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Supplementary Information for

Chemical disarming of isoniazid resistance in Mycobacterium tuberculosis

Kelly Flentie[1], Gregory A. Harrison[1], Hasan Tükenmez[2], Jonathan Livny[3], James A. D. Good[4,5], Souvik Sarkar[4,5], Dennis X. Zhu[1], Rachel L. Kinsella[1], Leslie A. Weiss[1], Samantha D. Solomon[1], Miranda E. Schene[1], Mette R. Hansen[4,5], Andrew G. Cairns[4,5], Martina Kulén[4,5], Torbjörn Wixe[4,5], Anders E. G. Lindgren[4,5], Erik Chorell[1,4,5], Christoffer Bengtsson[4,5], K. Syam Krishnan[4,5], Scott J. Hultgren[1,6], Christer Larsson[2,5], Fredrik Almqvist[4,5*], and Christina L. Stallings[1*]

Correspondence to:
Christina Stallings
stallings@wustl.edu
Fredrik Almqvist
fredrik.almqvist@umu.se

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Supplementary Information Text

Materials and Methods

Bacterial strains and growth conditions

For each experiment, WT *Mtb* Erdman was inoculated from a freezer stock into Middlebrook 7H9 liquid media supplemented with 60 µl/l oleic acid, 5 g/l bovine serum albumin, 2 g/L dextrose, 0.003 g/L catalase (OADC), 0.5% glycerol, and 0.05% Tween 80. Actively growing *Mtb* was then inoculated into Sauton's liquid medium (0.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 4.0 g/L L-asparagine, 6% glycerol, 0.05g/L ferric ammonium citrate, 2.0 g/L citric acid, 0.01% (w/v) $ZnSO_4$) and used for experiments. Viable CFU were enumerated on Middlebrook 7H10 or 7H11 agar media supplemented with OADC and 0.5% glycerol. Δ*cydA Mtb* was generated using specialized transduction (1). The homology region upstream of *cydA* was amplified using Erdman genomic template DNA by RK9: GAAGCTTGTGATACCGAACTGCCACCGCGAAATGTC and RK10: GAGATCTGCGGCCACATAAACGTCAAGGG, and the downstream homology region was amplified by RK11: GTCTAGAGACGACGAGGTAGCACCATTG and RK12: GGGTACCGGTCACCCACGTCATGATCTTG. A HindIII/SmaI fragment of the upstream region and a XbaI/KpnI fragment of the downstream region were cloned upstream and downstream of a hygromycin resistance marker (Hyg^R). We recombined this cassette into the genome of a specialized transducing phage and transduced WT Erdman *Mtb* with this engineered phage. By selecting for hygromycin resistant *Mtb*, we identified *Mtb* strains that had replaced the *cydA* gene with Hyg^R by homologous recombination. Successful replacement of the *cydA* gene was confirmed by Southern blot.

Compound Preparation

The 2-pyridone library contained a mix of compounds with a diverse substitution pattern in positions C2 and C6-C8. These were prepared by previously published methodology (2-7). C10 was prepared as previously described (8, 9). For experiments involving agar media containing C10, we developed a C10-imidazole salt (C10-IMD) with improved solubility by the following method. HPLC-purified C10 was dissolved in methanol in room temperature to which 1 equivalent of imidazole from a separately prepared imidazole (IMD) stock solution (20 mg/mL) in methanol was added. The reaction mixture was stirred at room temperature for 2 hours in a closed round-bottom flask. After 2 hours, the solvent was evaporated under vacuum and the resulting product was mixed well with 10 mL of Acetonitrile-Water (1:3). The resulting mixture was subjected to freeze drying overnight, resulting in C10-IMD, a white solid. C10-IMD was used in place of C10 in the following figures: Fig. 2D, Fig. 3A-F, Fig. 4A, D-E, and Fig. 5B. The concentration of IMD was normalized in all samples in Fig. 2D, Fig. 3D-F, and Fig. 4A. In Fig. 4E-F, the 0 μM C10 ±INH samples have 25 μM IMD, and in all experiments the concentration of DMSO is normalized in all samples.

Antibiotics

Q203 was synthesized and provided by Enamine (New Jersey). Clofazimine, Hygromycin B, isoniazid, ethambutol, streptomycin, and rifampicin were all purchased from Sigma-Aldrich.

Hypoxia-induced pellicle formation and tolerance assays

Sauton's media was inoculated with stationary phase *Mtb* at a 1:100 dilution with or without C10. Culture vessels were closed tightly to restrict oxygen for 3 weeks, at which point seals on the vessels were opened, and for biofilm assays, the cultures were incubated for 2 additional weeks before pictures were taken and/or CFU were enumerated. For tolerance assays, when hypoxic culture vessels were opened and re-aerated, H_2O_2 or antibiotics was pipetted into the

media at the indicated concentrations. To enumerate viable CFU after 2 weeks of exposure to the indicated stress, bacteria were harvested from each well, centrifuged to pellet, and resuspended in 1% Tween 80 in phosphate-buffered saline (PBS). Glass beads were added to each tube and tubes were shaken overnight at room temperature to disassociate bacterial clumps. Serial dilutions were plated to enumerate viable CFU.

katG sequencing

To sequence the *katG* gene in isolated colonies from INH plates, we inoculated bacteria into liquid medium containing 0.5 μg/ml INH. Genomic DNA was isolated from these cultures and *katG*-specific primers were used to amplify segments of the *katG* gene and to sequence the amplicons. Sequences were compared to the WT *Mtb* Erdman *katG* sequence. Primer sequences: katG1: GCGGGTTGTGGTTGATCG, katG2: CAGCACGGCAAAGGATTC, katG3: GACAGTCAATCCCGATGC, katG4: AGCCCAAGCCCATCTG, katG5: TCGTGGATGCGGTAGGTG. Sequencing was performed by Genewiz, South Plainfield, NJ.

Preparation of RNA and RNA-sequencing

cDNA libraries were prepared from total RNA using the RNAtag-Seq protocol (10). Briefly, RNA samples were fragmented, depleted of genomic DNA, and dephosphorylated prior to their ligation to barcoded adaptors. Barcoded RNAs were pooled, depleted of rRNA using RiboZero (Epicentre) and converted to Illumina cDNA libraries in 3 steps: (i) reverse transcription of the RNA primed from the ligated adapter; (ii) degradation of the RNA and ligation of a second adaptor to the single-stranded cDNA; (iii) PCR amplification of the cDNA with primers targeting the ligated adapters and carrying the full sequence of the Illumina sequencing adaptors. The aerobic RNA libraries were sequenced on HiSeq2500 using 1x50 reads. The hypoxic RNA libraries were sequenced on HiSeq 2000 to yield 25b PE reads.

RNA-Sequencing data analysis

For aerobic RNA-sequencing Salmon was used in mapping-based mode to map and quantify RNA sequencing reads (11). A transcriptome index was created using the *M. tuberculosis* H37Rv transcriptome genome assembly ASM19595v2. Count tables were imported into R using tximport (12). Differential expression analysis was performed in R using DESeq2 (13). For hypoxic RNA-sequencing, sequencing reads were aligned to the H37Rv genome (RefSeq NC_000962), and the overall fragment coverage of genomic regions corresponding to features such as open reading frames and rRNAs based on RefSeq annotations was conducted by using bioinformatic pipelines developed in house as previously described (14). Differential-expression analysis was conducted with DESeq (15). Transcript functional categorization was performed by determining the "functional category" assigned on Mycobrowser (https://mycobrowser.epfl.ch/).

Multiplate alamar blue assays (MABAs).

Mtb was inoculated from a freezer stock into 7H9+OADC and grown to mid-log phase. Logarithmically growing *Mtb* was then inoculated into Sauton's medium in 96 well plates with wells containing increasing concentrations of C10 at an ODλ_{600} of 0.0008, corresponding to approximately 4x10^5 CFU/mL in 200 µL per well. Plates were incubated at 37˚C for 1 week, at which point 32.5 µL of a resazurin-tween mixture (8:5 ratio of 0.6mM Resazurin in 1XPBS to 20% Tween 80) was added and the plate was incubated at 37˚C overnight. Production of fluorescent resorufin was measured by removing samples from the plate, mixing with formalin to kill the *Mtb*, and measuring the fluorescence on a Tecan M200 Pro plate reader with excitation λ_{ex} = 530 nm and emission λ_{em} = 590 nm. For each assay, media alone served as a negative control and untreated *Mtb* was included as a positive control. The % inhibition was calculated as

the ((positive control - negative control) - (fluorescence of the sample - negative control))/(positive control - negative control) x 100%.

ATP Quantification.

Mtb was inoculated into Sauton's medium ± compounds at an $OD\lambda_{600}$ of 0.1 and incubated in a roller apparatus for 24 hours. An aliquot of the culture was then heat inactivated at 95°C for 20 min and diluted 1:100. Samples were then mixed with BacTiter Glo (Promega) reagent at a 1:1 ratio, and luminescence was quantified on a Tecan M200 Pro plate reader (integration = 1 second). Luminescence was normalized to the OD_{600} of the culture to account for changes in bacterial growth.

CellROX assay to measure ROS.

Mtb was inoculated into Sauton's medium ± compounds at an $OD\lambda_{600}$ of 0.1 and incubated in a roller apparatus for 24 hours. Then 5 mL of the culture were pelleted and resuspended in 0.5 mL of media containing 10 μM CellROX Green reagent (Thermofisher). Samples were incubated shaking for 37°C for 1 hour, and then washed twice with PBS containing 0.05% Tween 80 and resuspended in 4% paraformaldehyde to kill the *Mtb*. Fluorescence was measured using a Tecan M200 Pro plate reader with excitation λ_{ex} = 485 nm and emission λ_{ex} = 520 nm. Fluorescence was normalized to the OD_{600} of the culture to account for changes in bacterial growth.

Data and Statistics

Determination of the significance of differences in measurements was performed with Prism (Graphpad Software, Inc) by calculating p values using unpaired Student's t test (to compare two groups) or using a one- or two-way ANOVA with Tukey's multiple comparison test (to compare more than two groups), as indicated. To compare CFU/mL data, the data was first log_{10}-

transformed and then a t test, one-way ANOVA, or two-way ANOVA was used to compare between groups. To determine the IC_{50} values in the MABA, each replicate was graphed separately and the IC_{50}'s were determined for each replicate individually. Then IC_{50}'s were averaged and the standard deviation was determined. Only relevant statistical comparisons are depicted in figures, but complete statistical comparisons for all data can be found in table S1.

Fig. S1.

C10 inhibits *Mtb* biofilm formation. *Mtb* was incubated in low oxygen in Sauton's medium in the presence of a range of C10 concentrations for 3 weeks, then re-aerated and incubated for 2 additional weeks. Representative image from at least 3 replicates is shown.

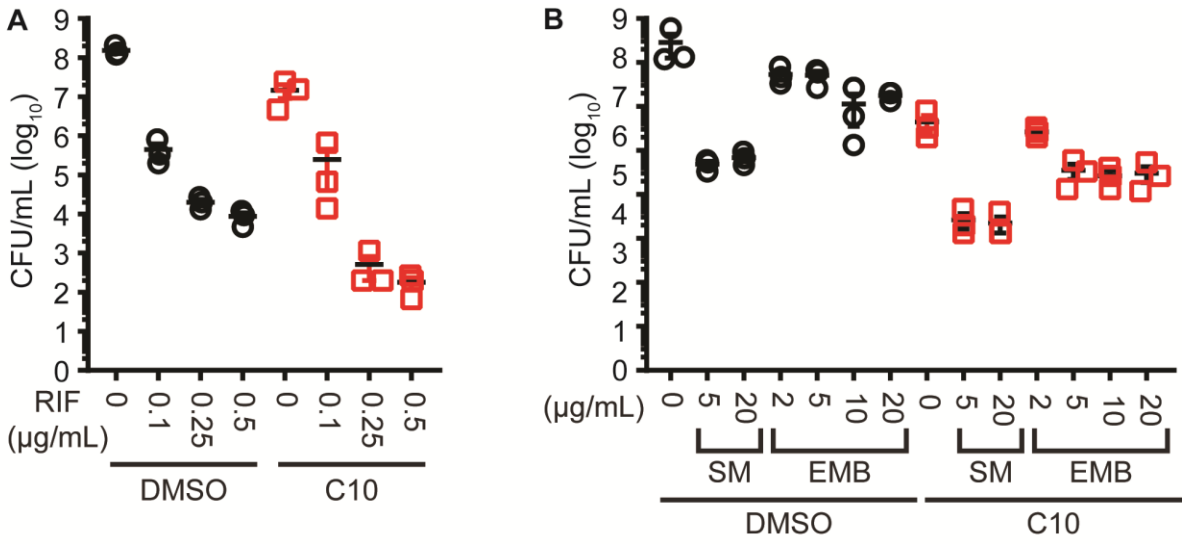

Fig. S2.

C10 does not block the development of hypoxia-induced tolerance to rifampicin (RIF), streptomycin (SM), or ethambutol (EMB). *Mtb* was cultured in low oxygen conditions ±50 μM C10 for 3 weeks, then re-aerated and treated with the indicated concentration of (A) RIF or (B) SM or EMB for an additional 2 weeks before CFU/mL were enumerated. Mean ±SEM between biological triplicates is graphed for each sample. Complete statistical comparisons for all data are in table S1.

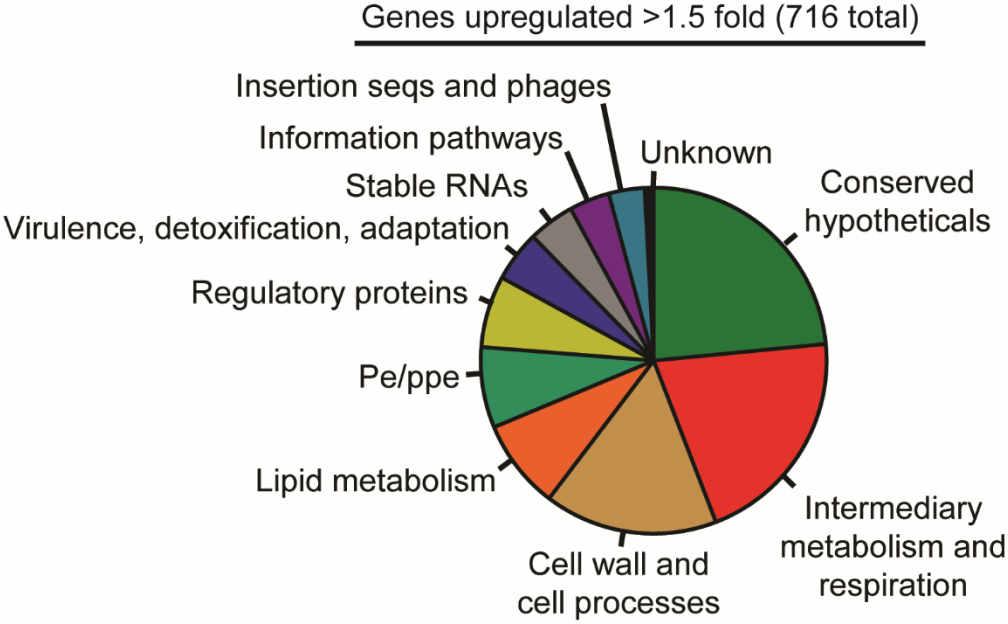

Fig. S3.

Functional categories of genes up-regulated >1.5-fold in C10-treated cultures during hypoxia. RNA-seq was performed on *Mtb* treated with 50 μM C10 for 2 weeks in hypoxic conditions. The functional categories based on gene annotations in Mycobrowser for the genes significantly ($p_{adj} < 0.05$) up-regulated > 1.5-fold are presented in a pie chart.

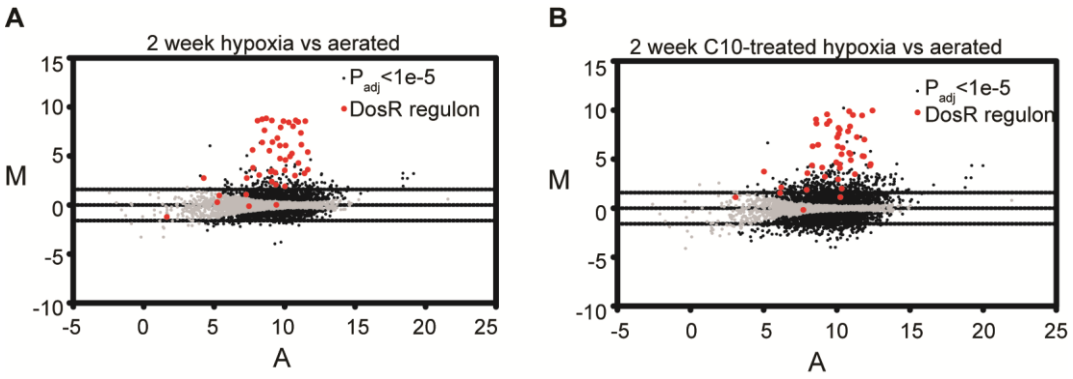

Fig. S4.

C10 treatment does not inhibit the induction of the DosR regulon in hypoxic *Mtb*. (A-B) MA plots of *Mtb* transcript abundance after 2 weeks of incubation in low oxygen conditions in the (A) absence or (B) presence of 50 μM C10. Each dot represents an annotated *Mtb* gene with the $\log_2$ relative transcript abundance in hypoxic conditions divided by that of aerated conditions (M) plotted against the $\log_2$ of its average abundance in both conditions (A). M and A values are based on data from 3 biological replicates. The dotted lines mark 3-fold differential abundance.

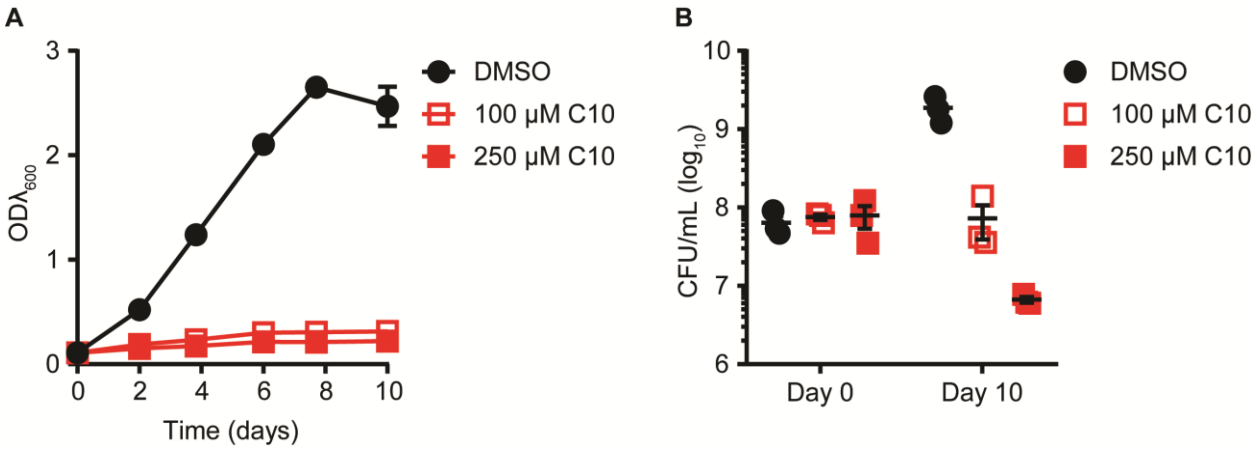

Fig. S5.

C10 inhibits growth of *Mtb* at high concentrations. (A-B) WT *Mtb* was incubated in Sauton's media the presence of DMSO, 100 μM, or 250 μM C10 and (A) the ODλ$_{600}$ was monitored over 10 days. (B) Viable bacteria were enumerated by plating CFU on agar media containing no drugs on Day 0 and Day 10 of the treatment. n=3, complete statistical comparisons are presented in *SI Appendix* Table S1.

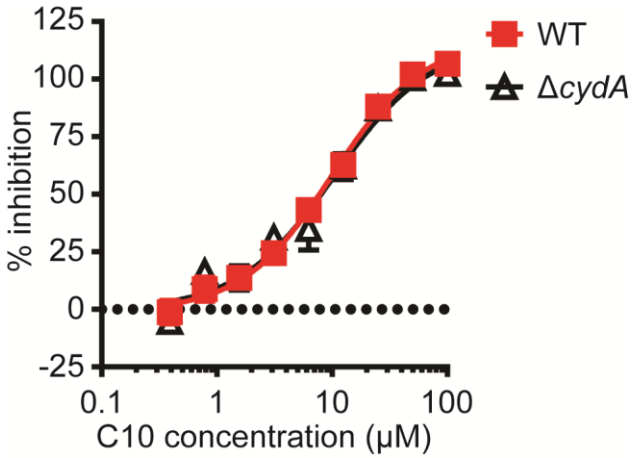

Fig. S6.

C10 inhibits respiration in Δ*cydA Mtb* very similarly to WT. Either WT (n = 2) or Δ*cydA* (n = 4) *Mtb* was incubated with increasing concentrations of C10 and respiration was measured in the MABA.

Table S1.

Complete statistical comparisons for all data.

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Table S2.

katG mutations in *Mtb* colonies that grew on Sauton's media plates containing 0.5 μg/ml INH (pictured in 2C). AA stands for amino acid.

DNA mutation	Consequence
Insertion	Frameshift, AA6
Missense	W328L
Deletion	Frameshift, AA477
Missense	A172T
Missense	A144E
Deletion	Frameshift, AA673
Deletion	Frameshift, AA401

Table S3.

RNA-seq data for *Mtb* cultured in aerobic conditions and exposed to 5 μM or 25 μM C10 for 48 hours. Genes are listed by the Rv number, as based on the H37Rv genome, with the associated Gene Name, Functional Category, Gene Product, and Predicted Function from Mycobrowser. Log_2 fold change and linear fold change describe the change in gene expression for each gene in the C10-treated samples divided by DMSO treated samples. The p_{adj}-value is listed.

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Table S4.

Genes significantly upregulated 1.5-fold ($p_{adj} < 0.05$) in *Mtb* treated with 5 μM or 25 μM C10 for 48 hours in aerated planktonic conditions. Genes are listed by the Rv number, as based on the H37Rv genome, with the associated Gene Name, Functional Category, Gene Product, and Predicted Function from Mycobrowser. Log_2 fold change and linear fold change describe the change in gene expression for each gene in the C10-treated samples divided by DMSO treated samples. The p_{adj}-value is listed.

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Table S5.

Fold change of the transcript levels for ETC-related enzymes in C10-treated *Mtb* compared to DMSO-treated control samples in both aerobic and hypoxic conditions. Aerobic *Mtb* cultures were treated with either 5 μM or 25 μM C10, whereas hypoxic *Mtb* cultures were treated with 50 μM C10. Genes are organized into enzyme class based on if they encode subunits of a dehydrogenase, a terminal oxidase, ATP synthase, or a quinone biosynthesis enzyme. The enzyme that is encoded by each gene is indicated. When possible, the gene name is indicated. The corresponding gene Rv number is indicated for each gene. The f old change in gene expression and p_{adj}-value are listed for each gene in either aerobic or hypoxic conditions. Data is derived from the RNA-seq data in table S3 and table S6.

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Table S6.

RNA-seq data for *Mtb* cultured in hypoxic conditions in the presence of 50 μM C10 versus DMSO for 2 weeks. The gene identification column shows a gene identifier for each gene, as well as its annotated function. The Rv number and gene name are listed for each transcript when possible. Both the $\log_2$ fold change and the fold change describe the change in gene expression for each gene in the C10-treated samples divided by the DMSO treated samples. The p_{adj}-value is listed. The functional group of each gene is indicated as listed in Mycobrowser.

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Table S7.

Table of genes significantly upregulated 1.5-fold ($p_{adj} < 0.05$) in C10-treated cultures compared to DMSO-treated cultures after 2 weeks in low oxygen. Data is derived from the RNA-seq data in table S6. The gene identification column shows a gene identifier for each gene, as well as its annotated function. The Rv number and gene name are listed for each transcript when possible. Both the $\log_2$ fold change and the fold change describe the change in gene expression for each gene in the C10-treated samples divided by the DMSO treated samples. The p_{adj}-value is listed. The functional group of each gene is indicated as listed in Mycobrowser.

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Table S8.

RNA-seq data comparing expression of the hypoxia-responsive DosR regulon in *Mtb* cultured in hypoxic conditions for 2 weeks ±50 μM C10. Fold change represents gene expression in the hypoxic samples (±50 μM C10) divided by gene expression in aerobic *Mtb*.

	2 week hypoxic + DMSO: aerobic		2 week hypoxic + C10: aerobic	
Transcript	Fold change (log_2)	p value	Fold change (log_2)	p value
Rv0079	5.41	0	5.28	0
Rv0080	4.96	0	5.52	0
Rv0081	3.79	3.20E-142	4.36	6.29E-238
Rv0569	3.40	5.32E-155	3.21	9.85E-156
Rv0570	3.00	5.02E-173	3.47	0
Rv0571c	2.07	8.09E-60	2.95	5.68E-168
Rv0572c	2.75	3.53E-64	3.57	5.59E-147
Rv0573c	1.07	4.30E-10	1.88	2.93E-38
Rv0574c	3.06	4.33E-102	4.14	3.31E-251
Rv1733c	8.60	0	9.07	0
Rv1734c	-1.22	0.224905	1.12	0.015907
Rv1735c	0.98	9.16E-05	2.12	1.12E-24
Rv1736c	5.25	0	5.55	0
Rv1737c	6.08	0	6.16	0
Rv1738	8.76	0	8.64	0
Rv1812c	0.02	0.989474	1.13	2.25E-21
Rv1813c	5.62	0	6.32	0
Rv1996	7.36	0	7.26	0
Rv1997	6.06	0	6.32	0
Rv1998c	-0.11	0.587839	-0.17	0.353965
Rv2003c	3.50	1.04E-170	4.68	0
Rv2004c	4.59	0	5.64	0
Rv2005c	4.74	0	5.50	0
Rv2006	1.91	5.12E-62	2.00	6.29E-76
Rv2007c	6.83	0	7.58	0
Rv2028c	5.54	0	7.23	0
Rv2029c	6.42	0	8.02	0
Rv2030c	8.55	0	9.96	0
Rv2031c	8.56	0	9.88	0
Rv2032	8.62	0	8.89	0
Rv2623	8.47	0	9.47	0

Rv2624c	7.62	0	8.60	0
Rv2625c	8.43	0	9.50	0
Rv2626c	8.84	0	9.58	0
Rv2627c	7.92	0	8.19	0
Rv2628	6.39	0	6.47	0
Rv2629	3.61	0	4.35	0
Rv2630	3.31	3.93E-164	4.48	0
Rv2631	2.40	3.44E-76	4.10	0
Rv3126c	2.75	2.14E-17	3.73	1.14E-43
Rv3127	6.15	0	7.01	0
Rv3129	0.28	0.343218	1.54	9.82E-15
Rv3130c	8.64	0	8.34	0
Rv3131	8.03	0	7.84	0
Rv3132c	3.23	4.86E-208	4.50	0
Rv3133c	3.55	6.28E-226	4.9034	0
Rv3134c	4.28	0	5.3101	0

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Table S9.

RNA-seq data comparing expression of the CRP and Cmr regulons in *Mtb* cultured in aerobic or hypoxic conditions treated with 25 μM or 50 μM C10, respectively. H37Rv Gene number, gene name, log_2 Fold Change, linear Fold Change, and p_{adj} are presented for each gene in the CRP regulon (16) and for two different reported Cmr regulons (17, 18).

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