Humans serve as both host and reservoir for *Mycobacterium tuberculosis*, making tuberculosis a theoretically eradicable disease. How *M. tuberculosis* alternates between host-imposed quiescence and sporadic bouts of replication to complete its life cycle, however, remains unknown. Here, we identify a metabolic adaptation that is triggered upon entry into hypoxia-induced quiescence but facilitates subsequent cell cycle re-entry. Catabolic remodelling of the cell surface trehalose mycolates of *M. tuberculosis* specifically generates metabolic intermediates reserved for re-initiation of peptidoglycan biosynthesis. These adaptations reveal a metabolic network with the regulatory capacity to mount an anticipatory response.

*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), kills more humans than any other bacterium, yet humans remain its only major natural reservoir. Replication of the bacillus is slowed or arrested in most hosts, but resumes in some decades later, with a host response that is frequently fatal yet linked to the generation of infectious aerosols that enable completion of its life cycle. Such delayed replication has allowed infected hosts to have offspring before transmitting the infection. *M. tuberculosis* has thus come to depend on the regulation of cell cycle exit and re-entry as a critical mediator of pathogenicity and survival as a species.

Hypoxia is a physiological feature common to many of the intra- and extracellular niches of *M. tuberculosis* in humans and experimentally infected animals. In vitro, hypoxia arrests *M. tuberculosis*’s replication and renders phenotypic tolerance to nearly all clinically used TB drugs. However, *M. tuberculosis* also re-encounters atmospheric oxygen when inflammatory host tissue damage has become extensive enough to erode into the airways, facilitating re-entry into the cell cycle. Hypoxia and atmospheric oxygen have thus co-evolved as highly correlated but temporally sequential features of the physiology of *M. tuberculosis*.

Studies of *M. tuberculosis*’s response to hypoxia have revealed a broad and diverse range of responses, most of which have been considered in the physiological context of situational adaptation. Here, we report a functionally distinct set of metabolic adaptations that are triggered by, but facilitate survival through and recovery from, hypoxia. These adaptations thus reveal a predictive regulatory capability of *M. tuberculosis*’s metabolic network in which the temporal relationship between hypoxia and re-aeration has been encoded into an anticipatory response that links entry into and exit from dormancy.

Using our previously described *in vitro* system, we noted that exposure to hypoxia (1% O₂, 5% CO₂) triggered discrete accumulations of intermediates in the early portion of glycolysis, the pentose phosphate pathway and aminosugar biosynthesis. These accumulations occurred with glucose or acetate as a carbon source and reversed with re-aeration (Fig. 1 and Supplementary Fig. 1a,b). These changes were further linked to reciprocal, hypoxia-dependent decreases in levels of the downstream glycolytic intermediate, phosphoenolpyruvate (PEP) and upstream disaccharide, trehalose, but not further upstream glycogen polysaccharide stores (Supplementary Fig. 2a,b). These changes, albeit to varying degrees, were also durable to the experimental limit of this system (4 days) without a measurable loss of viability and dissociated from tricarboxylic acid (TCA) cycle activity, as tested by the inclusion of nitrate, a physiological alternative terminal electron acceptor capable of supporting near aerobic levels of TCA cycle activity in hypoxic environments (Supplementary Fig. 2c,d).

Upon transferring *M. tuberculosis* from unlabelled to uniformly ¹³C-labelled (¹⁵[₁³C]) glucose or acetate at the time of exposure to hypoxia, we noted that these metabolite accumulations predominately occurred within the unlabelled fraction of each pool (Fig. 1 and Supplementary Fig. 1a,b), suggesting catalysis of a pre-existing metabolic store. Trehalose is a highly abundant, non-reducing disaccharide of glucose that can serve as both carbohydrate store and bioprotectant. In *M. tuberculosis*, trehalose is also a core component of mycolyl glycolipids. Trehalose monomycolate (TMM) and di-mycolate (TDM), widely known as cord factor, form the outer lipid barrier of *M. tuberculosis*’s cell envelope and serve as potent triggers of host inflammation. We therefore hypothesized that the foregoing metabolic accumulations arose from hypoxia-induced catalysis of *M. tuberculosis*’s cell surface trehalose mycolates. Consistent with a recent lipidomic study, we observed a rapid and extreme depletion of *M. tuberculosis*’s TMMs and TDMs during hypoxia, while levels of sulfolipids and the plasma membrane phospholipid—phosphatidylethanolamine—were unchanged (Fig. 2a and Supplementary Fig. 3). Moreover, we observed link changes in intra-, but not extra-, cellular levels of unlabelled trehalose (which decreased) and free mycolates (which increased), consistent with the release, re-uptake and catalysis of mycolyl glycolipid-derived trehalose (Fig. 2a,b).

TDM is a ligand of the Mincle receptor of macrophages and a potent inducer of the pro-inflammatory cytokines interleukin-12 (IL-12) and tumour-necrosis factor (TNF). We hypothesized that the hypoxia-induced decreases in TDM and TMM levels might be accompanied by changes in levels of *M. tuberculosis*-elicited IL-12p40 and TNF secretion. Incubation of mouse bone-marrow-derived macrophages with equivalent numbers of paraformaldehyde-fixed *M. tuberculosis* cells revealed that hypoxia reversibly

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Supplementary Fig. 5a) Biochemical and genetic studies indicate that TreS is a catalytically competitive, trehalose synthase capable of reversibly interconverting maltose and trehalose, enabling conversion of trehalose to glucose in vivo\textsuperscript{14,15}. We therefore generated a \( \Delta \text{treS} \) deletion strain of \textit{M. tuberculosis} (\( \Delta \text{treS} \)) and genetically complemented counterpart to test the role and essentiality of \( \text{treS} \) in the foregoing response (Supplementary Fig. 5c). As expected, \( \Delta \text{treS} \) \textit{M. tuberculosis} exhibited a selective growth defect in media containing trehalose, but not glucose, as the sole exogenous carbon source (Supplementary Fig. 5d).

Metabolic profiling studies showed that \( \Delta \text{treS} \) \textit{M. tuberculosis} failed to exhibit the same metabolite accumulations observed in wild-type \textit{M. tuberculosis} and \( \text{treS} \) complemented strain at early time points after exposure to hypoxia (Fig. 3). \( \Delta \text{treS} \) \textit{M. tuberculosis} was also unable to adapt to, or recover from, incubation at 1% \( \text{O}_2 \) (Supplementary Fig. 5d). We could similarly reproduce these effects using a validated chemical inhibitor of TreS, validamycin A (Supplementary Fig. 6a–c)\textsuperscript{14}. Thus, while wild-type \textit{M. tuberculosis} exhibited a modest, hypoxia-induced repression of \( \text{treS} \), this effect was not observed in \( \Delta \text{treS} \) \textit{M. tuberculosis}. These data do not support a role for \( \text{treS} \) in the foregoing response to hypoxia (Supplementary Fig. 6d).

The foregoing results indicate that hypoxia triggers a catabolic remodelling of \textit{M. tuberculosis}’s immunostimulatory cell envelope, part of which is shunted into a stable accumulation of pentose phosphate pathway intermediates. The pentose phosphate pathway furnishes reducing equivalents for reductive biosynthesis and anti-oxidant defence and precursors for nucleotide and trehalose metabolism.
To a discrete (24 h) exposure of ampicillin, a covalently reactive inhibitor and functional probe of peptidoglycan biosynthesis. As expected, hypoxic *M. tuberculosis* exhibited a phenotypic tolerance to ampicillin and streptomycin compared to aerobic counterparts (as reported by time to outgrowth following treatment and subculture into fresh antibiotic-free medium). Re-aerated *M. tuberculosis*, in contrast, exhibited a return to near aerobic levels of susceptibility to ampicillin, but not streptomycin, during the first 24 h of re-aeration (Supplementary Fig. 8a,b). These results thus link the observed biochemical response to an early, specific and essential role for de novo peptidoglycan biosynthesis during recovery from hypoxia.

Growing evidence indicates that genetic regulatory systems have evolved the capacity to encode anticipatory responses to conserved spatiotemporal features of the environment that can prepare organisms for a given condition in advance of its arrival. Metabolic enzymes catalyse biochemical reactions that typically occur on the order of seconds to minutes, but serve physiological activities that operate across a far broader range of timescales. "Metabolic enzymes are a well-documented determinant of activities that operate across a far broader range of timescales. The capacity to encode anticipatory responses to conserved spatiotemporal features of the environment that can prepare organisms for a given condition in advance of its arrival has evolved the capacity to encode anticipatory responses to conserved spatiotemporal features of the environment that can prepare organisms for a given condition in advance of its arrival."
Traditional stimulus-specific responses help meet the challenge of unpredictability, in part, through probabilistic, bet-hedging mechanisms. In contrast, our studies identify a deterministically encoded mechanism in which the temporal relationship between hypoxia and environmental oxygen has been encoded into an anticipatory metabolic response, where hypoxia serves as a cue for the subsequent arrival of oxygen.

Peptidoglycan is an essential component of the cell wall required for both structural integrity and growth of virtually all bacteria, including \textit{M. tuberculosis}. \textit{De novo} synthesis of peptidoglycan begins with the condensation of two thermodynamically activated substrates, PEP and UDP-\textit{N}-acetylglucosamine, by MurA, the first committed enzyme of the pathway. However, hypoxic \textit{M. tuberculosis} maintains adenosine triphosphate (ATP) at levels approximately five times lower than those of replicating counterparts. The hypoxia-induced shunting of catabolized trehalose mycolates into activated pentose phosphate intermediates may thus help \textit{M. tuberculosis} overcome a key thermodynamic barrier to cell cycle re-entry and completion of its life cycle, consistent with recently emerging reports of additional O$_2$-sensitive mechanisms of MurA regulation.

The mechanisms used to accumulate, store and release trehalose in \textit{M. tuberculosis} remain to be determined. Existing evidence implicates the Antigen 85 complex, which generates trehalose as a by-product of the biosynthesis of the mycolic acid-based outer cell envelope; the LpqY-SugA-SugB-SugC ABC transporter, which catalyses the specific, retrograde transport of extracellular trehalose; the TreS-encoded trehalose synthase, described here; and glyceraldehyde-3-phosphate dehydrogenase, which was recently shown to regulate flux between upper and lower glycolysis via its universally conserved requirement for high Pi (inorganic phosphate) concentrations.

Mechanism notwithstanding, growing evidence indicates that the lesions in which \textit{M. tuberculosis} resides within the lung are both diverse and dynamic. The evolution of anticipatory responses may thus help \textit{M. tuberculosis} bridge the challenge of navigating an often decades-long life cycle on an intermediary basis.

We previously reported that, unlike other bacteria, replicating \textit{M. tuberculosis} operates its intermediary metabolism in a modular and compartmentalized manner by co-catabolizing different carbon sources to distinct metabolic fates. Our discovery of metabolism of cell surface-derived trehalose to functionally compartmentalized pentose phosphate intermediates now expands this view to another pathway and \textit{M. tuberculosis} in a hypoxic, non-replicating state. This work more broadly demonstrates a metabolically encoded regulatory capability that enables a critical transition point in a pathogen’s life cycle.
Methods

M. tuberculosis filter culture and metabolite extraction. M. tuberculosis strains H37Rv, treS knockout (ΔtreS KO) and the complemented strain (ΔtreS::pitoS) 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 requirement), 0.04% Tylosapox (broth only), 0.5 g l⁻¹ BSA and 0.085% NaCl. M. tuberculosis-laden filters used for metabolic profiling were generated as previously described⁷ and incubated at 37 °C for 5 days to reach the mid-log phase of growth. M. tuberculosis-laden filters were then transferred onto chemically identical medium containing fresh 13C acetate (or glucose) or [U-13C] acetate (or glucose)-m7H10. The M. tuberculosis-laden filters were metabolically quenched by plunging filters into a mixture of acetonitrile/methanol/H2O (40:40:20) precooled to ~40 °C, and metabolites were extracted by mechanical lysis with 0.1 mm zirconia beads in a Precellys tissue homogenizer for 3 min (6,500 r.p.m.) twice under continuous cooling at or below 2 °C. Lysates were clarified by centrifugation and then filtered across a 0.22 μm filter. The residual protein content of metabolite extracts (BCA instructions and validated by a resazurin indicator, which decolourized at oxygen environment) was measured using a Palladium catalyst, achieving a palladium atmosphere of 1% O2 (as reported by a palladium indicator strip) and 5% CO2, levels similar to those encountered in the tuberculous lungs of infected animals, within 4 h. We previously showed that, in this system, M. tuberculosis neither underwent net replication nor death in response to incubation at 1% O2, and resumed growth upon re-aeration. We further showed that this system was associated with both the reversible biphasic induction of dosR, a previously validated transcriptional marker of hypoxia, and accompanying reductions of levels of ATP and nicotinamide adenine dinucleotide (NAD).

LC-MS metabolic profiling. Liquid chromatography–mass spectrometry (LC-MS)–based metabolomics was used as described in ref. 7. Extracted metabolites were separated on a Cogent Diamond Hydride type C column (gradient 3) and the mobile phase consisted of solvent A (ddH2O with 0.2% formic acid) and solvent B (acetonitrile with 0.2% formic acid). The mass spectrometer used was an Agilent Accurate Mass 6220 time of flight (TOF) coupled to an Agilent 1200 liquid chromatography (LC) system. Detected ions were deemed metabolites on the basis of unique accurate mass–retention time identities. Metabolite identities were extracted using a calibration curve generated with varying concentrations of standard metabolites to confirm the identity of the extracted metabolites. Metabolite identities were also confirmed by tandem mass spectrometry (MS/MS) using a Q-TOF Ultima system with a nitrogen atmosphere. The average ± s.e.m. of three experimental replicates (n = 3) and representative of two independent experiments.

In vitro hypoxia model. In vitro hypoxia was achieved using a type A Bio-bag environmental chamber (Becton Dickinson), as specified by the manufacturer’s instructions and validated by a resazurin indicator, which decolourized at oxygen concentrations of 1.0% or less. M. tuberculosis-laden filters were transferred to fresh chemically equivalent m7H10 immediately before insertion or after recovery from a sealed hypoxic chamber. M. tuberculosis-laden filters recovered from hypoxic chambers were immediately plunged into the precooled quenching solution as described in ref. 7. This system gradually depletes oxygen and generates CO2 via a palladium catalyst, achieving 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. We previously showed that, in this system, M. tuberculosis neither underwent net replication nor death in response to incubation at 1% O2, and resumed growth upon re-aeration. We further showed that this system was associated with both the reversible biphasic induction of dosR, a previously validated transcriptional marker of hypoxia, and accompanying reductions of levels of ATP and nicotinamide adenine dinucleotide (NAD).

Figure 4 | Biochemical shunting of hypoxia-induced metabolic stores into de novo peptidoglycan biosynthesis during re-aeration. Metabolic tracing of hypoxia-induced [12C] metabolic stores of glycolytic, pentose phosphate pathway and amino sugar biosynthesis into de novo peptidoglycan biosynthesis following re-aeration at 20% O2 for 0, 0.5, 1, 1.5, 4, 8 and 24 h. Changes are shown in stacked bar graph format relative to accumulated levels observed following incubation at 1% O2 for 48 h (designated 0 h) for either [12C] (unlabelled species) or total ([12C] + all [13C] species) of indicated metabolite. Grey font indicates undetected intermediates. α-Glu, α-glutamate; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; L-Ala, L-alanine; MurNGlyc, glycolyl N-acetylmuramic acid; PEP, phosphoenolpyruvate; Pi, inorganic phosphate; UDP, uridine diphosphate; PPi, inorganic pyrophosphate. All values are the average ± s.e.m. of three experimental replicates (n = 3) and representative of two independent experiments.
matrix-associated ion suppression effects. The abundance of extracted metabolite ion intensities was extracted using Profiner 6.0 and Qualitative Analysis 6.0 (Agilent Technologies). The clustered heat map and hierarchical clustering trees were generated using Cluster 3.0 and Java TreeView 1.0.

LC-MS lipidomic analysis. Total lipid extraction was performed with minor modifications as described previously.29 Total lipids were extracted using a Zirconia beads in a Precellys tissue homogenizer for 3 min (6,500 r.p.m.) twice under continuous cooling at or below 2 °C. PBS was used for both washing twice and Zirconia beads. After centrifugation, lipid extracts were decanted, and bacteria were subjected to two additional extractions using CHCl₃/CH₂OH (1:1, vol/vol) and CHCl₃/CH₂OH (1:2, vol/vol) with pooling of all extracts and evaporation with N-EVAP (Organamation Associates) using the low boiling point mixture point set. Dried lipids were resuspended in a minimum volume of CHCl₃/CH₂OH (1:1, vol/vol) and dried under nitrogen in preweighed vials, then reweighed in triplicate on a microbalance (Mettler Toledo, XP205); values were reported when fully dried as shown by replicate measurements showing less than 1% variance. Using 2 mg of lipid extract, replicate measures showed a variance of 20 µg, providing mass errors below 1% for lipid-determined species. Extracts were then redissolved in CHCl₃/CH₂OH (1:1, vol/vol) at 1 mg ml⁻¹. LC-MS grade solvents (Fisher) and clean borosilicate glassware (Fisher), amniot alveoli (Supelco) and Teflon-lined caps (Fisher) were used.

Intrabacterial trehalase and glycogen assays. Intrabacterial trehalase and glycogen concentrations under varying metabolic states (replicating, non-replicating and re-aeration) were measured by using Glucose (GO) Assay Kit (Sigma-Aldrich). The GO assay kit allows quantitative determination of glucose content present in bacterial extracted materials. Extracted glucose is oxidized to glucic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide then reacts with o-dianisidine in the presence of peroxidase to form a coloured product, reaction with sulfuric acid with which forms a more stable coloured product. The intensity of the coloured final product can be measurable at 540 nm which is proportional to the glucose amount extracted. Extractions of intrabacterial trehalase and glycogen were similarly performed as described for the extraction of M. tuberculosis trehalose. M. tuberculosis trehalase- laden filters were incubated for 5 days on m7H10, transferred to fresh chemically identical m7H10 under either ambient, hypoxic or re-aerated after incubation under 24 h hypoxia and harvested the cytosolic materials by mechanical lysis with 0.1 mm Zirconia beads in a Precellys tissue homogenizer for 3 min (6,500 r.p.m.) twice under continuous cooling at or below 2 °C. PBS was used for both washing twice and extracting cytosolic fraction. Extracted cytosolic fraction containing both trehalase and glycogen was used as a substrate for intermediate enzyme mixture containing either trehalase or amyloglucosidase to produce glucose, proportional to the amount of either trehalase or glycogen, respectively. Intrabacterial glucose production was quantitated by using Glucose (GO) Assay kit. Known concentration of authentic trehalase or glycogen was used for generation of standard curves and a positive control.

¹³C pulse chase labelling of cell envelope TMM. To selectively ¹³C label M. tuberculosis trehalase myoclates, we applied the following experimental approach (Supplementary Fig. 4). To fully ¹³C labe 16. The extracellular trehalomalase released by M. tuberculosis was expressed as an extracellular trehalomalase that is able to hydrolyse trehalose into glucose, which was previously demonstrated to be metabolically similar. Colony-forming units (c.f.u.) were determined by plating on m7H10 with supplements (0.2% glycerol, 0.2% glucose, 0.5 g l⁻¹ BSA and 0.085% NaCl). All cell viability analyses were performed in triplicate in two independent experiments.

Macrophase cytokine enzyme-linked immunosorbusent essays (ELISAs). Mouse bone-marrow-derived macrophages (BMMs) were collected from eight- to ten-week-old C57BL/6 mice and differentiated in cell culture as described in ref. 30. A total of 2 × 10⁶ macrophages per well in 24-well plates were exposed to single-cell suspensions of paraformaldehyde-fixed M. tuberculosis recovered from replicating, non-replicating (hypoxic) or re-aerated cultures taken from the early log phase of growth at a multiplicity of infection (MOI) of 3 and 5. Four hours following exposure, cells were washed twice with PBS to remove extracellular bacteria and replaced with fresh medium. Supernatants from M. tuberculosis-exposed macrophages were collected 24 h later, and cytokines IL-12 and TNF-α were measured by ELISA (R&D Systems). Supernatant from mock-treated macrophages was used as a control. Three independent experiments were performed for each infection condition. Bacterial input was determined by plating of serial dilutions on m7H10 plates at 37 °C and enumerating c.f.u.s after 2 weeks of incubation. We fixed M. tuberculosis under replicating, non-replicating and re-aerated states with 4% formaldehyde followed by washing with PBS twice macrophage infection. Bacterial input was determined before fixing.

Measurement of trehalase secretion from M. tuberculosis culture. As previously described, the filter culture system used for metabolomics studies was modified by replacing the underlying m7H10 agar medium with a plastic insert containing chemically equivalent m7H9 liquid medium (without Tyloxapol) in direct contact with the underside of the M. tuberculosis-laden filter. Growth on top this new device enabled timed start-stop measurements of trehalase secretion by sampling the cell-free liquid medium. A time-dependent trehalase secretory was sampled in the medium following exposure to and incubation hypoxia and measured using LC-MS.

Measurement of intrabacterial NADPH/NADP ratios. M. tuberculosis-laden filters, as used for metabolomics profiling, were separately measured to generate
intrabacterial NADPH and NADP content and their ratio. NADPH and NADP concentrations were measured using a FluroNADP/NADPH detection kit (Cell Technology). The metabolism of M. tuberculosis was rapidly quenched by plunging bacilli into the first solvent in the kit (cooled to <4 °C).

Statistical analysis. Analyses were performed using the analysis of variance (ANOVA) test. A P value of less than 0.05 was considered statistically significant.

Data availability. The data that support the findings of this study are either available within this Letter and its supplementary information files or upon reasonable request to the corresponding author.

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Author contributions

H.E. and Z.W. designed, conducted and analysed lipidomic profiling studies. E.L. and D.B.M. conducted and analysed metabolomic profiling studies. H.E. and P.R. conducted macrophage cytokine release assays. H.E., Z.W. and R.M. conducted antibiotic susceptibility assays. K.Y.R. initiated and directed this research.

Supplementary information

Supplementary information is available with the manuscript and at www.nature.com/reprints.

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Additional information

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Competing interests

The authors declare no competing financial interests.