Trehalose Recycling Promotes Energy-Efficient Biosynthesis of the Mycobacterial Cell Envelope

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ABSTRACT The mycomembrane layer of the mycobacterial cell envelope is a barrier to environmental, immune, and antibiotic insults. There is considerable evidence of mycomembrane plasticity during infection and in response to host-mimicking stresses. Since mycobacteria are resource and energy limited under these conditions, it is likely that remodeling has distinct requirements from those of the well-characterized biosynthetic program that operates during unrestricted growth. Unexpectedly, we found that mycomembrane remodeling in nutrient-starved, nonreplicating mycobacteria includes synthesis in addition to turnover. Mycomembrane synthesis under these conditions occurs along the cell periphery, in contrast to the polar assembly of actively growing cells, and both liberates and relies on the nonmammalian disaccharide trehalose. In the absence of trehalose recycling, de novo trehalose synthesis fuels mycomembrane remodeling. However, mycobacteria experience ATP depletion, enhanced respiration, and redox stress, hallmarks of futile cycling and the collateral dysfunction elicited by some bactericidal antibiotics. Inefficient energy metabolism compromises the survival of trehalose recycling mutants in macrophages. Our data suggest that trehalose recycling alleviates the energetic burden of mycomembrane remodeling under stress. Cell envelope recycling pathways are emerging targets for sensitizing resource-limited bacterial pathogens to host and antibiotic pressure.

IMPORTANCE The glucose-based disaccharide trehalose is a stress protectant and carbon source in many nonmammalian cells. Mycobacteria are relatively unique in that they use trehalose for an additional, extracytoplasmic purpose: to build their outer “myco” membrane. In these organisms, trehalose connects mycomembrane biosynthesis and turnover to central carbon metabolism. Key to this connection is the retrograde transporter LpqY-SugABC. Unexpectedly, we found that nongrowing mycobacteria synthesize mycomembrane under carbon limitation but do not require LpqY-SugABC. In the absence of trehalose recycling, compensatory anabolism allows mycomembrane biosynthesis to continue. However, this workaround comes at a cost, namely, ATP consumption, increased respiration, and oxidative stress. Strikingly, these phenotypes resemble those elicited by futile cycles and some bactericidal antibiotics. We demonstrate that inefficient energy metabolism attenuates trehalose recycling mutant Mycobacterium tuberculosis in macrophages. Energy-expensive macromolecule biosynthesis triggered in the absence of recycling may be a new paradigm for boosting host activity against bacterial pathogens.

KEYWORDS Mycobacterium, mycomembrane, oxidative stress, starvation, trehalose

The mycobacterial cell envelope is comprised of covalently bound peptidoglycan, arabinogalactan, and mycolic acids, as well as intercalated glycolipids and a thick capsule (1). The mycolic acids attached to the arabinogalactan and the noncovalent glycolipids, respectively, form the inner and outer leaflets of the mycomembrane, a
distinctive outer membrane present in members of the Corynebacterineae suborder. The mycomembrane is a key determinant of envelope permeability and home to a variety of immunomodulatory lipids and glycolipids (2–4). There is substantial evidence that the mycomembrane is remodeled in vivo and in response to host-mimicking stresses, conditions in which mycobacterial growth and envelope synthesis are presumed to be slow or nonexistent (3, 5–13). While these studies have elucidated bulk changes in mycomembrane composition, the dynamics and subcellular distribution of the molecular transitions have not been characterized. It is also unclear in most cases whether the alterations are solely catabolic, or whether anabolic reactions also contribute to changes in mycomembrane composition under stress.

Recycling pathways are likely to be at the nexus of stress-triggered mycomembrane reorganization. Mycolic acids are ligated to the nonmammalian disaccharide trehalose in the cytoplasm (14). Once transported to the periplasm, trehalose monomycolate (TMM) donates its mycolic acid to arabinogalactan, forming arabinogalactan mycolates (AGM), or to an acceptor TMM, forming trehalose dimycolate (TDM; Fig. 1A). Both processes release free trehalose. TDM can also be degraded by TDM hydrolase (TDMH) into TMM and free mycolic acids, the latter of which are an important component of biofilm extracellular matrix in mycobacteria (7, 15). While a salvage mechanism for mycolic acids is still under debate (16–19), recapture of trehalose occurs via the LpqY-SugABC transporter (20). Depending on the specific environmental demand, mycobacteria may funnel reclaimed trehalose back to central carbon metabolism to generate intermediates for glycolysis or the pentose phosphate pathway or to store it in the cytoplasm, possibly as a stress protectant or compatible solute (6, 21–23). An additional but unexplored potential fate for recaptured trehalose is direct reincorporation into TMM or other glycoconjugates destined for the cell surface. Thus, trehalose connects mycomembrane synthesis and turnover to the metabolic status of the mycobacterial cell.

We find that mycomembrane remodeling triggered by nutrient limitation comprises both synthesis and degradation of AGM and TDM. Remodeling continues in the absence of trehalose recycling. However, compensatory anabolism upsets the energy and redox balance of the cell in a manner indicative of futile cycling (24–28). Similar dysfunction has been proposed to enhance the efficacy of certain antibiotics (29, 30), and indeed, loss of LpqY sensitizes Mycobacterium tuberculosis to multiple drugs (31). M. tuberculosis ΔsugC and ΔlpqY strains are also known to be attenuated during infection (20, 32, 33). We show here that inefficient ATP metabolism is the primary mechanism of attenuation in macrophages. While previous studies identified multiple phenotypes for trehalose recycling mutants, they did not explain how the LpqY-SugABC system contributes to mycobacterial fitness. Our data indicate that trehalose recycling minimizes energy consumption and oxidative stress during mycomembrane adaptation to nutrient limitation. Given the energetic costs associated with de novo biosynthesis, recycling pathways for trehalose and other mycomembrane components may be particularly important for M. tuberculosis resilience to stress.

RESULTS

Mycomembrane synthesis and degradation are active under carbon limitation. Decreased TDM abundance has been reported for mycobacteria growing in biofilms or adapting to hypoxia or nutrient limitation (3, 5, 7, 23). Since uncontrolled TDM hydrolysis results in cell lysis (7, 34), we sought to understand the kinetics of TDM turnover under stress. TMM donates mycolic acids to other molecules of TMM, to form the TDM glycolipid, or to arabinogalactan, to form covalent arabinogalactan mycolates (AGM, Fig. 1A). The TMM-mimicking probe N-AlkTMM specifically incorporates into TDM because the amide linkage permits mycolic acid acceptance but not donation of the alkyne-appended lipid chain (35). To track TDM hydrolysis under carbon limitation, we performed a pulse-chase experiment in which we labeled M. smegmatis with N-AlkTMM for 12 h in low (0.02%)-glucose-supplemented 7H9 medium then washed the
Mycomembrane synthesis and degradation are active under carbon limitation. (A) Mycomembrane synthesis and degradation. TMM, trehalose monomycolate; TDM, trehalose dimycolate; AG, arabinogalactan; AGM, arabinogalactan mycolates; MA, free mycolic acids; TDMH, TDM hydrolase. (B) TDM turnover under nutrient deprivation. *M. smegmatis* was cultured in 0.02% glucose-supplemented medium in the presence of metabolic probes O-AlkTMM (primarily labels AGM), N-AlkTMM (labels TDM), or HADA (labels cell wall peptidoglycan). After 24 h, the cultures were washed and resuspended in probe-free medium. Aliquots were removed 0, 4, and 8 h into the chase and fixed with 2% formaldehyde. Alkynes were detected by copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction with carboxyrhodamine-110 azide. Fluorescence was quantitated by flow cytometry, with the median fluorescence intensities (MFIs) being normalized to the initial, 0-h time point for each probe. The experiment was performed three times in triplicate; the results of one representative experiment are shown. (C) Metabolic labeling of *M. smegmatis* in 0.02% glucose-supplemented medium with O-AlkTMM, N-AlkTMM, and alkDala (labels peptidoglycan). Alkynes were detected by CuAAC reaction with carboxyrhodamine-110 azide. Data were normalized to labeling in 2% glucose-supplemented medium and plotted from four independent experiments. (D) Quantitation of TLC of different mycomembrane components for *M. smegmatis* in 0.02% glucose-supplemented medium. TDM, trehalose dimycolate; CS-MA, free, culture supernatant mycolic acids; AGM-MA, mycolic acids released from arabinogalactan. TLC results were scanned and processed in ImageJ (99). The data are normalized to TLC results from samples taken from *M. smegmatis* cultured in 2% glucose-supplemented medium and plotted from three independent experiments. (For representative TLC results, see Fig. S2.) (E) PI staining of *M. smegmatis* during adaptation to low carbon. *M. smegmatis* was cultured in 0.02% glucose-supplemented medium. Aliquots were removed at 13, 24, and 48 h and incubated with PI. Fluorescence was quantitated by flow cytometry, and the MFI was plotted. The experiment was performed three times in triplicate; the results of one representative experiment are shown. (F) O-AlkTMM labeling of *M. smegmatis AGM* in 2 or 0.02% glucose-supplemented medium. (Left) Fluorescence microscopy. Scale bars, 5 μm. (Right) The cellular fluorescence was quantitated for cells lacking visible septa from three independent experiments. The signal was normalized to both cell length and total fluorescence intensity. Cells were oriented such that the brighter pole is on the right-hand side of the graph. A.U., arbitrary units. (G) Quantification of trehalose from supernatants of *M. smegmatis* wild-type and ΔsugC strains cultured in 2 or 0.02% glycerol-supplemented medium. The experiment was performed at least three times in triplicate; the results of one representative experiment are shown. Error bars, standard deviations. The statistical significance of 0.02% versus 2% glucose or glycerol samples from three independent experiments was assessed by two-tailed Student t test. *, P < 0.05; **, P < 0.005.
sample before transferring it to 7H9 lacking both the probe and glucose (Fig. 1B, left). Alkyne-labeled TDM was detected on fixed cells at 0, 4, and 8 h posttransfer by copper-catalyzed azide-alkyne cycloaddition (CuAAC) with a fluorescent azide label. We found that TDM labeling decreased by ~3-fold in this time period (Fig. 1B, right). Fluorescence derived from D-amino acid-labeled cell wall peptidoglycan remained steady, however, consistent with limited bacterial growth under this condition (Fig. 1B, right; see also Fig. S1A in the supplemental material).

Under acid stress, nonreplicating but metabolically active M. tuberculosis make new TDM (9). We found that N-AlkTMM uptake (no chase) increased ~2-fold in low-glucose medium (Fig. 1C). However, a decline in the steady-state abundance of TDM (Fig. 1D; see also Fig. S2B) suggested that enhanced synthesis is outweighed by the TDM turnover observed in the pulse-chase experiment (Fig. 1B, right).

We hypothesized that there were additional changes in mycomembrane metabolism. O-AlkTMM is also a TMM-mimicking probe but features an ester-linked lipid chain. While the molecule can serve as either an alkyne-lipid donor or acceptor, ~90% of labeling from this probe is present in the M. smegmatis AGM cellular fraction (35). O-AlkTMM uptake was enhanced in low-glucose medium to a greater extent than N-AlkTMM (Fig. 1C). The fluorescence signal derived from this probe was also more persistent than N-AlkTMM in a no-probe, no-glucose chase (Fig. 1B).

A variety of carbohydrates can serve as mycolate acceptors, including glucose (36, 37). High levels of glucose in the growth medium might therefore suppress O-AlkTMM labeling of the cell surface by competing with arabinogalactan. While in our labeling window M. smegmatis grew faster in 7H9 medium with high (2%) versus medium (0.2%) glucose supplementation, O-AlkTMM-derived fluorescence in the high-glucose condition was lower (see Fig. S1B). However, O-AlkTMM labeling was similar for M. smegmatis in 0.2 or 0.02% glucose or acetate (see Fig. S1B), despite sluggish or absent bacterial replication under the low carbon conditions (see Fig. S1A). Thus, incorporation of O-AlkTMM into AGM is suppressed in high glucose, likely because the alkyne-fatty acid from the probe is transferred to the unanchored glucose and washed away. Nonetheless our data indicate that substantial AGM synthesis occurs in growth-limiting amounts of glucose or acetate. Since the steady-state abundance of the molecule did not change in carbon-limited medium (Fig. 1D; see also Fig. S2C), these experiments also suggest that AGM synthesis is balanced by the turnover that we observed by pulse-chase (Fig. 1B, right).

We previously showed that the fluorescent D-amino acid HADA as well as alkyne-D-alanine (alkDala) incorporate into M. smegmatis peptidoglycan via both cytoplasmic and L,D-transpeptidase enzymes (38). HADA and alkDala labeling roughly correlated with mycobacterial growth rate under different amounts of glucose or acetate (Fig. 1C; see also Fig. S1A and C in the supplemental material). Suppressed levels of peptidoglycan synthesis or remodeling during carbon limitation stood in contrast to active mycomembrane metabolism.

**AGM synthesis occurs along the periphery of the mycobacterial cell during carbon limitation.** TDM hydrolysis enhances envelope permeability in oleic acid- and glucose-deprived M. tuberculosis (3). Surprisingly, despite an analogous decrease in TDM abundance (Fig. 1D; see also Fig. S2B), M. smegmatis became less permeable to propidium iodide when cultured in glucose-limited medium (Fig. 1E). Global AGM levels have also been linked to mycobacterial permeability (39). Although AGM abundance was relatively unaffected in glucose-deprived medium (Fig. 1D; see also Fig. S2C), our data suggest that the apparent stasis belies active synthesis and degradation (Fig. 1B and C). We considered whether AGM remodeling might impact its spatial distribution, which in turn could alter cell permeability.

Mycobacteria growing in nutrient-replete medium construct their cell envelope in gradients that emanate from the poles and continue along the sidewall (35, 38, 40–48). While polar peptidoglycan synthesis promotes cell elongation, sidewall synthesis occurs in response to cell wall damage (38). We hypothesized that the AGM synthesis that we observe under carbon deprivation (Fig. 1C) is a cell-wide response, similar to
peptidoglycan repair. Quantitative fluorescence microscopy revealed that O-AlkTMM labeling of *M. smegmatis* growing in carbon-replete medium comprised polar gradients (Fig. 1F) as expected (35, 38). However, in slow- or nongrowing, carbon-deprived *M. smegmatis*, O-AlkTMM-labeled species were more evenly distributed around the periphery of the cell. This observation suggests that AGM synthesis fortifies the mycomembrane along the sidewall as mycobacteria adapt to carbon deprivation.

**Trehalose cycling supports mycomembrane metabolism during carbon starvation.** Mycomembrane synthesis centers on the mycolic acid donor trehalose monomycolate (TMM). Prior to its export to the periplasm, TMM is synthesized in the cytoplasm by the ligation of a mycolic acid to trehalose (50). *De novo* synthesis of mycolic acids and trehalose is both energy and resource intensive; recycling pathways for both molecules have been shown or proposed (18–20). We hypothesized that nutrient-starved mycobacteria might buffer the costs of TMM synthesis by enlisting recycling pathways. Since the recycling mechanism for mycolic acids is still controversial (16, 17), we focused on the role of trehalose uptake.

Trehalose released as a by-product of extracellular mycomembrane metabolism is recycled via the LpqY-SugABC transporter (20) (Fig. 2A). At least two different processes liberate trehalose: (i) ligation of mycolic acids from TMM to arabinogalactan to form AGM and (ii) transfer of mycolic acids from TMM to another molecule of TMM to form TDM (Fig. 1A). Breakdown of TDM by the TDM hydrolase (TDMH) yields TMM and mycolic acids (7, 15, 34), so subsequent use of TMM in the foregoing reactions would also release trehalose. Our metabolic labeling results suggested that all of these processes are active as *M. smegmatis* adapts to carbon limitation (Fig. 1). We were unable to measure extracellular trehalose levels in wild-type *M. smegmatis*, presumably because LpqY-SugABC rapidly internalizes the disaccharide (20). However, by using *M. smegmatis* ΔsugC, a strain that lacks a functional trehalose transporter, we were able to detect elevated levels of trehalose in the supernatant when bacteria were grown in carbon-limited conditions (Fig. 1G; note that we used glycerol as the carbon source as glucose interferes with the assay). We also found that free mycolic acids accumulated in the supernatant of low glucose cultures (Fig. 1D; see also Fig. S2D), as expected from TDM turnover. Together, our data indicate that trehalose is liberated upon reorganization of the mycomembrane.

Exogenously supplied trehalose can support mycobacterial growth (20) after it is transported by LpqY-SugABC (20) and metabolized by trehalase (21) or TreS (6, 50–52) (Fig. 2A). We recovered similar CFU for ΔsugC, Δtre, ΔtreS, and wild-type *M. smegmatis* strains from 1, 2, 4, and 6 days in low glucose (Fig. 2B and C). These data suggest that trehalose catabolism is not required for viability, nor does it fuel appreciable cell growth, under carbon deprivation. Given that both the optical density and CFU of *M. smegmatis* were steady (Fig. 2B and C; see also Fig. S1A), trehalose recovered from the mycomembrane also does not fuel appreciable cell growth under this condition.

In hypoxic and biofilm cultures of *M. tuberculosis*, TMM and TDM levels decrease (5, 6, 23). Glycolipid turnover occurs rapidly in the former, within 4 h (6), and slowly in the latter, within 16 days (23). We did not observe a net decrease in TMM for *M. smegmatis* or *M. tuberculosis* under carbon limitation (Fig. 2F and G) despite an increase in TMM-consuming AGM and TDM remodeling (Fig. 1C). We posited that TMM pools might be replenished by recycled trehalose. Metabolic incorporation of exogenous 6-azido-trehalose (6-TreAz) by *M. smegmatis* or *M. bovis* BCG requires uptake by LpqY-SugABC (53). We found that 6-TreAz labeling was enhanced in slow-growing, glucose-starved *M. smegmatis* (Fig. 2D) or oleic acid- and glucose-starved *M. tuberculosis* (Fig. 2E) (3). As incorporation of the metabolite was respectively abolished or diminished in ΔsugC *M. smegmatis* (Fig. 2D; see also Fig. S3A) (53) or *M. tuberculosis* (Fig. 2E), enhanced 6-TreAz labeling under carbon limitation indicates an increase in trehalose recycling.

6-TreAz recovered by the LpqY-SugABC transporter may remain intact in the cytoplasm, be catabolized, or be converted to azido-TMM and transported outside the cell (Fig. 2A) (53). Although it has not been reported, it is possible that the probe
incorporates into other trehalose-bearing molecules in the mycobacterial envelope (21). To tune our detection for the cell surface, we selected DBCO-Cy5 as the fluorescent, azide-reactive label because the localized charge on the sulfonated cyanine dye confers poor membrane permeability (54). The enhanced 6-TreAz labeling that we observed for \textit{M. smegmatis} and \textit{M. tuberculosis} during carbon limitation (Fig. 2D and E) strongly suggests that at least some of the recycled trehalose is converted into an envelope component(s). Given that (i) TMM and TDM are the only known trehalose-containing glycoconjugates shared by both \textit{M. smegmatis} and \textit{M. tuberculosis} and that (ii) TDM cannot be labeled by 6-TreAz (53), we conclude that TMM is the most likely target. As steady-state TMM levels remained relatively constant in both species (Fig. 2F and G; see Fig. S3B and C), enhanced conversion of 6-TreAz to azido-TMM further

**FIG 2** Trehalose cycling supports mycomembrane metabolism during carbon limitation. (A) Potential fates of recycled trehalose in catabolism (trehalase [Tre] or TreS) or in trehalose monomycoate (TMM) biosynthesis. (B and C) Survival of wild-type, Δ sugC, complemented Δ sugC (CΔ sugC), Δ treS, and Δ tre \textit{M. smegmatis} strains in 0.02% glucose-supplemented medium. Tenfold serial dilutions were plated at the indicated time points. The experiment was performed two times with similar results; the results of one experiment are shown. (D and E) 6-TreAz labeling of wild-type and Δ sugC \textit{M. smegmatis} (Msmeg) and \textit{M. tuberculosis} (Mtub) cultured in low- or high-carbon medium. Azides were detected by strain-promoted azide-alkyne cycloaddition (SPAAC) with DBCO-Cy5 label. The fluorescence was detected by flow cytometry, with MFI values from controls lacking 6-TreAz (but subjected to SPAAC) subtracted from the sample MFI. The experiment was performed at least three times in triplicate; the results of one representative experiment are shown. (F and G) TMM abundance in \textit{M. smegmatis} and \textit{M. tuberculosis} cultured in low- or high-carbon medium. TLC results were scanned and processed in ImageJ (99). The data are normalized to the TLC results from mycobacteria cultured in high-carbon medium and plotted from two (\textit{M. tuberculosis}) or three (\textit{M. smegmatis}) independent experiments. (For representative TLC results, see Fig. S3B and C.) Error bars, standard deviations. The statistical significance of low- versus high-carbon samples was assessed by two-tailed Student t test. *, \( P < 0.05 \).
suggests that trehalose recycling under carbon deprivation helps to maintain TMM levels. These data are consistent with a model in which trehalose cycles in and out of the cell to remodel the mycomembrane in carbon-deprived mycobacteria.

**Mycomembrane reorganization under carbon deprivation can occur in the absence of trehalose cycling.** Our experiments suggest that trehalose cycling contributes to mycomembrane reorganization during carbon limitation. However, loss of trehalose import by LpqY-SugABC did not impact the abundance of TMM, TDM or AGM (see Fig. S2B, S2C, S3B, S3C, S4B, and S4C); synthesis of AGM or TDM (see Fig. S4D); turnover of TDM (compare Fig. 1B, right, to Fig. S4E); or permeability (see Fig. S4F). The absence of measurable changes in mycomembrane metabolism or composition were consistent with earlier work showing that *M. tuberculosis ΔsugC* and ΔlpqY strains do not have detectable changes in the glycolipid composition of their mycomembranes compared to wild type (20). These data also indicate that mycomembrane reorganization can occur in the absence of trehalose recycling.

**Trehalose recycling promotes redox and energy homeostasis under carbon limitation.** While trehalose recycling was dispensable for *M. smegmatis* and *M. tuberculosis* mycomembrane remodeling and survival under carbon limitation, we hypothesized that it might be important for withstanding other stressors. We first sought to determine whether blocking trehalose recycling disrupts redox homeostasis. We tested this hypothesis under growth-limiting (see Fig. S1) (3) carbon limitation since trehalose recycling is enhanced under this condition (Fig. 2D and E).

*M. smegmatis* and *M. tuberculosis ΔsugC* strains were sensitized to exogenously applied hydrogen peroxide and/or to reactive oxygen species (ROS)-potentiating vitamin C (55) (Fig. 3A and B; see also Fig. S5A and B). Loss of trehalose recycling also enhanced the fluorescence of dihydroethidium (DHE), an indicator dye of endogenous cellular superoxide (Fig. 3C) (56). Propidium iodide staining remained unchanged (see Fig. S4F), suggesting that the effect was not due to nonspecific differences in uptake, efflux, or cell size. In *M. smegmatis*, the total pool of cytoplasmic thiol antioxidants was modestly enhanced in the absence of *sugC* (see Fig. S5C). We hypothesized that the increase in free thiols in the *sugC* mutant might be an adaptation to counteract the higher basal levels of superoxide. Consistent with a drive to maintain a reduced thiol pool (57) (58), we observed increased NADP:NADPH (see Fig. S5D) in *M. smegmatis ΔsugC*. Taken together, our data suggest that trehalose recycling that occurs during carbon limitation supports redox balance.

A possible endogenous source of ROS in the bacterial cell is respiration, which in turn can be estimated by the oxidation of the methylene blue dye (59). In carbon-limited medium, we observed more methylene blue decolorization for the ΔsugC mutant (Fig. 3D), indicating that respiration is enhanced in the absence of trehalose recycling. Notably, however, the mutant had lower levels of ATP than the wild type (Fig. 3E). These data are consistent with a model in which trehalose recycling maintains redox balance in carbon-limited mycobacteria by minimizing ATP consumption and respiration (Fig. 3F). Alternatively, or additionally, redox balance may enable energy homeostasis under this condition.

**Trehalose anabolism disrupts redox balance under carbon limitation.** Cytoplasmic trehalose can protect against ROS directly, in plants, fungi, and other bacteria (60–63), or indirectly, via TreS-dependent catabolism in mature *M. tuberculosis* biofilms (23). To test whether either of these potential mechanisms could account for recycling-promoted redox homeostasis, we measured the total trehalose pools, endogenous ROS levels, and exogenous ROS sensitivity of mutants defective in trehalose catabolism or anabolism. There are several metabolic pathways for trehalose in mycobacteria: OtsA and OtsB convert phosphorylated glucose intermediates to trehalose; TreY and TreZ degrade the glucose polymer α-glucan into trehalose; TreS converts trehalose to maltose; trehalase degrades trehalose into glucose (Fig. 2A and 4A; see also Fig. S6A). We found that changes to the size of the trehalose pool that were due to perturbations in catabolism (see Fig. S6G and H) or anabolism (see Fig. S6B) did not correlate with endogenous ROS levels (see Fig. S6C) or sensitivity to exogenous ROS (see Fig. S6D, E,
FIG 3 Trehalose recycling promotes redox and energy homeostasis under carbon limitation. (A and B) Sensitivity of carbon-deprived wild-type, ΔsugC, and complemented ΔsugC (CΔsugC) *M. smegmatis* (A) or *M. tuberculosis* (B) strains to hydrogen peroxide. Tenfold serial dilutions were plated. White triangles highlight the most sensitive strain or condition. The sensitivity of each strain or condition was assessed at least three independent times; representative data are shown. (C) Staining of *M. smegmatis* cultured in 0.02% glucose-supplemented medium by superoxide indicator dye dihydroethidium (DHE). Fluorescence was detected by flow cytometry, and the MFI was plotted. The experiment was performed three times in triplicate; the results of one representative experiment are shown. (D) Oxygen consumption of *M. smegmatis* cultured in 0.02% glucose-supplemented medium. Strains were incubated with or without methylene blue, and the absorbance at 665 nm was measured. The absorbance from untreated samples was subtracted and then values were normalized to those of the wild-type. The data are plotted for three independent experiments performed in triplicate. (E) ATP levels of *M. smegmatis* cultured in 0.02% glucose-supplemented medium. Protein concentration-normalized cell lysates were incubated with BacTiter-Glo reagent, and the luminescence was measured in relative light-forming units (RLU). The experiment was performed at least three times in triplicate; the results of one representative experiment are shown. (F) Cartoon summary of Fig. 3 and Fig. S5. Error bars, standard deviation. For panels C to E, the statistical significance of ΔsugC or complement strains versus the wild type from at least three independent experiments was assessed by a two-tailed Student t test. *, P < 0.05.
and F). These experiments indicated that the mycobacterial redox balance does not depend solely on the size of the trehalose pool or on trehalose catabolism during short-term carbon limitation.

How might trehalose recycling promote redox homeostasis under nutrient limitation? We noted that mycomembrane synthesis continues unabated in the ΔsugC mutant (see Fig. S4D) and that TMM remains at wild-type levels (Fig. 2F and G). The synthetic lethal interactions between otsA and treYZ or lpqY-sugABC in *M. tuberculosis* (64) suggest functional redundancy between the pathways encoded by these genes. The TreYZ pathway does not require energy to break down \( \alpha \)-glucan into trehalose but OtsA and OtsB convert phosphorylated glucose intermediates to trehalose. In glucose-limited conditions, trehalose biosynthesis via the OtsAB pathway may also require additional ATP to drive gluconeogenesis. We considered whether induction of ATP-expensive trehalose anabolism might explain the oxidative stress that occurs in the absence of LpqY-SugABC.

Four lines of evidence support the first part of this model, e.g., that loss of recycling stimulates ATP-consuming trehalose biosynthesis. First, the *M. smegmatis* ΔsugC strain has lower ATP levels than the wild type (Fig. 3E). Second, we observed enhanced metabolism of fluorescently labeled glucose in the mutant (see Fig. S7). Third, while the expression of otsA did not change and the expression of one of the two *M. smegmatis*
otsB homologs, (MSMEG_6043) was not detectable, the expression of the other otsB homolog, MSMEG_3954, was enhanced ~4-fold in the absence of sugC (Fig. 4B). Finally, the levels of glucose-6-phosphate—the end product of gluconeogenesis—were elevated in the ΔsugC strain but suppressed in the ΔotsA strain (Fig. 4C), respectively, consistent with increased and decreased flux through this pathway.

We next tested the second part of our model, e.g., whether induction of trehalose anabolism upsets redox balance in carbon-deprived mycobacteria. Given the synthetic lethal interaction between sugC and otsA (64), we opted to deplete the trehalose pool by inducible trehalase overexpression. We compared the hydrogen peroxide sensitivity of strains that overexpress trehalase in wild-type, ΔotsA, and ΔtreYZ backgrounds. Loss of OtsA, but not of TreYZ, rescued the sensitivity of M. smegmatis to hydrogen peroxide upon trehalase overexpression (Fig. 4D). These experiments indicate that trehalose replenishment by the OtsAB pathway can sensitize carbon-starved mycobacteria to ROS. Taken together, our data suggest that trehalose recycling limits energy consumption and oxidative stress during carbon limitation by alleviating the need for de novo biosynthesis.

**Trehalose recycling promotes M. tuberculosis survival in macrophages.** Deletion of sugC or lpqY inhibits M. tuberculosis replication in the acute phase of murine infection (20). Transposon insertions in sugABC or lpqY also attenuate pooled M. tuberculosis growth in interferon-gamma (IFN-γ)-activated or resting C57BL/6 bone marrow-derived macrophages (BMDM) (32). While it is likely that progressive carbon starvation underlies the in vivo and macrophage defects of trehalose recycling mutants, the precise mechanism(s) have not been clear. Our in vitro experiments support a model in which trehalose anabolism compensates for the loss of trehalose recycling but exacts energetic and redox costs. Since one consequence of IFN-γ activation is ROS production by the macrophage (65, 66), we first sought to test whether the magnitude of trehalose recycling mutant attenuation was different in the presence or absence of the cytokine. We confirmed that the M. tuberculosis ΔsugC mutant was defective for growing in immortalized BMDM and that this phenotype was reversed by genetic complementation (Fig. 5A and B). However, the IFN-γ-dependent decrease in the ΔsugC strain fitness relative to the wild type was very modest (see Fig. S8A), suggesting that sensitivity to ROS or to other, downstream stresses such as reactive nitrogen intermediates, acidic pH, and nutrient limitation (67, 68) does not fully account for attenuation in macrophages.

We next sought to determine whether dysfunctional energy metabolism compromises the fitness of trehalose recycling mutants during infection. To do this, we took a chemical-genetic epistasis approach. Bedaquiline inhibits ATP production by targeting the F1F0 ATP synthase (69, 70). Bedaquiline-treated M. tuberculosis is transiently able to maintain ATP levels by increasing oxidative and substrate-level phosphorylation (71, 72). Loss of trehalose recycling also results in ATP depletion (Fig. 3E) and enhanced respiration (Fig. 3D) in vitro. If these perturbations to (energy) metabolism are responsible for trehalose recycling mutant attenuation, we reasoned that bedaquiline should inhibit wild-type, ΔlpqY, and ΔsugC M. tuberculosis strains similarly, e.g., that the drug should not be additive with either of the mutations. Indeed, we found that the loss of lpqY or sugC was additive with treatment with rifampin, an antibiotic that does not impair mycobacterial energy metabolism (73, 74), but not with bedaquiline (Fig. 5C; see also Fig. S8B). Taken together, our data suggest that energy dysfunction that accompanies loss of trehalose recycling attenuates M. tuberculosis in macrophages.

**DISCUSSION**

Hints of mycomembrane plasticity began to appear in the early 1900s, when it was recognized that acid-fastness—a hallmark staining property still used for microscopy-based diagnosis of M. tuberculosis—varied with nutrient supply (75–77). More recent work supports the idea that the mycomembrane is reconfigured in vivo and in response to host-mimicking stresses (3, 5–13). The mechanisms by which these cell surface alterations occur are still emerging but have been attributed primarily to catabolic...
pathways (3, 6). We took advantage of recent advances in metabolic labeling (35, 78) to show that mycomembrane remodeling under in vitro carbon deprivation also involves anabolic reactions (Fig. 1C), a counterintuitive result since mycobacterial replication (see Fig. S1A) and presumably the overall metabolic activity are sluggish. Our data collectively indicate that the net result of such reactions is decreased TDM and spatial rearrangement of AGM (Fig. 6). We previously showed that synthesis of peptidoglycan along the nonexpanding sidewall of M. smegmatis is enhanced in response to cell wall damage (38). AGM synthesis under carbon starvation also occurs along the cell periphery (Fig. 1F), further supporting the notion that mycobacteria can edit their cell surface in a growth-independent fashion.

The adaptive consequences of mycomembrane remodeling are manifold (21, 79, 80). For example, bulk decreases in TDM and AGM abundance are known to increase mycobacterial cell permeability, which in turn enhances nutrient uptake and antimicrobial susceptibility (3, 4, 39). Although we do not observe gross changes in the amount of AGM under nutrient deprivation (Fig. 1D), the primary site of synthesis shifts from the pole to sidewall (Fig. 1F). The concomitant reduction in permeability (Fig. 1E)—despite an overall decrease in TDM abundance—suggests that the subcellular distribution of AGM also contributes to the barrier function of the mycobacterial cell envelope. Beyond enabling edits to the structural components of the mycomembrane,
remodeling reactions liberate smaller molecules that influence cell physiology. Free trehalose released by TDM and AGM synthesis can be recycled into glycolysis or pentose phosphate intermediates or act as a stress protectant or compatible solute in the cytoplasm (6, 21–23). Our data suggest that it can also be directly refashioned into trehalose-containing, cell surface glycolipids (Fig. 2D and E), likely TMM. Free mycolic acids generated by TDM hydrolysis are components of biofilm matrix (7) and, like trehalose, serve as carbon sources (81). We speculate that they may additionally be reused together with recycled trehalose to make TMM.

How do mycobacteria power mycomembrane remodeling when faced with a loss of nutrients? The three isoforms of the TMM-consuming antigen 85 complex (Ag85C), encoded in M. tuberculosis by fbpA, fbpB, and fbpC, have partially redundant acceptor specificities (39, 82). However, only fbpC is upregulated in nutrient-starved M. tuberculosis (83, 84), making Ag85C an obvious candidate for performing synthetic reactions under that condition. Perhaps the more interesting question, however, is the source of the energetically expensive TMM building blocks. Breakdown of TDM by TDMH furnishes free mycolic acids and TMM, the latter of which could serve as a donor for sidewall AGM synthesis (7, 15). While such a pathway would not require ATP, it would be limited by the amount of TDM loss that can be tolerated without lysis (7, 34) or reduced resilience to host stress (3). Our data suggest that M. smegmatis and M. tuberculosis also generate TMM in the cytoplasm from recycled trehalose (Fig. 2D and E). An intracellular route of TMM generation would limit TDM loss, thereby preserving mycomembrane integrity. Use of recycled materials in turn would allow the mycobacterial cell to reap the benefits of sidewall AGM fortification while minimizing energy expenditure. In the absence of trehalose recycling, de novo synthesis supplies the sugar and mycomembrane remodeling continues unabated (see Fig. S4). The cost of from-scratch, OtsAB-mediated anabolism is not apparent under standard in vitro culture conditions but sensitizes M. smegmatis and M. tuberculosis to ROS (Fig. 3) and may contribute to defective M. tuberculosis growth during infection (Fig. 5) (20).

FIG 6 Model for the role of trehalose recycling in mycomembrane remodeling under nutrient or host stress. (Bottom left) Mycobacteria growing under carbon-replete conditions synthesize peptidoglycan (PG; green) and arabinogalactan mycolates (AGM; red) primarily at the poles of the cell. (Bottom right) Mycobacteria respond to growth-limiting carbon deprivation by turning over trehalose dimycolate (TDM) and synthesizing AGM along the entire cell periphery. Peptidoglycan metabolism, in contrast, is relatively inactive. (Top left) In carbon-deprived wild-type cells, the TMM building blocks are obtained at least in part from trehalose recycled by LpqY-SugABC. Trehalose may also be funneled to central carbon metabolism via TreS- or trehalase (Tre)-mediated catabolism. (Top right) In carbon-deprived mutants unable to recycle trehalose, TMM is supplied by de novo trehalose synthesis (dark arrow), which in turn depletes ATP, drives respiration, and confers ROS sensitivity.
Trehalose is a cytoplasmic stress protectant and compatible solute and, in many types of bacteria, a carbon source (62, 85, 86). Mycobacteria and related organisms are relatively unique in using trehalose for extracellular purposes, to build their outer cell envelope. As the sugar fluxes in and out of central metabolism and the mycomembrane via several synthetic (OtsAB and TreYZ) and degradative (TreS and trehalase) processes, trehalose utilization may be particularly vulnerable to perturbations that induce redox and metabolic imbalances. Like carbon-limited ΔsugC M. smegmatis or M. tuberculosis ΔtreS strains, biofilm cultures of M. tuberculosis ΔtreS have disruptions in energy and redox homeostasis (23). However, our data suggest that the mechanisms are distinct. In mature biofilms, trehalose is shunted away from TMM and TDM synthesis into glycolytic and pentose phosphate intermediates in a TreS-dependent manner (23). In contrast, we find that TMM levels are maintained during the time frame of our experiment, either by LpqY-SugABC, in wild-type organisms, or by de novo synthesis, in ΔsugC mutants (Fig. 6). While biofilm M. tuberculosis ΔtreS mutants are likely more sensitive to ROS because they are depleted for the antioxidant precursor γ-glutamylcysteine (23), carbon-limited M. smegmatis ΔsugC mutants have higher levels of ROS-counteracting, cytoplasmic thiols (see Fig. S5C). Finally, biofilm M. tuberculosis ΔtreS is hypersensitive to ATP-depleting bedaquiline (23), whereas intracellular ΔsugC and ΔlpqY mutants are more tolerant (Fig. 5C). These and other metabolite data are most consistent with the idea that enhanced ROS production and susceptibility (Fig. 3) in the absence of trehalose recycling stems from increased anabolism of the sugar rather than decreased catabolism. While we focus here on mycomembrane remodeling that occurs within 1 to 3 days of adaptation to carbon-limited medium, the TreS-dependent, trehalose-catalytic shift occurs in 4- to 5-week-old biofilms. Under our conditions, the loss of TreS has no impact on ROS susceptibility (see Fig. S6E). While we cannot rule out stress- or species-specific differences between the two studies, we favor a model in which the adaptive role of trehalose changes over time: early fortification of the cell envelope, to protect against immediate environmental insults, and later rewiring of central carbon metabolism, to maintain ATP and antioxidant levels. Trehalose recycling maintains redox and ATP homeostasis in the second case by driving glycolysis and the pentose phosphate pathway and in the first case by providing energetically inexpensive substrates for mycomembrane remodeling, thereby easing the demand for the products of these metabolic pathways.

The presence of a retrograde transporter enables trehalose to cycle in and out of the cell and serve as a metabolic node between the mycomembrane and cytoplasm. Recycling of the sugar is known to enhance M. tuberculosis survival in a mouse model of tuberculosis. It is widely hypothesized that the in vivo growth defects of trehalose recycling mutants stem from progressive carbon starvation (20, 21, 50). Nutrient deprivation coupled with loss of trehalose catabolism may indeed reduce fitness in vivo. However, our data suggest a more complex model, namely, that futile trehalose cycling consumes ATP and stimulates compensatory, ROS-generating respiration (Fig. 6). The energy and redox phenotypes of a trehalose recycling mutant resemble those elicited by other futile cycles (24–28) and some bactericidal antibiotics (29, 71, 72, 87, 88). Enhanced bacterial respiration has been proposed to increase drug efficacy (29, 30), and indeed, the loss of trehalose recycling sensitizes M. tuberculosis to multiple antibiotics (31). Here, we found that disrupted energy metabolism is the primary mechanism of attenuation for trehalose recycling mutant M. tuberculosis in macrophages (Fig. 5). Dysfunction triggered by forced de novo synthesis of energy-expensive macromolecules may be a fruitful avenue for potentiating both immune and antibiotic activity against bacterial pathogens, including those that inhabit growth-limiting, nutrient-deprived host niches.

MATERIALS AND METHODS

Bacterial strains and culture conditions. M. smegmatis mc²155 was grown in Middlebrook 7H9 growth medium (HiMedia, India) supplemented with Tween 80 (7H9T) and glucose (2 or 0.02%) at 37°C unless otherwise specified in the text. Two-day-old primary cultures of M. smegmatis grown in...
TABLE 1 Strains used in this study

| Strain                        | Source (references)            |
|-------------------------------|--------------------------------|
| Immortalized C57BL/6 BMDM     | Christopher Sassetti (93)       |
| M. smegmatis                  |                                 |
| mc215S                        |                                 |
| ΔsugC                         |                                 |
| ΔsugC pm361-sugC              |                                 |
| ΔotsA                         |                                 |
| ΔtreYZ                        |                                 |
| ΔtreS                         |                                 |
| Δtre                         |                                 |
| ΔotsA pYAB-tre                |                                 |
| ΔtreYZ pYAB-tre               |                                 |
| pYAB                          |                                 |
| pYAB-tre                      |                                 |
| M. tuberculosis               |                                 |
| H37Rv                         | Rainer Kalscheuer (20)          |
| ΔsugC                         | Rainer Kalscheuer (20)          |
| ΔlpqY                         | Rainer Kalscheuer (20)          |
| ΔsugC pm306-sugC              | Rainer Kalscheuer (20, 95)      |
| E. coli XL-1 Blue             | Agilent Technologies            |

2% glucose were normalized to an optical density at 600 nm (OD<sub>600</sub>) of 0.1 in fresh 7H9T supplemented with 2 or 0.02% glucose and allowed to grow for 24 h. M. tuberculosis H37Rv strains (gifts from Rainer Kalscheuer) were grown in Middlebrook 7H9 medium (BD Difco, Franklin Lakes, NJ) supplemented with Tween 80 and OADC (BD BBL, Sparks, MD). For starvation of M. tuberculosis, cultures grown in 7H9T-OADC to an OD<sub>600</sub> of 0.8 to 1.0 were collected by centrifugation and washed once with 7H9T and resuspended in 7H9T (starvation medium) to a normalized OD<sub>600</sub> of 1. To prepare a strain that expresses tre, the gene that encodes trehalase, under an acetamide-inducible promoter, we PCR amplified tre from genomic DNA of M. smegmatis by using 4535For_Acet (TGATGTGCTCAGAGTTCTGCAACAGACCGAGCC) and 4535Rev_Acet (GGCCTGATCTAGACATCGGGGCGTCCGC) primers. The resulting PCR product was ligated in pYAB033 vector (a gift from Yasu Morita) at the XbaI site and transformed in E. coli XL-1 Blue strain. The colonies were screened by colony PCR and the obtained plasmid was confirmed by sequencing. Bacteria used in this study are listed in Table 1.

**ROS sensitivity.** M. smegmatis grown in 0.02% glucose for 24 h were normalized to OD<sub>600</sub> of 1. The cultures were then treated with 0.15% H<sub>2</sub>O<sub>2</sub> for 10 min at 37°C with shaking. The trehalase overexpression strains were grown for 20 h in 0.02% glucose and then induced with 0.2% acetamide for an additional 10 h before being treated with 0.1% H<sub>2</sub>O<sub>2</sub> for 10 min at 37°C with shaking. After H<sub>2</sub>O<sub>2</sub> treatment, 3 μl of 10-fold serial dilutions made in phosphate-buffered saline (PBS) was spotted onto 7H9-2% glucose agar. For the thiourea rescue experiment, cultures were pretreated with 50 mM thiourea for 45 min prior to H<sub>2</sub>O<sub>2</sub>. For M. tuberculosis, cultures in starvation medium were grown for 5 days, normalized to an OD<sub>600</sub> of 0.1 in fresh starvation medium, and then treated with 0.4% of H<sub>2</sub>O<sub>2</sub> for 2 h at 37°C with shaking. After H<sub>2</sub>O<sub>2</sub> treatment, 5 μl of 10-fold serial dilutions made in PBS were spotted on 7H10-OADC agar plate. For the vitamin C experiment, M. tuberculosis cultures in starvation medium were normalized to an OD<sub>600</sub> of 0.1 in fresh starvation medium. The cultures were then treated with 20 mM vitamin C for 2 days. After vitamin C treatment, 5 μl of 10-fold serial dilutions made in PBS were spotted onto 7H10-OADC agar.

**Macrophage infections.** Immortalized C57BL/6 BMDM (iBMDM; a gift from Christopher Sassetti) were seeded at 10<sup>5</sup> cells/well in 24-well tissue culture plate and incubated at 37°C overnight. M. tuberculosis was added at 5:1 multiplicity of infection (MOI; bacteria/iBMDM) and incubated for 4 h. After incubation, the coculture was washed twice with high-glucose Dulbecco modified Eagle medium (DMEM; Genesee Scientific, San Diego, CA) to remove extracellular M. tuberculosis, and fresh 5 mM DMEM-FBS-HEPES medium was added (fetal bovine serum [Genesee Scientific, San Diego, CA] and HEPES [Gibco, Paisley, PA]). IFN-γ (PeproTech, Rocky Hill, NJ) was added or not at 25 ng/ml concentration. For antibiotic susceptibility experiments, cocultures were treated or not with 5 μg/ml of bedaquiline (BDQ) or rifampin (RIF) for 2 days of the infection. The infected iBMDM were incubated for 0 to 5 days and then washed once with PBS and lysed with 0.05% Triton X-100 in PBS. After lysis, 10 or 50 μl of 10-fold serial dilutions made in PBS were respectively spotted or spread onto 7H10-OADC agar to determine the CFU.

**Bliss scoring.** Bliss interaction scores (89) for pairs of mutant-drug interactions were obtained by subtracting the expected values for inhibition from the observed values. The expected values were calculated using the formula E<sub>ab</sub> = E<sub>a</sub> + E<sub>b</sub> – E<sub>a,b</sub>, where E<sub>a</sub> is the effect of the mutation (ΔsugC or ΔlpqY) and E<sub>b</sub> is the effect of the drug.
is the effect of the antibiotic (BDQ or RIF). Statistically significant combinations that produced Bliss scores ≥ 0 were interpreted as nonadditive interactions.

**DHE staining.** *M. smegmatis* grown for 24 h in 7H9T–0.02% glucose was normalized to an OD$_{600}$ of 1 with the same medium and then treated with 5 μM dihydroethidium (DHE; Sigma, St. Louis, MO) for 30 min at 37°C. Fluorescence was analyzed by flow cytometry.

**Total thiol abundance.** The protocol for measuring the total thiol content was adopted from (30). Briefly, 10 ml of *M. smegmatis* grown for 24 h in 7H9T–0.02% glucose was centrifuged at 2,500 × g for 5 min and washed with buffer containing 50 mM Tris-Cl (pH 8) and 5 mM EDTA, and the cell pellets were normalized by wet weight. Bacteria were resuspended in the same buffer and lysed by bead beating. Lysates were centrifuged at 16,000 × g for 15 min at 4°C, and 5,5′-dithiobis(2-nitrobenzoic acid) was added to 100 μl of supernatants to a final concentration of 0.05 mM. The total thiol content was estimated by determining the absorbance (λ) at 412 nm.

**Methylene blue.** *M. smegmatis* grown for 24 h in 7H9T–0.02% glucose was adjusted to an OD$_{600}$ of 0.25. Cultures were split in two; one of these was treated with 0.005% methylene blue and aliquoted to a 96-well plate. The plate was sealed with Microseal B adhesive sealing films (Bio-Rad, UK) and incubated at 37°C for 4 h with shaking. The seal was then removed, and the absorbance (λ) at 665 nm was measured. The difference between the absorbance (λ) values at 665 nm for treated and untreated samples was plotted.

**ATP, glucose-6-phosphate, and NADP/NADPH quantitation.** The ATP concentration was measured by using a BacLight-Glo (Promega, Madison, WI) luminescence kit. The glucose-6-phosphate (G6P) concentration and the NADP/NADPH ratio were respectively measured with an Amplite (AAT Bioquest, Sunnyvale, CA) colorimetric G6P assay and colorimetric NADP/NADPH ratio assay kits. *M. smegmatis* grown for 24 h in 7H9T–0.02% glucose was washed once with PBS. The pellets were resuspended in PBS and lysed by bead beating. Lysates were normalized by total protein concentration using a BCA protein assay kit (Pierce, Rockford, IL) and then processed according to the manufacturer’s protocol.

**Trehalose quantitation.** For intracellular trehalose detection, *M. smegmatis* grown for 24 h in 7H9T–0.02% glucose was washed once with PBS. Cell pellets were normalized by wet weight and then resuspended in chloroform-methanol (1:1) for overnight incubation with shaking. The suspension was centrifuged at 10,000 × g for 5 min, and the organic fraction was collected in a new tube. One part chloroform and one part water were added to the organic fraction and mixed vigorously in a shaker for 15 min. Suspensions were centrifuged, and the upper aqueous layers were processed according to the manufacturer’s instructions for the trehalose assay kit (Megazyme, Ireland). For extracellular trehalose detection, *M. smegmatis* were grown for 24 h in 7H9T supplemented with 2 or 0.02% glucose. Cultures were normalized to an OD$_{600}$ of 1 prior to centrifugation. The upper layer was collected and filtered through a 0.2-μm syringe. Filters were processed as described above to detect trehalose.

**Lipid extraction and TLC.** For extractable lipid analysis, 10 ml of culture was washed with PBS, and cell pellets were normalized by wet weight (*M. smegmatis*) or by OD$_{600}$ (*M. tuberculosis*). To obtain TDM and TMM, cell pellets were extracted with chloroform-methanol (2:1). The extracted lipids were separated by thin-layer chromatography (HPTLC silica gel; Millipore, Billerica, MA) with chloroform-methanol-H$_2$O (80:20:2) and 1-propanol-water (6:1) for TDM and TMM, respectively (35, 90). Lipids were extracted from 100 ml of culture as described previously (91). The protocol for measuring the total thiol content was adopted from (30). Briefly, mycolic-arabinogalactan-peptidoglycan (mAGP) complex was extracted from 100 ml of culture as described previously (91). The pellet was resuspended in PBS plus 0.05% Tween 80 (PBST) by water bath sonication. To extract mycolic acids from mAGP, the suspension was treated with 5% tetrabutyrammonium hydroxide (TBAH) overnight with shaking. The extracted mycolic acids were separated by treatment with an equal volume of dichloromethane, followed by treatment with an equal volume of 0.25 M HCl and washed with water as described previously (91). To extract free mycolic acids from culture supernatants, the OD$_{600}$ of *M. smegmatis* grown for 24 h in 7H9T–0.02% glucose was normalized to 1 with 7H9T. The normalized cultures were centrifuged at 10,000 × g for 5 min and supernatants were collected and passed through a 0.2-μm syringe filter. Supernatants (1 ml) were treated with 5% TBAH for 1 h, followed by an equal amount of dichloromethane and overnight incubation at room temperature with shaking. The suspension was then centrifuged at 10,000 × g, and the lower organic layer was removed. The organic layer was evaporated, and the pellet was mixed with 40 μl of chloroform-methanol (2:1). Mycolic acids were separated by TLC using chloroform-methanol (96:4) as described previously (7). Next, 5% molybdophosphoric acid in ethanol was used to develop the TLC results.

**Fluorescent glucose labeling.** *M. smegmatis* cultured in 0.02% glucose-supplemented 7H9T was normalized to an OD$_{600}$ of 1.0 in fresh medium and treated with a 5 μM concentration of the fluorescent glucose analogue 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminomethyl)-2-deoxyglucose (2-NBDG; Abcam, Cambridge, MA) for 2 h at 37°C with shaking. The cultures were centrifuged at room temperature for 5 min and 4,000 rpm and then washed twice with PBST. After normalizing to the wet weight, the pellets were extracted with chloroform-methanol (2:1) overnight. The organic extracts were separated from the cell suspension by centrifugation at room temperature for 15 min and 12,000 rpm and then treated with 1 volume of H$_2$O for 15 min at room temperature. The aqueous and organic layers were separated from each other suspension by centrifugation at room temperature for 5 min at 12,000 rpm and then subjected to TLC using chloroform-methanol-H$_2$O (80:20:2) and 1-propanol-ethanol acetate–water (6:1:3),
respectively. The TLC fluorescence was recorded by the ImageQuant system (GE Healthcare) or developed using 5% H2SO4 in ethanol.

Propidium iodide. We assessed propidium iodide (PI) uptake as described previously (92). Briefly, 50 μg/ml PI was added to M. smegmatis that had been cultured in 0.02 or 2% glucose. After incubation for 15 min at 37°C, the samples were washed once with PBS, and the fluorescence was measured by flow cytometry.

Cell envelope labeling. Probes used in this study include alkDala (50 μM), HADA (500 μM), O-AlkTMM (50 μM), N-AlkTMM (250 μM), and 6-TreAz (50 μM). M. smegmatis labeling was performed mainly as described previously (38). Briefly, the OD600 was normalized to 1 in the same medium. Cultures were shaken in the presence of probes for 30 min at 37°C for M. smegmatis. After incubation, the cultures were washed twice with PBST and fixed or not fixed with 2% formaldehyde at room temperature for 10 min. After fixation, the cultures were washed with PBST. Alkynes were detected by CuAAC reaction with carboxyrhodamine-110 azide (Click Chemistry Tools, Scottsdale, AZ). Azides were detected on live, unfixed cells by SPAAC reaction with DBCO-Cy5 (Click Chemistry Tools). Finally, the cultures were washed three times with PBST, and the fluorescence was measured by flow cytometry. For M. tuberculosis, the OD600 values for carbon-starved and unstarved cultures were normalized to 1 in the same media. Cultures were shaken in the presence of probes for 3 h at 37°C and then washed twice with PBST and subjected to SPAAC overnight at 37°C. The cultures were washed three times with PBST and fixed with 4% formaldehyde overnight at room temperature prior to removal from the BSL3 facility.

Microscopy analysis. Fluorescence microscopy and image quantitation were performed exactly as described previously (38).

qRT-PCR. M. smegmatis was cultured in 0.02% glucose medium for 24 h. Cell pellets were resuspended in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) prior to bead-beating (MP Biochemicals lysis matrix B). After bead beating, 300 μl of chloroform was added to each tube. The tubes were centrifuged at 14,000 rpm for 15 min at 4°C. The upper aqueous layer was removed and resuspended in 600 μl of isopropanol in a fresh tube. The tube was kept at −20°C for 1 h to overnight and then centrifuged for 20 min at 4°C and 14,000 rpm to precipitate the RNA. The RNA-containing pellet was washed once with 75% ethanol by centrifugation for 5 min at 4°C and then resuspended in RNase-free H2O. Next, 20 μg of RNA was treated with 2.5 μl of Turbo DNase (Ambion, Carlsbad, CA) in a final volume of 100 μl. The reaction mixture was incubated for 2 h at 37°C. The RNA was then cleaned up according to the manufacturer’s instructions for the RNasy minikit (Qiagen). cDNA synthesis was carried out with 5 μg of the cleaned-up RNA according to the manufacturer’s instructions for SuperScript IV reverse transcriptase (Invitrogen). The cDNA was then used for qRT-PCRs (iTaq Universal SYBR green Supermix; Bio-Rad, Hercules, CA). We used the sigA gene as our internal control. The primers are listed in Table 2.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.6 MB.
FIG S2, TIF file, 1.1 MB.
FIG S3, TIF file, 1.3 MB.
FIG S4, TIF file, 1.2 MB.
FIG S5, TIF file, 2.4 MB.
FIG S6, TIF file, 1.2 MB.
FIG S7, TIF file, 2.5 MB.
FIG S8, TIF file, 0.3 MB.

TABLE 2 Primers and sequences

| Primer       | Sequence                        |
|--------------|---------------------------------|
| 4535For_Acet | TGATGTGCTCTAGAGATTCAGCAACTGAGCC |
| 4535Rev_Acet | GGCTGATCTGACATCGGGGCGCTCGGG    |
| RT-otsA-For  | ACTACACAAAGGCGACTGAC           |
| RT-otsA-Rev  | TCGCGATGATAGCTCTCGAC           |
| RT-otsB-For  | AACAGAGGCTGTCGTAACCT           |
| RT-otsB-Rev  | AGGGTCTGCTGTAGGACTG            |
| RT-otsB-For  | GTGAGTCTTCCGGGGAGTCT           |
| RT-otsB-Rev  | AATCGGATGTACGACGACG            |
| RT-treY-For  | CTCTCGACGTATCGGTTGC            |
| RT-treY-Rev  | AGGATGGGGGAGAGATACAC           |
| RT-trez-For  | CTCGACTACCTGTCGATCTC           |
| RT-treZ-Rev  | ACCCTCGTAGGTTTCTGTGA           |
| ForsigA      | GGCTACAAAGTTCGAGCCT            |
| RevsigA      | CCGAGCTTGTGATACCTTC           |
Trehalose Recycling Supports Energy and Redox Homeostasis

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REFERENCES

1. Puffal J, Garcia-Heredia A, Rahlwes KC, Siegrist MS, Morita YS. 2018. Spatial control of cell envelope biosynthesis in mycobacteria. Pathog Dis 76. https://doi.org/10.1093/femspd/fty027.

2. Lee WB, Kang JS, Choi WY, Zhang Q, Kim CH, Choi UY, Kim-Ha J, Kim YJ. 2016. Mincle-mediated translational regulation is required for sterol, fatty acid production and inflammation resolution. Nat Commun 7:11322. https://doi.org/10.1038/ncomms11322.

3. Yang Y, Kulkia K, Montelaro RC, Reinhart T, Jaderem A, Ojha AK. 2014. A hydrolase of trehalose dimycolate induces nutrient influx and stress sensitivity to balance intracellular growth of Mycobacterium tuberculosis. Cell Host Microbe 15:153–163. https://doi.org/10.1016/j.chom.2014.01.008.

4. Gebhardt H, Meniche X, Tropis M, Kramer R, Daffe M, Morbach S. 2007. The key role of the mycolic acid content in the functionality of the cell wall permeability barrier in Corynebacterinae. Microbiology (Reading) 153:1424–1434. https://doi.org/10.1099/mic.0.003541-0.

5. Galagan JE, Minch K, Peterson M, Lyubetskaya A, Azizi E, Sweet L, Gomes A, Rustad T, Dolganov G, Glotova I, Abeel T, Mathwinney B, Kennedy AD, Allard R, Brabant W, Krueger A, Jaini S, Honda B, Yu WH, Hickey MJ, Zucker J, Garay C, Weiner B, Sisk P, Stolte C, Winkler JK, Van de Peer Y, Iazzetti P, Camacho D, Dreyfuss J, Liu Y, Dorhui A, Mollenkopf HJ, Drogaris P, Lamontagne J, Zhou Y, Piquenot J, Park ST, Raman S, Kaufmann SH, Mohney RP, Chelsky D, Moody DB, Shenman DR, Schoolsen G, 2013. The Mycobacterium tuberculosis regulatory network and hypoxia. Nature 499:178–183. https://doi.org/10.1038/nature12337.

6. Esh H, Wang Z, Li T, Rath P, Morris R, Branch Moody D, Rhee KY. 2017. Metabolic anticipation in Mycobacterium tuberculosis. Nat Microbiol 2:17084. https://doi.org/10.1038/nmicrobiol.2017.84.

7. Ojha AK, Trivelli X, Guerardel Y, Kremer L, Hatfull GF. 2010. Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms. J Biol Chem 285:17380–17389. https://doi.org/10.1074/jbc.M110.121813.

8. Kieser KJ, Rubin EJ. 2014. How sisters grow apart: mycobacterial growth and division. Nat Rev Microbiol 12:550–558. https://doi.org/10.1038/nrmicro3199.

9. Kieser KJ, Rubin EJ. 2014. How sisters grow apart: mycobacterial growth and division. Nat Rev Microbiol 12:550–558. https://doi.org/10.1038/nrmicro3199.

10. Bhandari S, Scherman MS, Jones V, Crick DC, Beilis JT, Brennan PJ, McNeil MR. 2011. Detailed structural and quantitative analysis reveals the spatial organization of the cell walls of in vivo grown Mycobacterium leprae and in vitro grown Mycobacterium tuberculosis. J Biol Chem 286:23168–23177. https://doi.org/10.1074/jbc.M110.210534.

11. Dubberger CL, Rubin EJ, Boutte CC. 2020. The mycobacterial cell envelope: a moving target. Nat Rev Microbiol 18:47–59. https://doi.org/10.1038/s41579-019-0273-7.

12. Quemard A. 2016. New insights into the mycolate-containing compound biosynthesis and transport in mycobacteria. Trends Microbiol 24:725–738. https://doi.org/10.1016/j.tim.2016.04.009.

13. Holmes NJ, Kavunja HW, Yang Y, Vannest BD, Ramsey CN, Gepford DM, Banahene N, Poston AW, Piligan BF, Ronning DR, Ojha AK, Swarts BM. 2019. A FRET-based fluorogenic trehalose dimycolate analogue for probing mycomembrane-remodeling enzymes of mycobacteria. ACS Omega 4:4348–4359. https://doi.org/10.1021/acs.omega.9b00130.

14. Dunphy KY, Senaratne RH, Masuzawa M, Kendall LV, Riley LW. 2010. Attenuation of Mycobacterium tuberculosis dysfunctionally disrupted in a fatty acyl-coenzyme A synthetase gene fadD5. J Infect Dis 211:1232–1239. https://doi.org/10.1086/651452.

15. Willburn KM, Fliewerger RA, VanderVen BC. 2018. Cholesterol and fatty acids grease the wheels of Mycobacterium tuberculosis pathogenesis. Pathog Dis 76. https://doi.org/10.1093/femspd/fty021.

16. Cantrell SA, Leavell MD, Marianovic O, Lavarone AT, Leary JA, Riley LW. 2013. Free mycolic acid accumulation in the cell wall of the mecI operon mutant strain of Mycobacterium tuberculosis. J Microbiol 51:619–626. https://doi.org/10.1007/s12275-013-0992-y.

17. Forrellad MA, McNeil M, Santangelo M, d’I P, Blanco FC, Garcia E, Klepp LI, Huff J, Niederweis M, Jackson M, Bigi F. 2014. Role of the Mce1 transporter in the lipid homeostasis of Mycobacterium tuberculosis. Tuberculosis (Edinb) 94:170–177. https://doi.org/10.1016/j.tube.2013.12.005.

18. Kalscheuer R, Weinick B, Veeraraghavan U, Besra GS, Jacobs WR Jr. 2010. Trehalose-recycling ABC transporter LpqY-SugA-SugB-SugC is essential for virulence of Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 107:21761–21766. https://doi.org/10.1073/pnas.1014652108.

19. Nobre A, Alarico S, Maranja A, Mendes V, Empadinhas N. 2014. The molecular biology of mycobacterial trehalose in the quest for advanced tuberculosis therapies. Microbiology (Reading) 160:1547–1570. https://doi.org/10.1099/micro.0.075895-0.

20. Shleeva MO, Trutneva KA, Demina GR, Sorokoumova GM, Laptinskaya PK, Shumkova ES, Karpelyants AS. 2017. Free trehalose accumulation in dormant Mycobacterium smegmatis cells and its breakdown in early resuscitation phase. Front Microbiol 8:524. https://doi.org/10.3389/fmicb.2017.00524.

21. Lee JJ, Lee SK, Song N, Nathan TO, Swarts BM, Eoh H. 2019. Transient drug-tolerant and permanent drug-resistance rely on the trehalose-catalytic shift in Mycobacterium tuberculosis. Nat Commun 10:2928. https://doi.org/10.1038/s41467-019-10975-7.

22. Brynilden MP, Winkler JA, Spina CS, MacDonald IC, Collins JJ. 2013. Potentiating antibacterial activity by predictably enhancing endogenous microbial ROS production. Nat Biotechnol 31:160–165. https://doi.org/10.1038/nbt.2458.

23. Adolfsen KJ, Brynilden MP. 2015. Futile cycling increases sensitivity...
toward oxidative stress in Escherichia coli. Metab Eng 29:26–35. https://doi.org/10.1016/j.ymben.2015.02.006.

26. Koebmann BJ, Westerhoff HV, Snoep JL, Nilsson D, Jensen PR. 2002. The glycolytic flux in Escherichia coli is controlled by the demand for ATP. J Bacteriol 184:3909–3916. https://doi.org/10.1128/JB.184.18.3909-3916.2002.

27. Mok WW, Park JO, Rabinowitz JD, Bryndlsen MP. 2015. RNA futile cycling in model persister derived from MzaF accumulation. mBio 6:e01588-15. https://doi.org/10.1128/mBio.01588-15.

28. Izailov M, Madhavane R, Burgard A, Postier B, Didonato R, Jr, Sun J, Schilling CH, Lovley DR. 2008. Geobacter sulfurreducens strain engineered for increased rates of respiration. Metab Eng 10:267–275. https://doi.org/10.1016/j.mib.2008.06.005.

29. Yang JH, Bening SC, Collins JJ. 2017. Antibiotic efficacy-context matters. Curr Opin Microbiol 39:73–80. https://doi.org/10.1016/j.mib.2017.09.002.

30. Vilchez C, Hartman T, Weinick B, Jain P, Weisbrod TR, Leung LW, Freundlich JS, Jacobs WR, Jr. 2017. Enhanced respiration prevents drug tolerance and drug resistance in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 114:4495–4500. https://doi.org/10.1073/pnas.1704731114.

31. Danelishvili L, Shulzhenko N, Chinson JJ, Babrak L, Hu J, Morgun A, Burrows G, Bermudez LE. 2017. Mycobacterium tuberculosis proteome response to antituberculosis compounds reveals metabolic “escape” pathways that prolong bacterial survival. Antimicrob Agents Chemother 61. https://doi.org/10.1128/AAC.00430-17.

32. Rengarajan J, Bloom BR, Rubin EJ. 2005. Genome-wide requirements for Mycobacterium tuberculosis adaptation and survival in macrophages. Proc Natl Acad Sci U S A 102:8327–8332. https://doi.org/10.1073/pnas.0503272102.

33. Sassetti CM, Rubin EJ. 2003. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci U S A 100:12989–12994. https://doi.org/10.1073/pnas.2134250100.

34. Yang Y, Bhatti A, Ke D, Gonzalez-Juarrero M, Lenaerts A, Kremer L, Guerandel Y, Zhang P, Ohja AK. 2013. Exposure to a cutinase-like serine esterase triggers rapid lysis of multiple mycobacterial species. J Biol Chem 288:382–392. https://doi.org/10.1074/jbc.M112.419754.

35. Foley HN, Stewart JA, Kavunja HW, Rundell SR, Swarts BM. 2016. Bioorthogonal chemical reporters for selective in situ probing of mycomembrane components in mycobacteria. Angew Chem Int Ed Engl 55:2053–2057. https://doi.org/10.1002/anie.201509296.

36. Isuzugama N, Nakata T, Talekar RS, McConnell MJ, Katoh K, Nakao H, Otsuba A, Behar SM, Yano I, Moody DB, Sugita M. 2008. Mycolyltransferase-mediated glycolipid exchange in mycobacteria. J Biol Chem 283:28835–28841. https://doi.org/10.1074/jbc.M805776200.

37. Gavaldà S, Bardou F, Laval F, Bon C, Malaga W, Chalut C, Guilhot C, Guerardel Y, Zhang P, Ojha AK. 2013. The key to tuberculosis control? Expert Rev Proteomics 10:543–555. https://doi.org/10.1586/14789290.10.3.543.

38. Newton GL, Buchmeier N, Fahey RC. 2008. Biosynthesis and functions of mycolothiol, the unique protective thiol of Actinobacteria. Microbiol Mol Biol Rev 72:471–494. https://doi.org/10.1128/MMBR.00008-08.

39. Kumar A, Farhana A, Gudry L, Saini V, Hondalus M, Stein AY. 2011. Redox homeostasis in mycobacteria: the key to tuberculosis control? Expert Rev Mol Med 13:e39. https://doi.org/10.15171/ermm.2011.0097.

40. Wang CJ, Hayes LG, Rodriguez-Rivera FP. 2017. An in vitro model for sequential study of shift-down of Mycobacterium tuberculosis through two stages of nonreplicating persistence. Infect Immun 85:2062–2069. https://doi.org/10.1128/IAI.00281-17.

41. Wang C, Pi L, Jiang S, Yang M, Shu C, Zhou E. 2018. ROS and trehalose regulate sclerotial development in Rhizoctonia solani AG-1A. Fungal Biol 122:322–332. https://doi.org/10.1016/j.funbio.2018.02.003.

42. Kalscheuer R, Babu Sait MR, Treuren WV, Martinson N, Kalscheuer R, Kana BD, Bertozzi CR. 2013. The antituberculosis drug ofsB2 in sputum with a method based on TAPI-2. https://doi.org/10.7554/eLife.02213.

43. Kamariza M, Shieh F, Eland CS, Peters JS, Chu B, Rodriguez-Rivera FP, Babu Sait MR, Treuren WV, Martinson N, Kalscheuer R, Kana BD, Bertozzi CR. 2018. Rapid detection of Mycobacterium tuberculosis in sputum with a solvatochromic trehalose probe. Sci Transl Med 10:aam6310.

44. Kalscheuer R, Koliwer-Brandl H. 2014. Genetics of mycobacterial trehalose metabolism. Microbiol Spectr 2. https://doi.org/10.1128/microbiolspec.MOB-00002-13.

45. Schubert K, Sieger M, Fierer F, Giacomelli G, Bohn K, Rieblinger A, Lindenthal L, Sachs N, Wanner G, Bramkamp M. 2017. The antituberculosis drug ethambutol selectively blocks apical growth in CMN group bacteria. mBio 8: e0213-16. https://doi.org/10.1128/mBio.0213-16.

46. Yang NJ, Hinner MJ. 2015. Getting across the cell membrane: an overview of how mycobacteria escape phagolysosomes of living macrophages. J Biol Chem 286:1641–1647. https://doi.org/10.1074/jbc.M115047200.

47. Kamariza M, Shieh F, Eland CS, Peters JS, Chu B, Rodriguez-Rivera FP, Babu Sait MR, Treuren WV, Martinson N, Kalscheuer R, Kana BD, Bertozzi CR. 2018. Rapid detection of Mycobacterium tuberculosis in sputum with a solvatochromic trehalose probe. Sci Transl Med 10:aam6310.
81. Rajni Rao N, Meena LS. 2011. Biosynthesis and virulent behavior of lipids produced by Mycobacterium tuberculosis. J Bacteriol 193:6144–6154. https://doi.org/10.1128/JB.05834-12.

82. Puech V, Guilhot C, Perez E, Tropis M, Armitige LY, Gicquel B, Daffe M. 2002. Evidence for a partial redundancy of the fibronectin-binding proteins for the transfer of mycoloyl residues onto the cell wall arabinogalactan-tum of Mycobacterium tuberculosis. Mol Microbiol 44:1109–1122. https://doi.org/10.1046/j.1365-2958.2002.02953.x.

83. Jamet S, Quentin Y, Coudray C, Teixier P, Laval F, Daffe M, Fichant G, Cam K. 2015. Evolution of mycolic acid biosynthesis genes and their regulation during starvation in Mycobacterium tuberculosis. J Bacteriol 197:3797–3811. https://doi.org/10.1128/JB.00433-15.

84. Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. 2002. Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. Mol Microbiol 43:717–731. https://doi.org/10.1046/j.1365-2958.2002.02279.x.

85. Zhao DQ, Li TT, Hao ZJ, Cheng ML, Tao J. 2019. Exogenous trehalose favors high temperature stress tolerance to herbaceous peony by enhancing antioxidant systems, activating photosynthesis, and protecting cell structure. Cell Stress Chaperones 24:247–257. https://doi.org/10.1007/s12192-019-00961-1.

86. Liu J, Ren X, Pan YT, Pustaskz I, Carroll D. 2003. New insights on trehalose: a multifunctional molecule. Glycobiology 13:17R–27R. https://doi.org/10.1093/glycob/cwg047.

87. Dwyer DJ, Belenky PA, Yang JH, MacDonald IC, Martell JD, Takahashi N, Chan CT, Lobritz MA, Braff D, Schwartz EG, Ye JD, Pati M, Vercruyse M, Ralifo PS, Allision KR, Khalil AS, Ting AY, Walker GC, Collins JJ. 2014. Antibiotics induce redox-related physiological alterations as part of their lethality. Proc Natl Acad Sci U S A 111:E2100–E9. https://doi.org/10.1073/pnas.1401876111.

88. Lobrit MA, Belenky P, Porter CB, Gutierrez A, Yang JH, Schwar EG, Dwyer DJ, Khalil AS, Collins JJ. 2015. Antibiotic efficacy is linked to bacterial cellular respiration. Proc Natl Acad Sci U S A 112:8173–8180. https://doi.org/10.1073/pnas.1509743112.

89. Bliss CL. 1956. The calculation of microbial assays. Bacteriol Rev 20:243–258. https://doi.org/10.1128/BR.20.4.243-258.1956.

90. Touchette MH, van Vlack EB, Bai L, Kim J, Cognetta AB, 3rd, Previti ML, Backus KM, Martin DW, Cravatt BF, Seeliger JC. 2017. A screen for protein–protein interactions in live mycobacteria reveals a functional link between the virulence-associated lipid transporter Lpz and the mycolyltransferase antigen 85A. ACS Infect Dis 3:336–348. https://doi.org/10.1021/acsinfecdis.6b00179.

91. Payne K, Sun Q, Sacchettini J, Hartfull GF. 2009. Mycobacteriophage lysin B is a novel mycolylarabinogalactan esterase. Mol Microbiol 73:367–381. https://doi.org/10.1111/j.1365-2958.2009.06775.x.

92. Sharma A, Pohane AA, Bansal S, Bajaj A, Jain V, Srivastava A. 2015. Cell penetrating synthetic antimicrobial peptides (SAMPs) exhibiting potent and selective killing of mycobacterium by targeting its DNA. Chem Eur J 21:3540–3545. https://doi.org/10.1002/chem.201404650.

93. Nambi S, Long JE, Mishra BB, Baker R, Murphy KC, Olive AJ, Nguyen HP, Shaffer SA, Sassetti CM. 2015. The oxidative stress network of Mycobacterium tuberculosis reveals coordination between radical detoxification systems. Cell Host Microbe 17:829–837. https://doi.org/10.1016/j.chom.2015.05.008.

94. Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR, Jr. 1990. Isolation and characterization of efficient plasmid transformation mutants of Mycobacterium smegmatis. Mol Microbiol 4:1911–1919. https://doi.org/10.1111/j.1365-2958.1990.tb00190.x.

95. Lobrit MA, Belenky P, Porter CB, Gutierrez A, Yang JH, Schwar EG, Dwyer DJ, Khalil AS, Collins JJ. 2015. Antibiotic efficacy is linked to bacterial celluar respiration. Proc Natl Acad Sci U S A 112:8173–8180. https://doi.org/10.1073/pnas.1509743112.

96. Bliss CL. 1956. The calculation of microbial assays. Bacteriol Rev 20:243–258. https://doi.org/10.1128/BR.20.4.243-258.1956.

97. Touchette MH, van Vlack EB, Bai L, Kim J, Cognetta AB, 3rd, Previti ML, Backus KM, Martin DW, Cravatt BF, Seeliger JC. 2017. A screen for protein–protein interactions in live mycobacteria reveals a functional link between the virulence-associated lipid transporter Lpz and the mycolyltransferase antigen 85A. ACS Infect Dis 3:336–348. https://doi.org/10.1021/acsinfecdis.6b00179.

98. Lobrit MA, Belenky P, Porter CB, Gutierrez A, Yang JH, Schwar EG, Dwyer DJ, Khalil AS, Collins JJ. 2015. Antibiotic efficacy is linked to bacterial celluar respiration. Proc Natl Acad Sci U S A 112:8173–8180. https://doi.org/10.1073/pnas.1509743112.

99. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–680. https://doi.org/10.1038/nmeth.2570.