Supplementary Information for Eoh et al.

Metabolic anticipation in *Mycobacterium tuberculosis*

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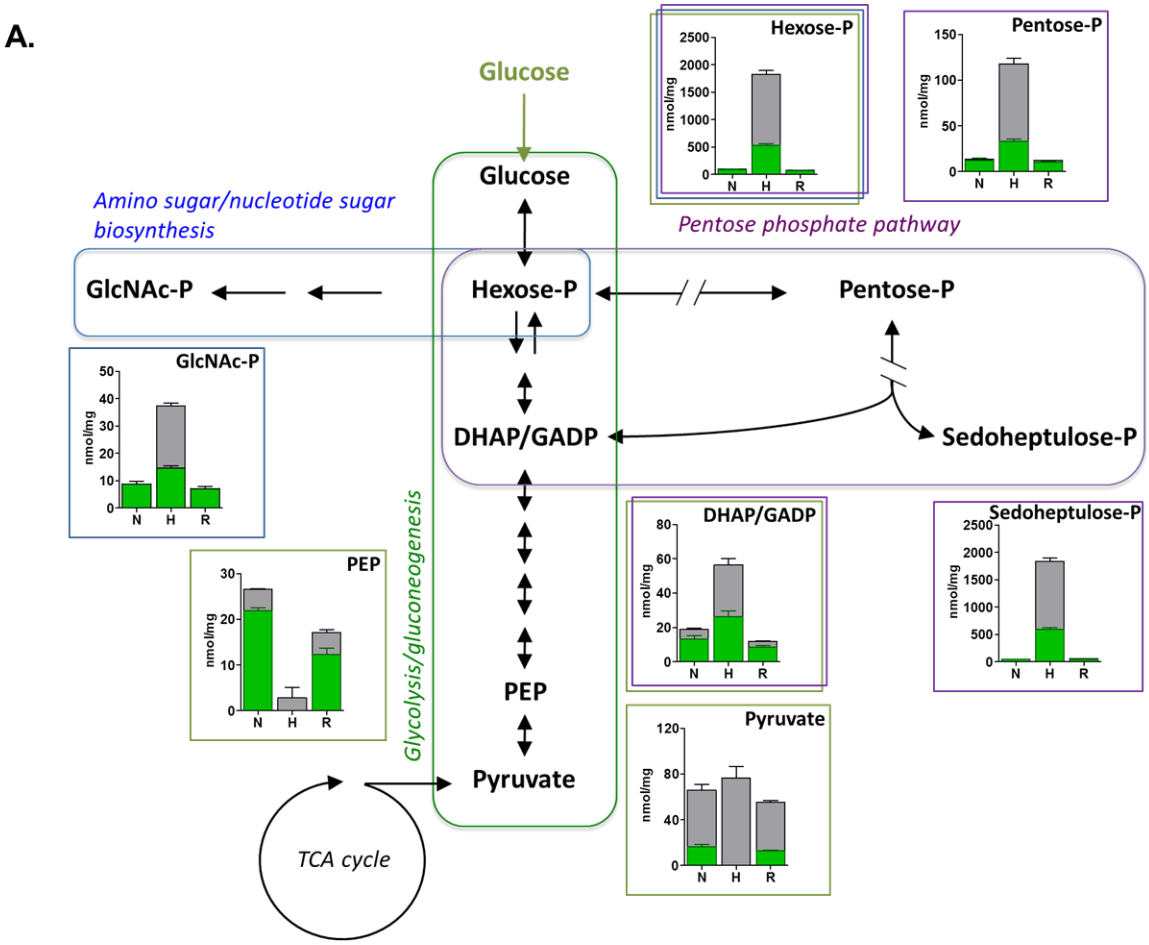
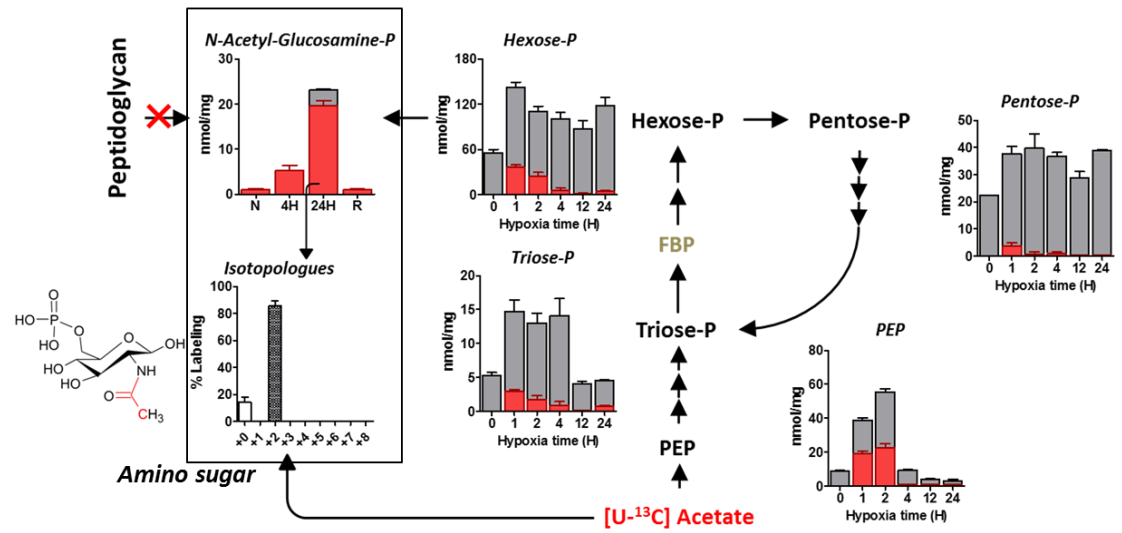
This document contains Supplementary Figures 1-8.


A.
Glucose
Glucose
Hexose-P
Pentose-P
Amino sugar/nucleotide sugar biosynthesis
Pentose phosphate pathway
GlcNAc-P
Hexose-P
Pentose-P
DHAP/GADP
Sedoheptulose-P
PEP
Pyruvate
Glycolysis/gluconeogenesis
TCA cycle
nmol/mg
N H R

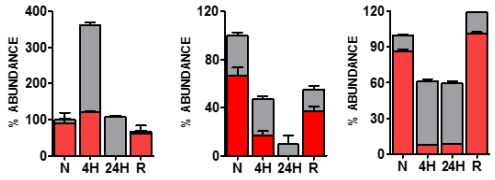

B.
Peptidoglycan
N-Acetyl-Glucosamine-P
Isotopologues
% Labeling
Amino sugar
Hexose-P
Triose-P
Hypoxia time (H)
Hexose-P
Pentose-P
FBP
Triose-P
PEP
[U-13C] Acetate
Pentose-P
PEP
N 4H 24H R
0 1 2 4 12 24

Supplementary Figure 1

A. Hypoxia-induced remodeling of upper glycolysis, amino sugar and nucleotide sugar biosynthesis and pentose phosphate pathways in *M. tuberculosis* in response to 1% O_2/5% CO_2. Intrabacterial pool sizes (nmol/mg protein) and isotopic labeling of *M. tuberculosis* intermediates in: upper glycolysis (***green box***); amino sugar biosynthesis (***blue box***); and pentose phosphate pathways (***purple box***)**,** incubated in [U-^{13}C]glucose-containing media for 24 h at 20% (normoxia; **N**); 4 h (**4H**) or 24 h (**24H**) at 1% O_2 (hypoxia; **H**); and 24 h at 20% O_2 following pre-incubation at 1% O_2/5% CO_2 for 24 h (re-aerated; **R**). Total bar heights indicate the intrabacterial concentration (nmol/mg protein), whereas the green area of each bar denotes the enrichment of ^{13}C labeling achieved following transfer to [U-^{13}C] glucose-containing m7H10 media under the condition indicated. All values are the average of three biological replicates (n=3) ±SEM and representative of 2 independent experiments. DHAP, dihydroxyacetone phosphate; GADP, glyceraldehyde phosphate; GlcNAc-P, N-acetyl glucosamine phosphate; hexose-P, glucose-6-phosphate and its isomers; pentose-P, ribose-5-phosphate and its isomers; PEP, phosphoenolpyruvate; sedoheptulose-P, sedoheptulose-7-phosphate and its isomers.

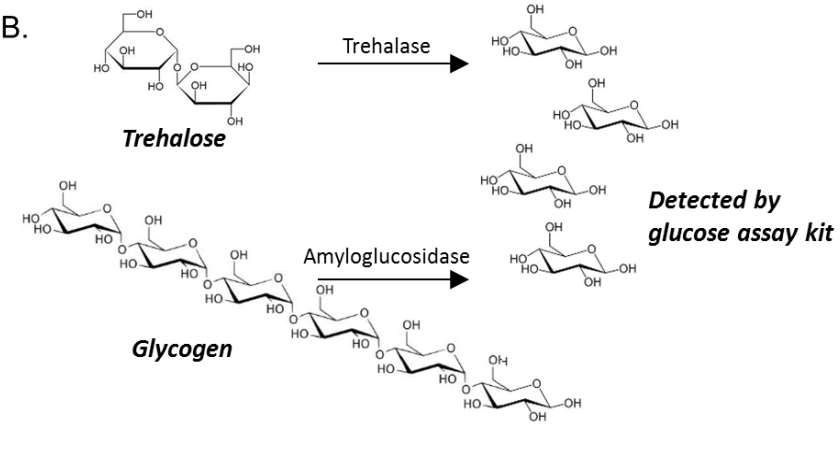
B. Kinetic ^{13}C isotopic tracing of glycolytic, amino and nucleotide sugar, and pentose phosphate pathway intermediates. Isotopologue analysis indicates that the observed accumulation of amino sugar (N-acetyl-glucosamine-P) predominantly (~90 %) consists in the M+2 isotopologue, indicative of *de novo* biosynthesis arising from the condensation of exogenous [U-^{13}C] acetate and endogenous hexose-P, rather than degradation of pre-existing peptidoglycan.

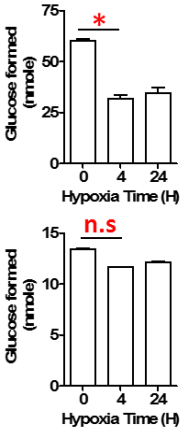
A.



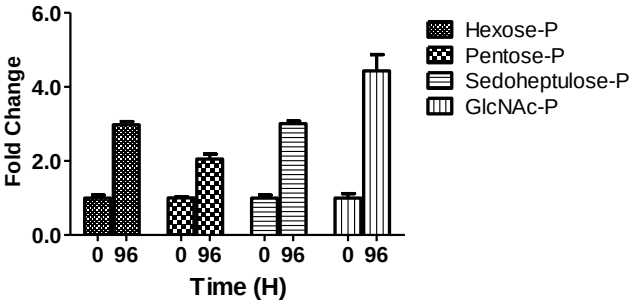
	R	4H	24H
Hex-P	1	5.45	5.85
Pent-P	1	3.22	4.78
S7P	1	6.58	9.54
T3P	1	3.62	1
PEP	1	0.46	0.09
Trehalose	1	0.6	0.57

B.

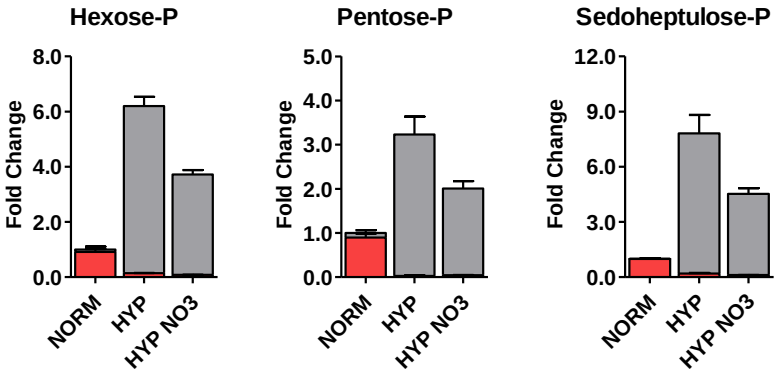




C.



D.



Supplementary Figure 2

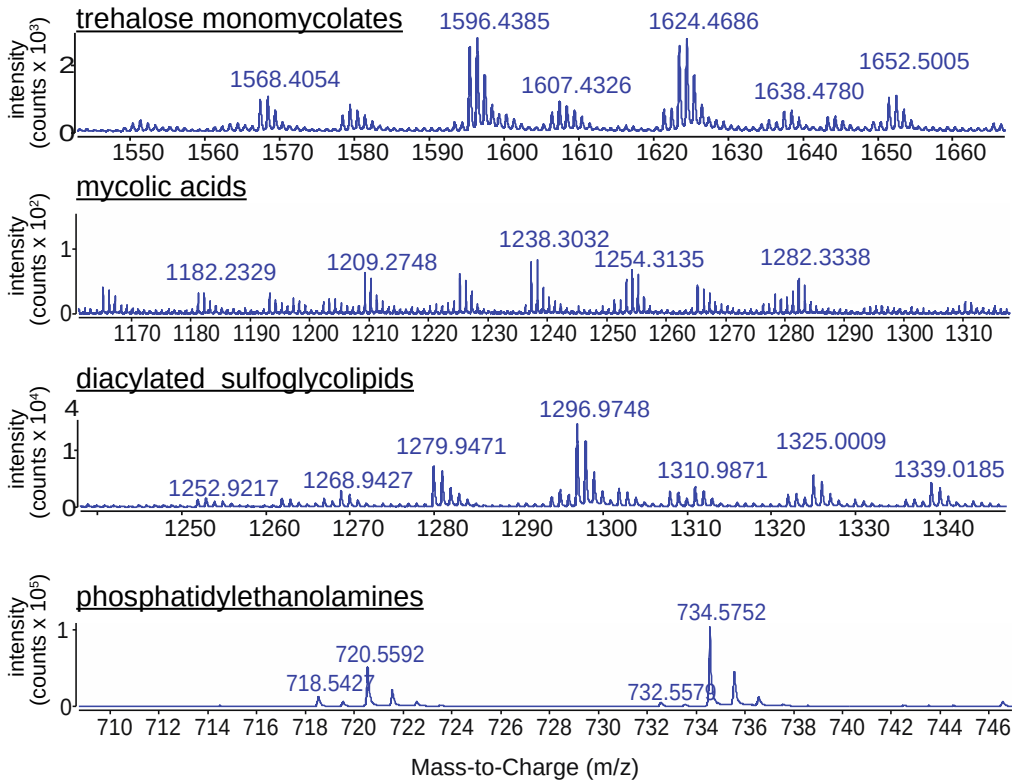
A. Metabolic pool sizes of downstream glycolytic intermediates, T3P and PEP and an upstream intermediate, trehalose following 4 (4H) or 24 h (24H) 1% O_2/5%CO_2 and 24h at 20% O_2 following pre-incubation at 1% O_2/5%CO_2 for 24h (R). Total bar heights indicate the overall intrabacterial concentration (relative to that of normoxic Mtb), whereas the red colored area of each bar denotes the enrichment of ^{13}C labeling achieved following transfer to [U-^{13}C] acetate-containing m7H10 media under the condition indicated. Table in right panel shows the fold changes of intermediates in glycolytic and pentose phosphate pathways at 4 (4H) and 24 h (24H) hypoxia condition relative to those of normoxic Mtb counterparts. T3P, triose 3-phosphate (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate).

B. Intrabacterial trehalose or glycogen concentration of *M. tuberculosis* in ambient air (0H) or 1% O_2 for 4 (4H) and 24 h (24H) as determined by "glucose assay kit." This kit consists in a coupled enzyme system using trehalase and amyloglucosidase. Production of glucose is quantitatively proportional to intrabacterial amount of trehalose and glycogen, respectively. The kit ultimately yields a colored final product that can be measured at 540 nm and is proportional to the glucose produced by the coupled enzymes. Intrabacterial trehalose concentration, but not glycogen, was diminished in *M. tuberculosis* under 1% O_2 vs ambient air. Shown values are expressed relative to those observed in normoxic Mtb, which was set to a reference value of 1. *, $p \leq 0.005$; and n.s, not significant by Student's unpaired t-test.

C. Metabolic alterations in upper glycolytic and pentose phosphate pathways following 96 hours (4 days) of incubation under hypoxia (1% O_2/5% CO_2). Shown values are expressed relative to those observed in normoxic Mtb, which was set to a reference value of 1.

D. Metabolic alterations in upper glycolytic and pentose phosphate pathways in the presence of nitrate, an alternate terminal electron acceptor capable of supporting TCA cycle activity at 1% O_2/5% CO_2. Shown values are expressed relative to those observed in normoxic Mtb, which was set to a reference value of 1.

A.



B.

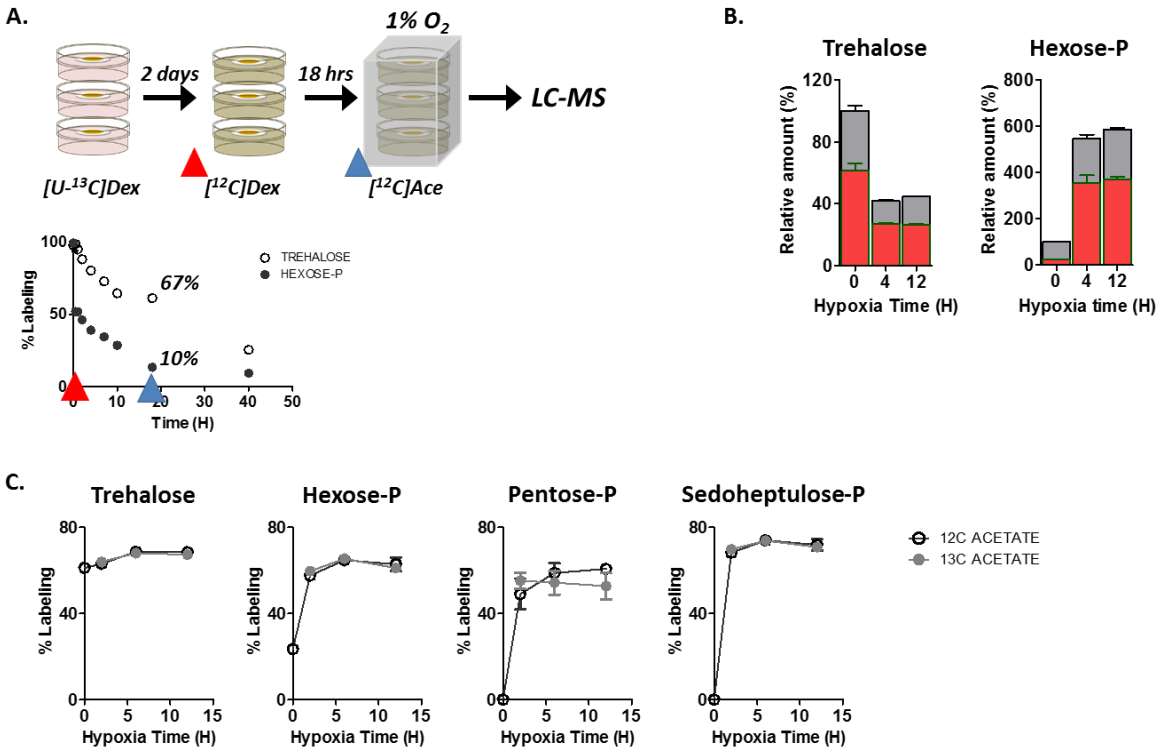
1. *M. tuberculosis* under replicating (20% O_2).
2. Nonreplicating *M. tuberculosis* exposed to 1% O_2 for 4 hr.
3. Nonreplicating *M. tuberculosis* exposed to 1% O_2 for 24 hr.
4. Reaerated *M. tuberculosis* for 24 hr after exposure to 1% O_2 for 24 hr.

Supplementary Figure 3

Hypoxia induced depletion of TDM content in *M. tuberculosis*.

A. Extracted ion chromatograms of *M. tuberculosis* phosphatidylethanolamine, free mycolic acid, trehalose dimycolate, and diacylated sulfoglycolipid isolated from replicating Mtb.

B. Analysis of the extractable TDM from *M. tuberculosis* incubated for 24 hr at 20% O_2 (1); 4 h at 1% O_2 (2); 24 h at 1% O_2 (3); and 24 hr at 20% O_2 following re-aeration of *M. tuberculosis* pre-incubated at 1% O_2 for 24 hr (4). Equal amounts of total cellular lipids were run in the solvent system (chloroform:methanol:water; 20:4:0.5; v/v/v). The TLC plate was sprayed with α-naphthol to specifically reveal glycolipids. Pink-stained compounds correspond to glycolipids; whitish-stained compounds migrating close to the origin are phospholipids. The labeled band shown in the TLC correspond to the R_f of a TDM standard.



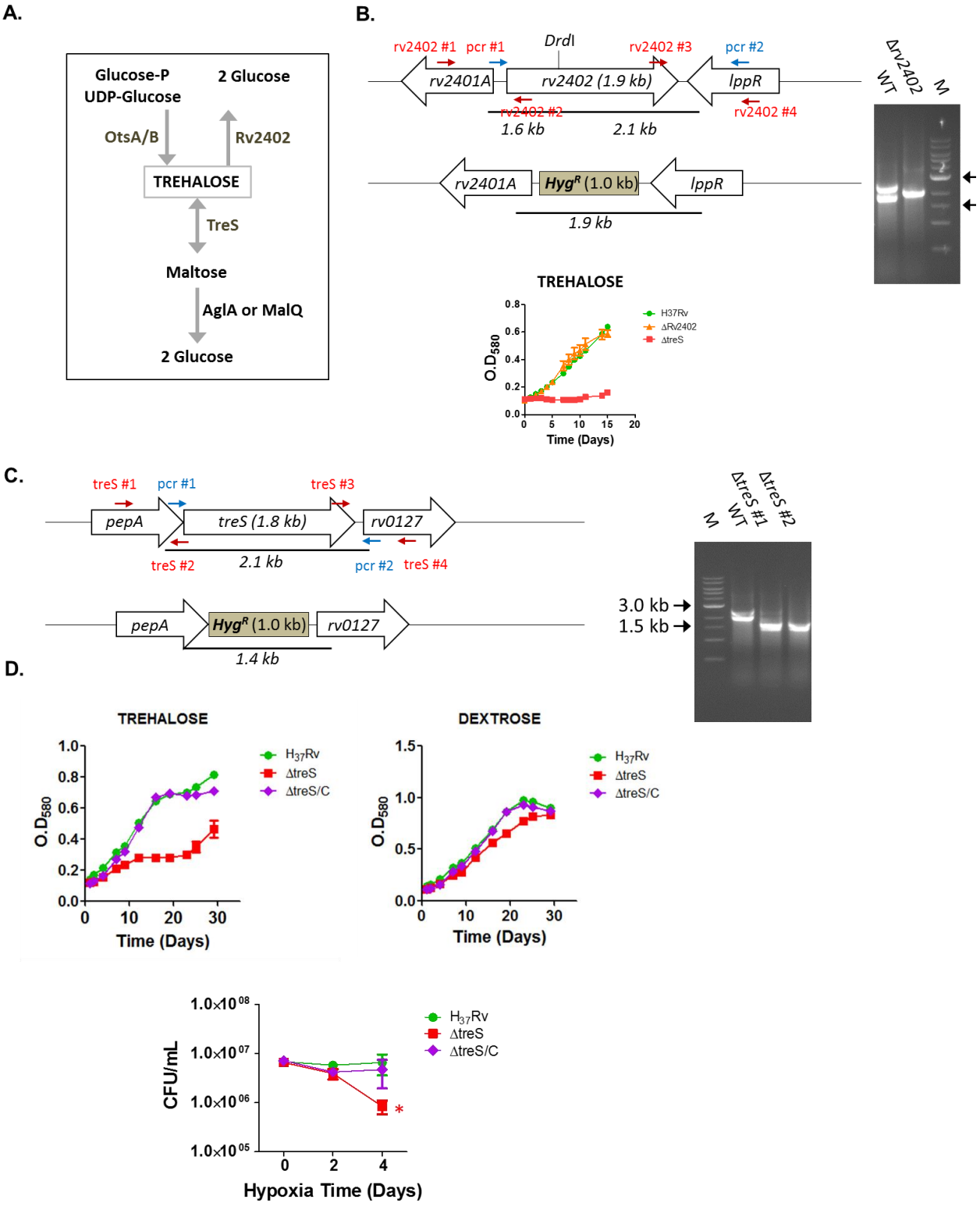
Supplementary Figure 4

Pulse-chase labeling of *M. tuberculosis* cell envelope reveals hypoxia-induced catabolism of TMM into intermediates of upper glycolysis, pentose phosphate pathway, and aminosugar biosynthesis.

A. Top, schematic diagram illustrating the labeling strategy used to selectively pre-label cell envelope TMM and follow the metabolic fate of labeled trehalose and hexose phosphate following exposure to 1% O_2/5%CO_2. Bottom, Time course plot demonstrating the extent of remaining 13C labeling in free hexose phosphate and trehalose pools following 48h incubation in [U-^{13}C] glucose, followed by 18 h of isotopic washout in unlabeled glucose. Shown values indicate % of total metabolite pools that consist in ^{13}C labeled species. Red and blue arrows indicate time points when the metabolic enrichment was measured.

B. Time course plot demonstrating the extent of labeling observed in free hexose phosphate and trehalose pools during incubation in 1% O_2/5%CO_2 for either 4 or 12 h after 48 h of incubation in [U-^{13}C] glucose, followed by 18 h of isotopic washout in unlabeled glucose. Shown values are expressed relative to those observed in normoxic Mtb, whose total pool sizes were set to a reference value of 100%.

C. Time course plot of ^{13}C enrichment of trehalose, hexose phosphate, pentose phosphate, and sedoheptulose phosphate following exposure to 1% O_2/5%CO_2 in the presence of unlabeled (open circle) or [U-^{13}C] (closed circle) acetate. Shown values indicate % of total metabolite pools that consist in ^{13}C labeled species. All values are means ± SEM of biological replicates (n=3) and representative of 2 independent experiments.


A.
Glucose-P
UDP-Glucose
2 Glucose
OtsA/B
Rv2402
TREHALOSE
TreS
Maltose
AglA or MalQ
2 Glucose
B.
rv2402 #1
pcr #1
DrdI
rv2402 #3
pcr #2
rv2401A
rv2402 (1.9 kb)
lppR
rv2402 #2
rv2402 #4
1.6 kb
2.1 kb
rv2401A
Hyg^R (1.0 kb)
lppR
1.9 kb
ΔRv2402
WT
M
TREHALOSE
O.D580
Time (Days)
H37Rv
ΔRv2402
ΔtreS
C.
treS #1
pcr #1
treS #3
pepA
treS (1.8 kb)
rv0127
treS #2
2.1 kb
pcr #2
treS #4
pepA
Hyg^R (1.0 kb)
rv0127
1.4 kb
M
WT
ΔtreS #1
ΔtreS #2
3.0 kb
1.5 kb
D.
TREHALOSE
DEXTROSE
O.D580
Time (Days)
H37Rv
ΔtreS
ΔtreS/C
CFU/mL
Hypoxia Time (Days)
*

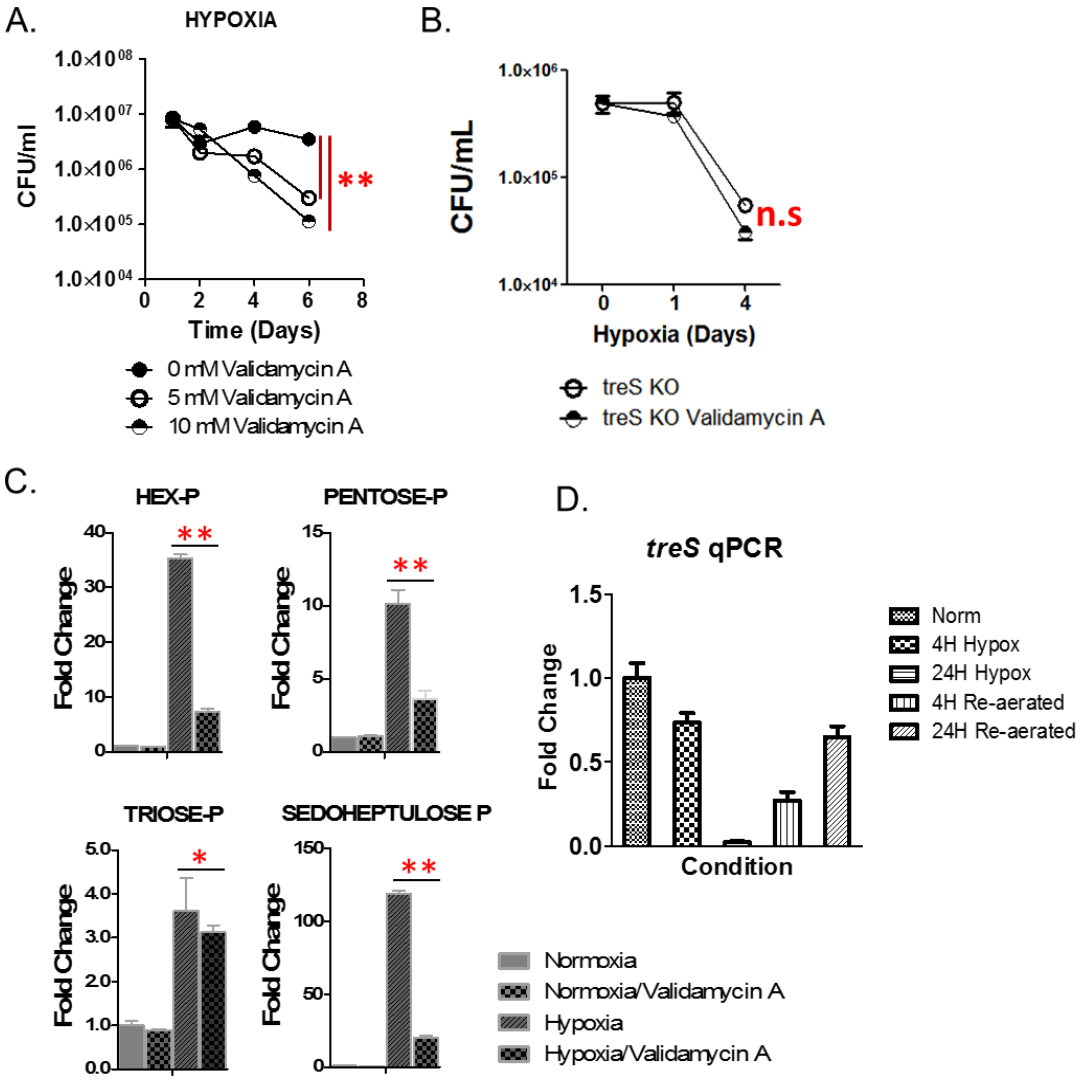
Supplementary Figure 5

A. Schematic diagram of annotated genes and enzymes involved in trehalose catabolism and biosynthesis in *M. tuberculosis*.

B. Generation of Δ*rv2402*. Strategy for deleting *rv2402* by homologous recombination and validation of Δ*rv2402* candidate by PCR using newly designed primer set (pcr #1 and pcr #2). PCR products were digested with *Drd*I and were at expected sizes. Growth curve indicated that Δ*rv2402* showed comparable growth to that of H37Rv wild type in m7H9 containing trehalose as a single carbon source.

C. Generation of Δ*treS*. Strategy for deleting *treS* (*rv0126*) by homologous recombination and validation of 2 independent Δ*treS* candidates by PCR using newly designed primer set (pcr #1 and pcr #2). PCR products were at expected sizes. Primer sequences used to generate and confirm indicates constructs are provided in Methods section.

D. Growth patterns (top right and left panels) and survival (bottom panel; recorded as CFU/ml) of wild type (H37Rv), *treS*-deficient (Δ*treS)* and complemented (Δ*treS*/C) *M. tuberculosis* in media containing either trehalose (left) or dextrose (right) as single carbon sources; and following incubation 1% O_2. *$p < 0.005$ by ANOVA with Bonferroni post-test correction. CFU= colony forming units. Primer sequences used to generate and confirm indicates constructs are described in Methods section


A.
HYPOXIA
CFU/ml
1.0x10 08
1.0x10 07
1.0x10 06
1.0x10 05
1.0x10 04
0
2
4
6
8
Time (Days)
**
0 mM Validamycin A
5 mM Validamycin A
10 mM Validamycin A
B.
CFU/mL
1.0x10⁶
1.0x10⁵
1.0x10⁴
0
1
4
Hypoxia (Days)
n.s
treS KO
treS KO Validamycin A
C.
HEX-P
PENTOSE-P
TRIOSE-P
SEDOHEPTULOSE P
Fold Change
**
*
Normoxia
Normoxia/Validamycin A
Hypoxia
Hypoxia/Validamycin A
D.
treS qPCR
Fold Change
1.5
1.0
0.5
0.0
Condition
Norm
4H Hypox
24H Hypox
4H Re-aerated
24H Re-aerated

Supplementary Figure 6

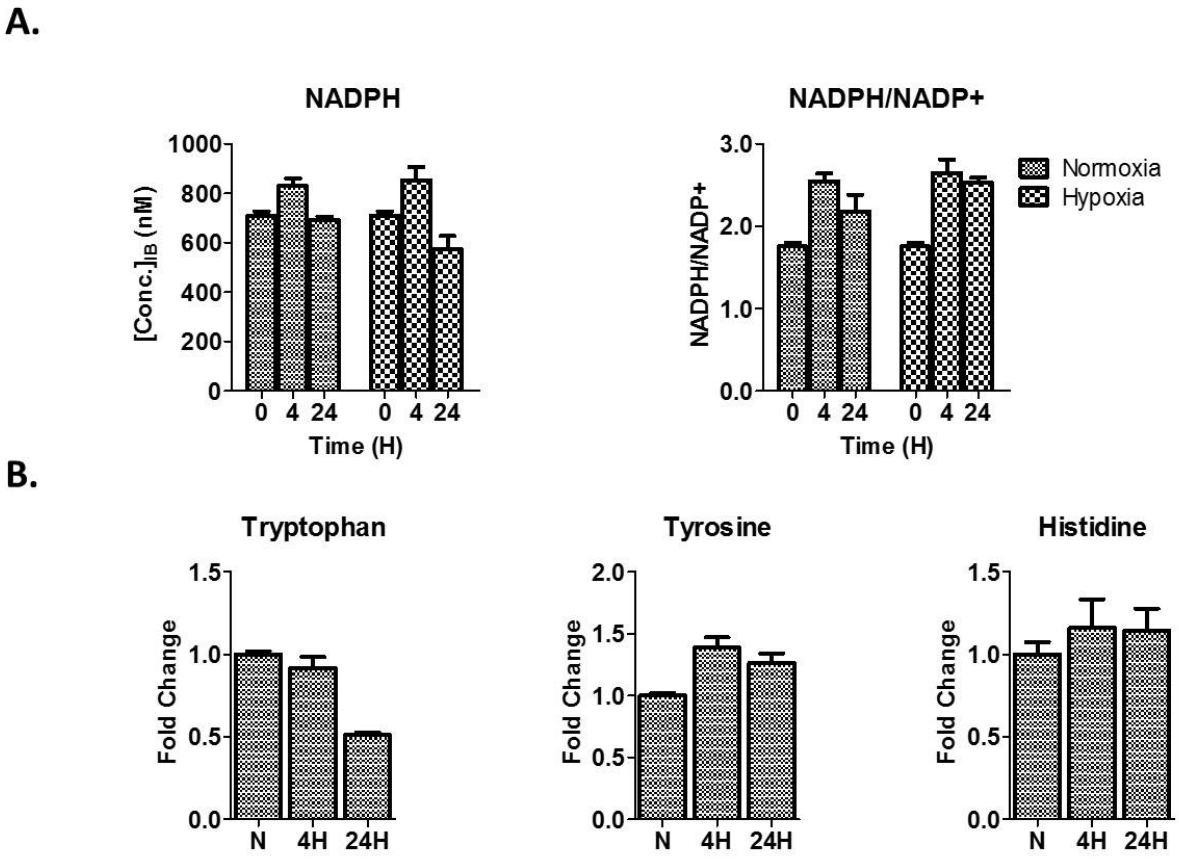
Chemical validation of TreS essentiality in response to hypoxia using the specific inhibitor validamycin A.

A. Viability of WT *M. tuberculosis* following treatment with validamycin A during adaptation to hypoxia vs. vehicle control. All values are means ± SEM of 3 biological replicates (n=3) and representative of 2 independent experiments. **p≤0.005 by 2-way ANOVA with Bonferroni post-test.

B. Viability of Δ*treS M. tuberculosis* following treatment with validamycin A during adaptation to hypoxia vs. vehicle control. All values are means ± SEM of 3 biological replicates (n=3) and representative of 2 independent experiments. n.s, not significant by Student's unpaired t-test.

C. Metabolic profiles of Mtb treated with the TreS inhibitor validamycin A under normoxia or hypoxia. Shown values are expressed relative to those observed in normoxic Mtb, which was set to a reference value of 1. All values are means ± SEM of 3 biological replicates (n=3) and representative of 2 independent experiments. *p≤0.05, **p≤0.005 by Student's unpaired t-test.

D. Relative changes of *treS* mRNA transcript under varying metabolic states including normal replicating (Norm), 4h (4H Hypox) or 24h (24H Hypox) hypoxia, and 4h (4H Re-aerated) or 24h (24H Re-aerated) reaeration after 24h hypoxia. Shown values are expressed relative to those observed in normoxic Mtb, which was set to a reference value of 1. All values are means ± SEM of 3 biological replicates (n=3) and representative of 2 independent experiments.

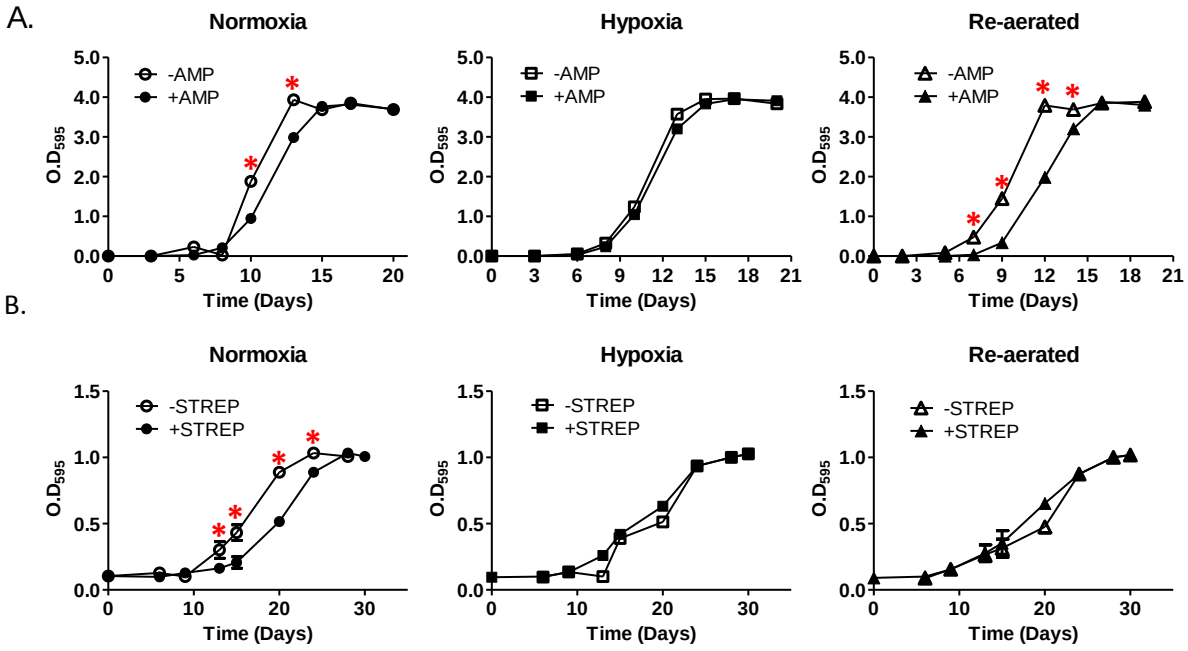


Supplementary Figure 7

Metabolic consequences of hypoxia-induced increase in pentose phosphate pathway intermediates.

A. Intrabacterial levels in NADPH and NADPH/NADP+ ratio were detected by LC-MS metabolomics. No statistically significant changes greater than 2-fold ($p<0.05$ by Student's unpaired t-test) were detected.

B. Relative pool size changes of four aromatic amino acids such as phenylalanine, tryptophan, tyrosine, and histidine were calculated. N= normoxia; 4H= 4 h incubation at 1% O_2/5%CO_2; 24H= 24 h incubation at 1% O_2/5%CO_2. Shown values are expressed relative to those observed in normoxic Mtb, which was set to a reference value of 1. All values were the average of 3 biological replicates ±SEM and representative of 2 independent experiments. No statistically significant changes greater than 2-fold ($p<0.05$ by Student's unpaired t-test) were detected.



Supplementary Figure 8

Metabolic state dependent sensitivity of *M. tuberculosis* against a peptidoglycan inhibitor, ampicillin.

A. Growth curves of *M. tuberculosis* incubated in ambient air and during re-aeration following 24h challenge with the covalent peptidoglycan biosynthesis inhibitor, ampicillin, at 8X MIC (100 ug/ml) and 1:100 subculture into antibiotic-free media.

B. Growth curves of *M. tuberculosis* in different metabolic states were also monitored in the presence of 2X MIC (1.25 µg/ml) streptomycin (STREP), a protein synthesis inhibitor, to show the functional specificity of hypoxia induced intermediates in *de novo* peptidoglycan biosynthesis during reactivation.

All values are the average of three biological replicates ± SEM and representative of two independent experiments. *p≤0.05 between antibiotic-treated (+AMP or +STREP) and vehicle control (-AMP or –STREP) by Student's unpaired t-test.