essential for mediating adaptation to O2 limited environments and during reversible shifts between aerobic and anaerobic respiration (13, 20–23). However, the metabolic changes accompanying *M. tuberculosis* exit and entry into cell cycle remain incompletely defined.

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**Metabolic Slowing and Remodeling of TCA Cycle Activity in *M. tuberculosis* at 1% O₂.** In response to hypoxia, *M. tuberculosis* decreased nutrient uptake and/or consumption by 80%, as reported by residual [U-13C] acetate levels in the medium (Fig. S2A). This decrease was accompanied by a slowdown in TCA cycle activity, as reported by the [13C]-labeling patterns of its intermediates (Fig. S2B–D), and reached a metabolic steady state after 12–24 h. In addition, all changes could be restored with reoxygenation (Fig. S3A and B). Canoinal TCA cycle activity results in the progressive assimilation of acetate-based C₂ units, manifest by the accumulation of higher order [13C₂]-based isotopologues when cells are grown in [U-13C] acetate-containing medium. The coordinate downshift of dominant [13C]-labeled isotopologues for all TCA cycle intermediates from +6 (or +4) to +2 [13C]-labeled forms (Fig. S4A and B) thus revealed hypoxia-induced slowing of TCA cycle activity. Consistent with this slowing, we observed an accompanying decrease in gluconeogenic carbon flow, reported by a near complete loss of [13C]-labeling of pyruvate (Fig. 1 and Figs. S3B and S4B). [13C]-Labeling analysis of TCA cycle activity of quiescent *M. tuberculosis* (preadapted to 1% O₂) confirmed a sustained downshift of [13C₂]-labeled isotopologues for TCA cycle intermediates compared with those from aerobic controls (Fig. S4A and B).

In addition to the broad quantitative decrease in TCA cycle activity described above, we discovered a more complex pattern of changes in the levels and labeling patterns of specific TCA cycle intermediates. In response to O₂ depletion, the level of α-ketoglutarate decreased threefold, whereas levels of succinate, malate, and aspartate (a surrogate of oxaloacetate) increased 6.5-, 1.4-, and 1.8-fold, respectively (Fig. 1). This pattern was indicative of an activation of *M. tuberculosis* isocitrate lyases (ICLs), which serve dual roles in the glyoxylate shunt and methylcitrate cycles, and have previously been reported to be up-regulated in *M. tuberculosis* (preadapted to 1% O₂) (Fig. S4), a sustained downshift of [13C₂]-labeled isotopologues for TCA cycle intermediates compared with those from aerobic controls (Fig. S4A and B).
viability by adding exogenous succinate to the culture medium as a functional means of inhibiting secretion or efflux of intracellular succinate by concentration-dependent mechanisms (40). We first confirmed that the concentrations of added succinate neither altered the pH of the extracellular medium nor affected M. tuberculosis growth under aerobic conditions. We next showed that exogenous succinate impaired secretion of intracellular succinate produced from metabolism of 13C-labeled acetate in hypoxic M. tuberculosis, as predicted (Fig. S7B). We finally showed that incubation with exogenous succinate selectively impaired both the membrane potential and survival of hypoxic, but not aerated M. tuberculosis, as reported by the fluorescent, membrane-permeable dye 3,3′-diethyloxycarbocyanide chloride (DiOC2) and CFUs, respectively (Fig. 3 B and C and Fig. S7C) (18, 41). These studies thus establish that succinate secretion is a specific and essential biochemical component of M. tuberculosis adaptive response to hypoxia.

### Metabolic Essentiality of Succinate Dehydrogenase Activity in Adaptation to Hypoxia

In addition to establishing a role for succinate in sustaining membrane potential, we also evaluated its canonical role as a substrate of succinate dehydrogenase (SDH). SDH couples the oxidation of succinate to the reduction of ubiquinone to ubiquinol and is the only TCA cycle enzyme that is a component of a reverse TCA cycle and demonstrating that ICL is essential for succinate production in hypoxic conditions. These studies thus established that M. tuberculosis ICL is an essential mediator of adaptation to hypoxia because it generates succinate, and possibly glycine, in a manner distinct from its canonical roles in fatty acid metabolism (27).

#### Succinate-Mediated Maintenance of Membrane Potential in Hypoxic M. tuberculosis

Watanabe et al. (24) reported that, lacking an alternate electron acceptor, anaerobically adapted M. tuberculosis could metabolize glucose through a reverse TCA cycle to offload unrespired reducing equivalents onto fumarate and generate succinate, whose secretion was proposed to help maintain membrane potential. We sought to understand if the increase in succinate production mediated by ICL, rather than by the reverse TCA cycle, might likewise provide M. tuberculosis a means of maintaining membrane potential when metabolizing not only glucose but also acetate in response to O2 depletion. To address this possibility, we first measured succinate secretion from cells; to this end, we modified our filter culture system by replacing the underlying agar medium with a plastic inset containing chemically equivalent liquid medium in direct contact with the underside of the bacteria-laden filter (Fig. S74). Growth atop this liquid medium was indistinguishable from that achieved on adjacent agar medium and enabled timed start-stop measurements of secretion by sampling the cell-free liquid medium. As shown in Figs. 3A, 3C, and S3, these studies showed a time-dependent accumulation of 13C2-labeled succinate in the medium following exposure to and incubation under hypoxic conditions.

Based on these findings, we next tested the impact of inhibiting hypoxia-induced succinate secretion on M. tuberculosis viability by adding exogenous succinate to the culture medium as a functional means of inhibiting secretion or efflux of intracellular succinate by concentration-dependent mechanisms (40). We first confirmed that the concentrations of added succinate neither altered the pH of the extracellular medium nor affected M. tuberculosis growth under aerobic conditions. We next showed that exogenous succinate impaired secretion of intracellular succinate produced from metabolism of 13C-labeled acetate in hypoxic M. tuberculosis, as predicted (Fig. S7B). We finally showed that incubation with exogenous succinate selectively impaired both the membrane potential and survival of hypoxic, but not aerated M. tuberculosis, as reported by the fluorescent, membrane-permeable dye 3,3′-diethyloxycarbocyanide chloride (DiOC2) and CFUs, respectively (Fig. 3 B and C and Fig. S7C) (18, 41). These studies thus establish that succinate secretion is a specific and essential biochemical component of M. tuberculosis adaptive response to hypoxia.

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E. coli. We tested the impact of inhibiting *M. tuberculosis* SDH on viability using the suicide inhibitor 3-nitropropionate (3NP) (42). Treatment with 3NP led to an accumulation, rather than a reduction of newly synthesized 13C succinate or accumulation of methylecitrinate cycle intermediates (Fig. S8 A and B). This suggested that under the conditions used, the dominant activity of 3NP in intact *M. tuberculosis* was as an inhibitor of SDH, rather than ICL. Although 3NP can inhibit isolated ICL (27), 3NP may act preferentially on SDH in intact *M. tuberculosis* because SDH is located at the membrane rather than in the cytosol. Treatment with 3NP resulted in a time-dependent loss of viability of both wild-type and ΔICL KO *M. tuberculosis* during adaptation to hypoxia that was not observed during reaeration of hypoxic conditions used, the dominant activity of 3NP in intact *M. tuberculosis* was as an inhibitor of SDH, rather than ICL. Although 3NP can inhibit isolated ICL (27), 3NP may act preferentially on SDH in intact *M. tuberculosis* because SDH is located at the membrane rather than in the cytosol. Treatment with 3NP resulted in a time-dependent loss of viability of both wild-type and ΔICL KO *M. tuberculosis* during adaptation to hypoxia that was not observed during reaeration of hypoxic *M. tuberculosis* or in aerobically cultured *M. tuberculosis* (Fig. 3 D and E and Fig. S8C). These findings establish that sustained metabolism of succinate through SDH is an additional essential component of *M. tuberculosis* metabolic adaptation to hypoxia.

Nitrate-Dependent Modulation of TCA Cycle Activity in Hypoxic *M. tuberculosis*. The specific biochemical conditions encountered by *M. tuberculosis* in the host are complex and heterogeneous (3, 4, 7, 9, 10). Nitrate is a natural component of human body fluids that arises in part from dietary sources and in part as a terminal autooxidation product of the nitric oxide produced by the three isoforms of nitric oxide synthase in diverse cells, including immune-activated smooth muscle, epithelial cells, and hematopoietic cells, such as macrophages infected with *M. tuberculosis* (11, 15, 43–45). Both nitric oxide and hypoxia increase *M. tuberculosis* nitrate reductase activity at the whole cell level. Nitrate reduction may enable *M. tuberculosis* to maintain respiratory activity in microaerophilic or hypoxic conditions (44–46). Nitrate is the second most efficient terminal electron acceptor after molecular oxygen and *M. tuberculosis* is the mycobacterial species most capable of using it. In addition, recent work has established the essentiality of nitrate during *M. tuberculosis* adaptation to the rapid onset of anaerobiosis and to acidic, microaerophilic environments (44, 45). We therefore characterized the impact of exogenous nitrate on *M. tuberculosis* TCA cycle activity during adaptation to hypoxia. As shown in Figs. 1 and 4A and Fig. S4B, provision of nitrate did not alter the pool sizes of most TCA cycle intermediates but did affect their isotopic labeling patterns and reduced the secretion of succinate, leading to larger intracellular pools, as predicted, and relief from the toxic effects of exogenous succinate observed in Fig. 4B. Feeding with [U-13C] acetate, for example, led to an upshift in the predominant isotopologue of succinate and malate, from that containing two to that containing four 13C atoms, indicative of an increase in glyoxylate shunt-based TCA cycle activity (Fig. S4B). Supplementation of nitrate in culture medium abolished succinate secretion and restored ATP levels, NADH/NAD ratios, and *M. tuberculosis* viability to near aerobic levels (Fig. 4 A and B and Fig. S1 G and H). Nitrate thus regulates both the metabolic and respiratory activity of *M. tuberculosis* during adaptation to hypoxia.

**Discussion**

Unlike most bacterial pathogens, *M. tuberculosis* spends the majority of its natural life cycle in a state of slowed or arrested replication imposed by humans, its only known natural host. *M. tuberculosis* thus faces the unusual challenge of needing to maintain a metabolic state of replicative quiescence for decades while remaining poised to reenter cell cycle at some point to transmit to a new host and propagate itself as a species (4, 7, 12, 13, 47). However, there has been little knowledge of the metabolic adaptations used by *M. tuberculosis* or other cells to transition in and out of cell cycle.

The present work sheds light on one unique set of such adaptations. Recent work showed that *M. tuberculosis* could operate a reductive TCA half cycle under anaerobic conditions, enabling it to metabolize glucose by generating succinate as an obligatorily secreted, fermentation product of fumarate, following reductive carboxylation of pyruvate or phosphoenolpyruvate to malate and/or oxaloacetate (24). However, evidence has strongly implicated lipids and fatty acids as key carbon sources metabolized by *M. tuberculosis* in the host (10, 25–28). A mechanism functioning only with carbohydrates could not readily accommodate the foregoing carbon sources that include even-chain fatty acids. Our studies thus extend these findings with the discovery of a different metabolic pathway (the glyoxylate shunt) that, in contrast to the above, is capable of supporting metabolism of both glycolytic and fatty acid carbon sources in response to O2 limitation, yet also produces succinate as its metabolic end product. Moreover, our studies reveal a broader multiplicity of previously unrecognized, essential metabolic roles for succinate during adaptation to O2 limitation. That is, as O2 is depleted, *M. tuberculosis* increases expression of the glyoxylate shunt enzyme isocitrate lyase, produces large amount of succinate, and can use this succinate to flexibly sustain membrane potential, ATP synthesis, and anaplerosis, depending on the specific conditions encountered (Fig. S9). Succinate is a bifunctional substrate of SDH, an enzyme that serves both the TCA cycle and ETC and couples carbon flow to ATP synthesis at an efficiency approximately two-thirds that of NADH. Increasing succinate production under O2 limiting conditions thus enables *M. tuberculosis* to sustain SDH activity and oxidative synthesis of ATP at a rate proportional to its respiratory capacity, while secreting some of the unused excess to maintain membrane potential when an alternative terminal electron acceptor is lacking and/or storing the remainder to enable the immediate resumption of carbon flow and ATP synthesis upon reoxygenation. The near neutral midpoint potential of the succinate/fumarate redox couple (\(\psi^i = +0.03\) V) makes it suitable to accumulate as a fermentation product of fumarate reductase when metabolizing glycolytic carbon substrates and oxygen is severely limited. Our studies thus reveal that *M. tuberculosis* uses succinate as a type of multifunctional “metabolic battery” capable of flexibly sustaining membrane potential, ATP synthesis, and TCA cycle precursors when O2 is depleted, while keeping its TCA cycle poised to resume oxidative activity upon access to a terminal electron acceptor, such as nitrate, although the specific mechanisms of nitrate-dependent respiration remain to be elucidated.

Multiple lines of microbiologic, immunohistologic, and biochemical evidence have established hypoxia as a feature of some niches faced by *M. tuberculosis* (3, 13). However, growing evidence has revealed that the extent of hypoxia associated with a given host varies widely based on the size, location, and composition of the microenvironment (31–33). Our studies modeled quiescent *M. tuberculosis* using an O2 environment of 1%, but the metabolic principle revealed may have broader significance,
namely, the ability of succinate to serve as a biochemical bridge between oxidative and fermentative metabolic states.

Our studies identified a previously unrecognized role for *M. tuberculosis* ICL in adaptation to hypoxia. Enzymatic and transcriptional profiling studies reported that *M. tuberculosis* increased ICL and glycine dehydrogenase activities in response to oxygen limitation, suggesting that *M. tuberculosis* may use ICL to replenish NAD in its oxidized form by offloading NADH reducting equivalents via a glyoxylate–glycine shunt (15). However, an *M. tuberculosis* strain lacking one of two ICL paralogs was unimpaired in its ability to survive hypoxic or anaerobic incubation (26). Subsequent work showed that a mutant lacking both ICL paralogs was unable to catabolize even- or odd-chain fatty acids (27). Our data now establish that *M. tuberculosis* ICLs (collectively, ICL) are essential for adaptation to hypoxia and that this essentiality is attributable to ICL’s ability to supply succinate as a substrate used to sustain hypoxic quiescence, rather than its canonical role in fatty acid catabolism. ICL-deficient *M. tuberculosis* is one of the most severely attenuated mutants tested in a mouse model of TB. Such attenuation may reflect the loss of multiple functions, some of which were revealed by metabolic studies and could not be foreseen by genetic or bioinformatic approaches (37, 38).

Replicating or not, all cells face the challenge of maintaining an energized membrane, ATP, and carbon precursors. The studies reported herein identify a metabolically unique and bioenergetically efficient mechanism of adapting to a potentially broad range of O₂ concentrations. Other microbes and cell types, such as tumor cells and host cells at inflammatory sites, occupy a similarly diverse range of O₂-limited niches. The mechanism described herein may thus pertain to them as well (48).

**Materials and Methods**

*M. tuberculosis* Filter Culture and Metabolite Extraction. *M. tuberculosis* strains H37Rv, Erdman, icl-knockout (∆ICL KO) and the complemented strain (ICL::pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL) were cultured in a biosafety level 3 facility at 37 °C in Middlebrook 7H9 broth (m7H9) or on 7H10 agar (m7H10) (Difco) supplemented with 0.2% acetate (or 0.2% glucose in case of Erdman-based mutant strains), 0.04% pICL). Culture filters were metabolically quenched by plunging filters into a mixture of acetonitrile/methanol/H₂O (40:40:20) pre-cooled to −20 °C. After shaking for 1 h, filters were extracted puriﬁed using the RNeasy kit (Qiagen) with the following steps: 2 mL of cold TRIzol (Gibco/BRL) and transferred to 2 mL screw-cap tubes containing 500 μL of 0.5 mm diameter Zirconia/silica beads (BioSpec Products). Cells were disrupted by 30-s pulses in a BioSpec Products bead beater three times. Lysates were then centrifuged at 13,000 × g for 10 min at 4 °C and supernatants were subsequently transferred to fresh tubes. The supernatant was then transferred to a tube containing 200 μL chloroform, inverted for 1 min, and centrifuged at maximum speed. The aqueous phase was then precipitated using 500 μL 80% ethanol. RNA was isolated using an RNeasy kit following manufacturer’s recommendations (Qiagen). The concentrations of extracted total RNA (μg/μL) were measured using a Nanodrop spectrophotometer. qRT-PCR was performed in 20-μL volumes using the iScript one-step RT-PCR kit with Syber green (Bio-Rad Laboratories) on a LightCycler 480 Real-Time PCR System (Roche). Reactions were set up as per the manufacturer’s instructions, using 10 ng of total RNA. The amplification procedure was as follows: cDNA synthesis for 30 min at 50 °C; RT inactivation for 5 min at 95 °C; and PCR cycling and detection (40 cycles) for 30 s at 95 °C, 30 s at annealing temperature (60 °C), and 30 s at 76 °C (acquiring signal at the end of this step). For all reactions, several no-RT and no-template controls were carried out and yielded no detectable signals. The primers used here are as follows: dosR-F, ggcagcagctgtgataggcttcgctctgct and dosR-R, gaaggag- ttacgctgctatccagggggt, icl-R, caaagctcagaaggaagct and icl-R, ttctcgtcagtt-catacg; siga-R, ctctctctgtagttgttatg; and siga-R, ttgctttcagcctttc.

**Measurement of Intrabacterial ATP Content and NADH/NAD Ratios.** *M. tuberculosis*-laden filters as used for metabolomic profiling were separately generated to measure intrabacterial ATP and NADH/NAD content. Intra-bacterial ATP concentrations were measured by BacTiter-Glo Microlod Cell Viability assay according to the manufacturer’s instructions (Promega). NAD and NADH concentrations were measured using a FluroNAD/NADH detection kit (Cell Technology). Metabolism of *M. tuberculosis* was rapidly quenching by plunging bacilli in the ﬁrst solvent in the kit.

**Membrane Potential Determination.** *M. tuberculosis* membrane potential was measured as previously described and adapted to our in vitro hypoxia apparatus (18, 41). Briefly, *M. tuberculosis* cultures were grown in m7H9
medium with 0.2% acetate to midlogarithmic phase and concentrated to an OD_{570} = 1.0 in fresh mTH9. M. tuberculosis cultures were then inoculated into 96-well microtiter plates and inserted into the hypoxia chamber already preinstalled with a squeezable, plastic Pasteur pipette loaded with 15 μM DiOC2 (which was fixated to the roof of the chamber as depicted in Fig S2B). After 48 h of hypoxic incubation, 15 μM DiOC2 was added to the well and incubated for 20 min at room temperature, followed by fixation with 1% formaldehyde for an additional 10 min (18). Cultures were then subsequently washed with fresh mTH9 to remove extracellular dye. As a positive control for membrane depolarization, cultures grown under ambient air conditions were treated with 5 μM of the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Invitrogen). DMSO was used as a vehicle control. The assay was performed in black with clear-bottom 96-well plates (Costar) and a SpectraMax MS spectrofluorometer (Molecular Devices) was used to measure green fluorescence (488 nm/530 nm) and shifts to red fluorescence (488 nm/610 nm), as a result of aggregation of dye molecules due to the presence of a large membrane potential. Membrane potential was measured as a ratio of red fluorescence (which depended on cell size and membrane potential) to green fluorescence (which depended on cell size alone). Each condition was measured in triplicate and each experiment was performed twice.

### Statistical Analysis

Analyses were performed by the ANOVA test. A P value of less than 0.05 was considered statistically significant.

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**References**


39. Alston TA, Mela L, Bright HI (1977) 3-Nitropropionate, the toxic substance of In- digofera, is a suicide inactivator of succinate dehydrogenase. Proc Natl Acad Sci USA 74(9):3767–3771.

Fig. S1. Multimodal adaptation of *Mycobacterium tuberculosis* to 1% O\(_2\). (A) *M. tuberculosis* viability during adaptation to 1% O\(_2\) reported by enumeration of colony forming units (CFUs) at indicated time points following transfer to 1% O\(_2\). (B) Reentry of *M. tuberculosis* adapted to 1% O\(_2\) into cell cycle following reaeration with 21% O\(_2\), as reported by CFU. (C) Time-dependent induction of *dosR* mRNA levels, a previously validated transcriptional marker of hypoxia (1), following transfer of *M. tuberculosis* to 1% O\(_2\). (D) Deinduction of *dosR* transcript levels, as reported by fold change relative to that measured at 24 h 1% O\(_2\). *dosR* mRNA levels were quantified by qRT-PCR, normalized to transcript levels of the housekeeping gene *sigA*, and expressed relative to those measured in *M. tuberculosis* incubated at 21% O\(_2\). (E) Relative intrabacterial ATP content of *M. tuberculosis* incubated for 24 h at 21% O\(_2\), 1% O\(_2\), and following 24 h of reaeration at 21% O\(_2\). (F) NADH/NAD ratio of *M. tuberculosis* incubated at 1% O\(_2\) for 24 h and 48 h. Time 0 represents values measured at 21% O\(_2\). (G) The effect of presence of an alternate terminal electron acceptor (nitrate, NO\(_3^-\)) of the electron transport chain on hypoxic *M. tuberculosis* metabolism. Intrabacterial ATP content of *M. tuberculosis* incubated for 24 h at 21% O\(_2\), 1% O\(_2\), and 1% O\(_2\) in the presence of 5 mM extracellular nitrate (H NO\(_3^-\)). (H) NADH/NAD ratio of *M. tuberculosis* incubated at 1% O\(_2\) in the presence of 5 mM extracellular nitrate. Time 0 represents values measured at 21% O\(_2\). *P* < 0.05 or **P** < 0.001. All values were the average of at least three experimental replicates in two independent experiments ±SEM.

Fig. S2. Metabolically active but slowing of *M. tuberculosis* adaptive to 1% $O_2$. (A) Relative depletion and/or consumption of extracellular acetate by *M. tuberculosis* incubated for 24 h at 21% $O_2$ (N), 1% $O_2$ (H), and at 21% $O_2$ following reaeration of *M. tuberculosis* preincubated at 1% $O_2$ for 48 h (R). Uptake and/or consumption were reported by LC-MS–based measurements of remaining $^{13}C$ acetate content of extracellular media (as described in the main text and Materials and Methods using the device depicted in Fig. S7A) and expressed relative to media-only control samples [Middlebrook 7H9 (m7H9)]. (B–D) Bulk metabolic activity of putative tricarboxylic acid (TCA) cycle in *M. tuberculosis* at 1% $O_2$ was measured by using the device depicted in Fig. S2B. The hypoxic chambers used are flexible plastic bags that contain a squeezable, plastic Pasteur pipette preloaded with fresh media containing $^{13}C$ labeled carbon affixed to the roof of the bag, as depicted. Following conversion of a resazurin indicator inside the bag to white (indicative of 1% $O_2$ condition, which was achieved by 12 h after initiation of the catalytic reaction), fresh media containing $^{13}C$ carbon was released onto hypoxic *M. tuberculosis* by squeezing the preinserted pipette without breaking the hypoxic seal or causing the resazurin indicator to turn pink. The bulk metabolic activity of *M. tuberculosis* at either 1% $O_2$ or 21% $O_2$ was reported by isotopic labeling measurements of succinate (C) and citrate (D) following “pulsed” addition of fresh [U-$^{13}C$] acetate-containing media to *M. tuberculosis* preadapted to 1% $O_2$ or 21% $O_2$, at the time, as indicated by black arrows, when *M. tuberculosis* adapted to hypoxia entered metabolically stationary phase (Fig. S3). The large block arrows in C and D indicate the $O_2$ concentration in the environment where *M. tuberculosis* incubated.
Fig. S3. Reversible M. tuberculosis metabolic responses adaptive to 1% O$_2$. (A) Clustered heat map describing the relative intrabacterial pool sizes of select metabolites (including TCA cycle intermediates and several amino acids) at 21% O$_2$ (normoxia), 1% O$_2$ (hypoxia), and following reaeration (reaerated) in a time-dependent manner up to 24 h. (B) Relative metabolic activity (as reported by relative isotopic labeling rates of metabolites) during incubation at 1% O$_2$ (black solid circles), 21% O$_2$ (gray squares), and following reaeration (gray triangles). Percentage isotopic labeling of metabolites was calculated by dividing the summed peak height ion intensities of all labeled isotopologue species by the ion intensity of both labeled and unlabeled species and expressed as percentages. All values were the average of at least three experimental replicates in two independent experiments ±SEM.
Fig. S4. Isotopomeric profiles of *M. tuberculosis* TCA cycle intermediates. *M. tuberculosis* was incubated in [U-^13^C] acetate-containing media for 24 h at 21% O_2 (N), 1% O_2 (H), and at 21% O_2 following reaeration of *M. tuberculosis* preincubated at 1% O_2 for 48 h (R). Individual ^13^C isotopomeric abundances of TCA cycle intermediates were expressed as percentages relative to the total abundance of all ion species (labeled and unlabeled) corresponding to the metabolite of interest. Isotopomeric distribution of TCA cycle intermediates, except for monoisotopic ^12^C mass (M+0) (A) or including monoisotopic ^12^C mass (M+0) (B). Isotopomeric composition of the metabolites at hypoxia in the presence of 5 mM extracellular nitrate (H NO_3) was also included in Fig. S4B. M+0, unlabeled; M+1, singly ^13^C labeled; M+2, doubly ^13^C labeled, and so on. Gray asterisks indicate ^13^C_2-labeled isotope species (indicative of incorporation of a single acetate unit), whereas black circles indicate ^13^C_4 or ^13^C_6-labeled species (indicative of accumulation of multiple acetate units arising from sustained TCA cycle activity). All values were average of independent experimental triplicates ±SEM. α-KG, α-ketoglutarate; ACO, aconitate; Asp, aspartate; FUM, fumarate; GLO, glyoxylate; ICL, isocitrate lyases; (ISO)CIT, (iso)citrate; MAL, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; SUC, succinate.
Fig. S5. Hypoxia-induced remodeling of *M. tuberculosis* TCA cycle activity when incubated with glucose as a defined carbon source. Intrabacterial pool sizes and isotopic labeling of TCA cycle-related intermediates in *M. tuberculosis* incubated in [U-\(^{13}\)C] glucose-containing media for 24 h at 21\% O\(_2\) (N), 1\% O\(_2\) (H), and at 21\% O\(_2\) following reaeration of *M. tuberculosis* preincubated at 1\% O\(_2\) for 48 h (R). Total bar heights indicate the intrabacterial concentration; the green colored area of each bar denotes the extent of \(^{13}\)C labeling achieved following transfer to [U-\(^{13}\)C] glucose-containing media under the condition indicated. All values were the average of at least three experimental replicates in two independent experiments ±SEM. Abbreviations are as in Fig. S4.

Fig. S6. Isotopomeric profiles of succinate extracted from *M. tuberculosis* treated with [U-\(^{13}\)C] aspartate in the presence of acetate in media for 24 h at either 21\% O\(_2\) (N) or 1\% O\(_2\) (H). M+0, unlabeled; M+1, singly \(^{13}\)C labeled, M+2, doubly \(^{13}\)C labeled succinate, and so on. Black circles indicate \(^{13}\)C\(_4\)-labeled succinate (predominant incorporation of \(^{13}\)C\(_4\) is consistent with en bloc incorporation and biosynthesis of succinate via reductive TCA cycle activity).
Fig. S7. The effect of succinate in extracellular media on *M. tuberculosis* metabolism. (A) Schematic diagram illustrating the device used to make timed start-stop measurements of secretion into the extracellular media which consists in a plastic inset containing chemically equivalent liquid media (m7H9) amenable to experimental sampling. (B) The effect of 2 mM succinate in extracellular medium on secretion of intracellular succinate from hypoxic (1% O$_2$) *M. tuberculosis*. Relative levels of secreted intracellular succinate measured using the device depicted in Fig. S7A with plates either containing or lacking 2 mM unlabeled extracellular succinate. To distinguish secreted intrabacterially synthesized succinate from exogenously supplied (unlabeled) extracellular succinate, *M. tuberculosis*-laden filters were preincubated on m7H10 containing [U-$^{13}$C] acetate for 2 d to label intracellular succinate within bacilli with $^{13}$C. *M. tuberculosis*-laden filters were then transferred to the devices shown in Fig. S7A and incubated for 20 h at 1% O$_2$. The amount of succinate secretion from *M. tuberculosis* bacilli during incubation in the presence or absence of unlabeled extracellular succinate was reported by the ion count intensities (IC) of $^{13}$C$_4$ succinate isotopologue using LC-MS. The addition of unlabeled extracellular succinate thus efficiently prevented secretion of intracellular succinate from hypoxic *M. tuberculosis* adapted to 1% O$_2$ (compare bar heights shown Left and Right). *P < 0.001 by ANOVA. All values were the average of at least three experimental replicates in two independent experiments ± SEM. (C) The effect of the same concentration of extracellular succinate on *M. tuberculosis* growth under aerobic conditions (in the presence of 0.2% acetate).

Fig. S8. Metabolic effect of 3-Nitropropionate (3NP) on *M. tuberculosis* metabolism and survival. (A) Metabolomic profiles of select TCA cycle intermediates in the presence of 200 μM 3NP at 21% O$_2$, for times as indicated. MAL, malate. (B) Isotopomeric profiles of succinate extracted from *M. tuberculosis* treated with 200 μM 3NP in [U-$^{13}$C] glucose-containing media for 24 h at 21% O$_2$. Red asterisks indicate $^{13}$C$_2$-labeled succinate; black circles indicate $^{13}$C$_4$-labeled succinate. (C) Viability of ΔICL KO following incubation at 1% O$_2$ in the absence or presence of 200 μM 3NP in 0.2% glucose-containing media. *P < 0.001 by ANOVA. All values were the average of at least three experimental replicates in two independent experiments ± SEM.
Fig. S9. Multifunctional essentiality of succinate during adaptation to 1% O$_2$. Hypoxia triggers activation of isocitrate lyase activity, leading to an increase in succinate levels that can be used to support membrane potential, ATP synthesis, and anaplerosis during adaptation to, residence in, and recovery from hypoxia-induced quiescence. $\alpha$KG, $\alpha$-ketoglutarate; $\Delta\psi$, membrane potential; ICL, isocitrate lyase; SDH, succinate dehydrogenase; 3NP, 3-nitropropionate.