Probing the Mycobacterial Trehalome with Bioorthogonal Chemistry

Supporting Information

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I. Abbreviations

Ac = acetyl

ADC = Liquid medium supplement containing albumin, dextrose, catalayse

AG = arabinogalactan

Ag85 = antigen 85

AzTDM = azide-labeled trehalose dimycolate

AzTMM = azide-labeled trehalose monomycolate

BARAC = biarylazacyclooctynone

BCG = Bacillus Calmette-Guérin

BM = BARAC-methyl

BSA = bovine serum albumin

Bz = benzoyl

CL = capsular layer

DAPI = 4',6-diamidino-2-phenylindole

DIC = differential interference contrast

DMF = *N*,*N*-dimethylformamide

DMSO = dimethylsulfoxide

ESI = electrospray ionization

FITC = fluorescein isothiocyanate

FT-ICR = Fourier transform ion cyclotron resonance

HPAEC-PAD = high-pH anion-exchange chromatography with pulsed amperometric detection

HR = high resolution

BCG = *Mycobacterium bovis* BCG-Pasteur

MFI = mean fluorescence intensity

MIC = minimum inhibitory concentration

MM = mycomembrane

MS = mass spectrometry

Msmeg = *Mycobacterium smegmatis* mc^2155

Mtb = *M. tuberculosis*

OADC = Liquid medium supplement containing oleic acid, albumin, dextrose, catalayse

OD_{600} = optical density measured by absorbance at a wavelength of 600 nm

PCR = polymerase chain reaction

PBS = phosphate-buffered saline

PBSB = PBS 1x with 0.5% bovine serum albumin

PG = peptidoglycan

PM = plasma membrane

Pyr = pyridine

SI = Supporting information

TDM = trehalose dimycolate

Tf = trifluoromethanesulfonyl

THF = tetrahydrofuran

TLC = thin layer chromatography

TMM = trehalose monomycolate

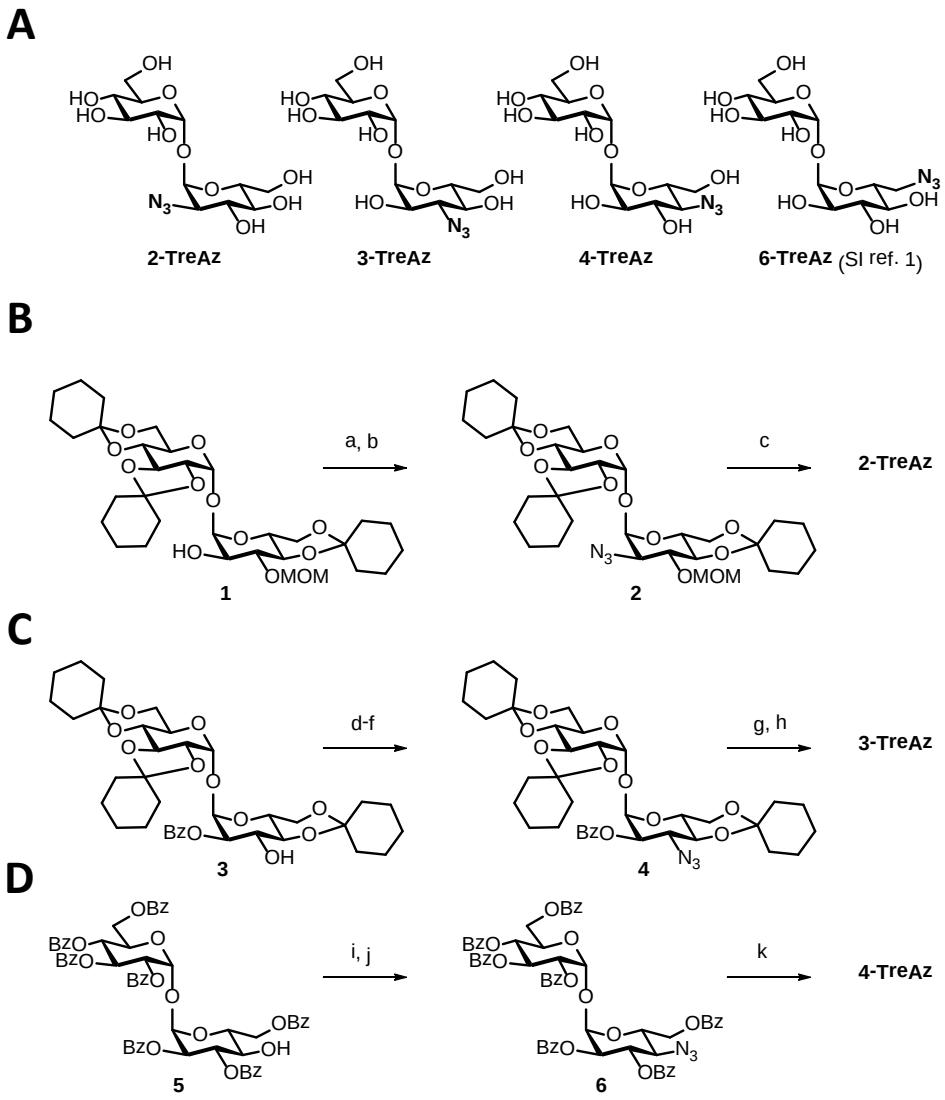
T = tesla

Tre = trehalose

TreAz = azide-modified trehalose

WT = wild type

II. Supplementary Schemes and Figures



Scheme S1. Synthesis of azide-modified trehalose (TreAz) analogues. (A) Compounds used in this study. 6-TreAz was synthesized as reported by Hannessian,[1] and the remaining analogues were synthesized according to (B–D). See page S19 for detailed synthetic methods and characterization data for all intermediates and final products. Conditions: (a) Tf_2O, pyr; $NaNO_2$, DMF, 65 °C, 63%; (b) Tf_2O, pyr; LiN_3, DMF, DMSO, 60 °C, 45%; (c) 10% aq. HCl–THF (1:1), 69%; (d) $(COCl)_2$, DMSO, CH_2Cl_2, –78 °C; then Et_3N, –78 °C → rt, 97%; (e) $NaBH_4$, NaOAc, MeOH, CH_2Cl_2, –60 °C, 73%; (f) Tf_2O, pyr; LiN_3, DMF, DMSO, rt, 81%; (g) NaOMe/MeOH, CH_2Cl_2, 84%; (h) 10% aq. HCl–THF (1:1), 65%; (i) Tf_2O, pyr; $NaNO_2$, DMF, rt, 66%; (j) Tf_2O, pyr, CH_2Cl_2; NaN_3, 15-crown-5, DMF, 60 °C, 72%; (k) NaOMe/MeOH, CH_2Cl_2, 83%.

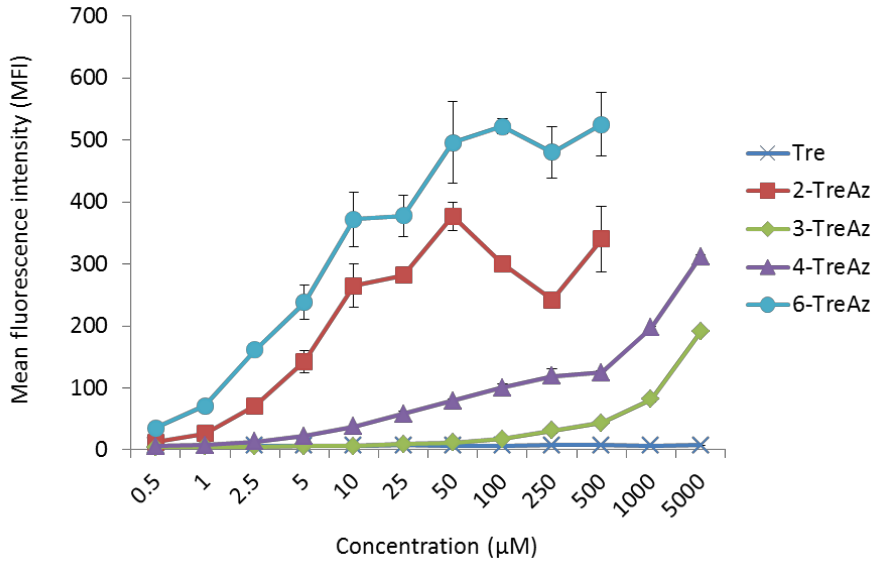


Figure S1. Concentration dependence of TreAz labeling in *Msmeg*. According to the general procedure in the methods section (page S27), *Msmeg* was cultured in the presence of varying concentrations of TreAz or trehalose, reacted with BARAC-Fluor (1 µM, 30 min), fixed, and analyzed by flow cytometry. 2- and 6-TreAz labeling was detectable over background at concentrations as low as 1 µM, and saturation of labeling occurred at approximately 50 µM. 3- and 4-TreAz required higher concentrations (250-500 µM) for moderate labeling, but could be improved by increasing the concentration. At 5000 µM, 3- and 4-TreAz labeled *Msmeg* with high efficiency. Data represents means of triplicates ± standard deviation, and are representative of at least two independent experiments.

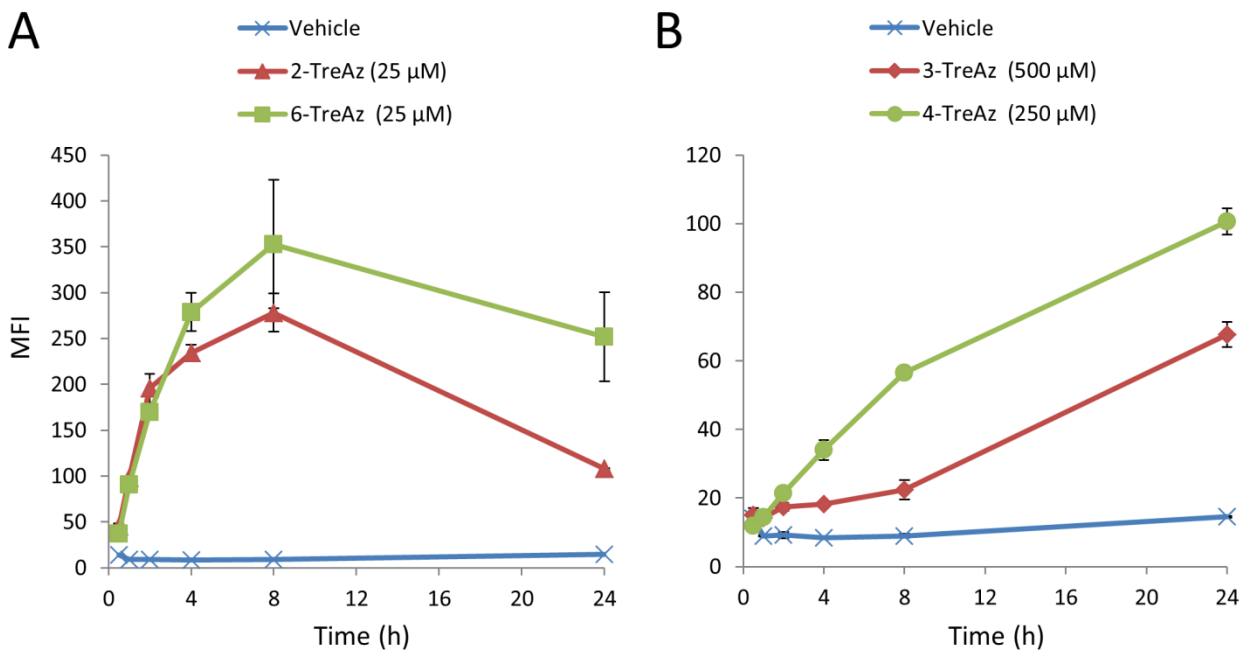


Figure S2. Time dependence of TreAz labeling in *Msmeg*. According to the general procedure in the methods section (page S27), *Msmeg* was grown in the presence or absence of TreAz in culture tubes. At different time points (0.5-24 h) aliquots were removed, immediately fixed, and stored at 4 °C. After samples for all time points were collected, fixed bacteria were reacted with BARAC-Fluor (1 µM, 30 min) and analyzed by flow cytometry. (A) 2- and 6-TreAz behaved similarly, with significant labeling occurring in as little as 30 min. Maximum labeling for both compounds was achieved in 4–8 h, while longer incubation times (24 h) led to reduced fluorescence in the population, possibly due to intracellular accumulation of compounds to inhibitory levels. (B) 3- and 4-TreAz labeling increased with time up to 24 h. Culture density for each time point: 0 h, OD_{600} = 0.4; 0.5 h, OD_{600} = 0.5; 1 h, OD_{600} = 0.6; 2 h, OD_{600} = 0.8; 4 h, OD_{600} = 1.7; 8 h, OD_{600} = 4.1; 24 h, OD_{600} = 4.0. Data represents means of triplicates ± standard deviation, and are representative of at least two independent experiments.

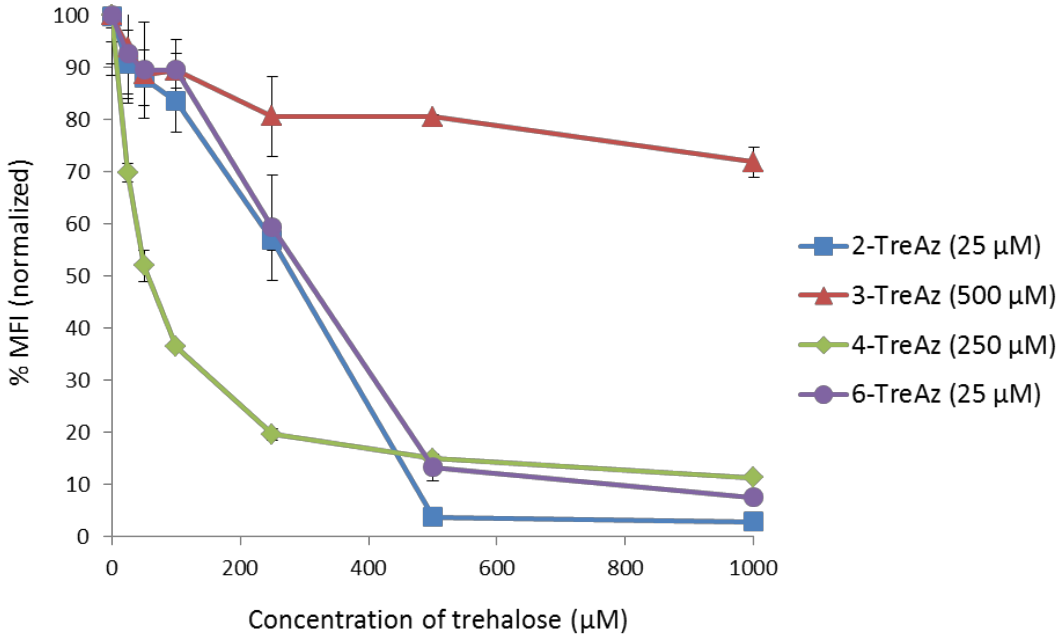


Figure S3. Competition of TreAz labeling by trehalose in *Msmeg*. According to the general procedure in the methods section (page S27), *Msmeg* was cultured in the presence of TreAz along with increasing concentrations of trehalose, then reacted with BARAC-Fluor (1 µM, 30 min), fixed, and analyzed by flow cytometry. Labeling with 2-, 4-, and 6-TreAz was competed out by 500 µM trehalose. 4-TreAz was the most sensitive to competition, while 2- and 6-TreAz exhibited a lag in response to exogenous trehalose until it reached 250 µM. 3-TreAz was not affected by trehalose, even at concentrations up to 5000 µM (not shown), which is consistent with the previously reported Ag85-based probe FITC-Tre.[2] On the y-axis, MFI is normalized to show percent of maximum fluorescence for each analogue. Data represents means of triplicates ± standard deviation, and are representative of at least two independent experiments.

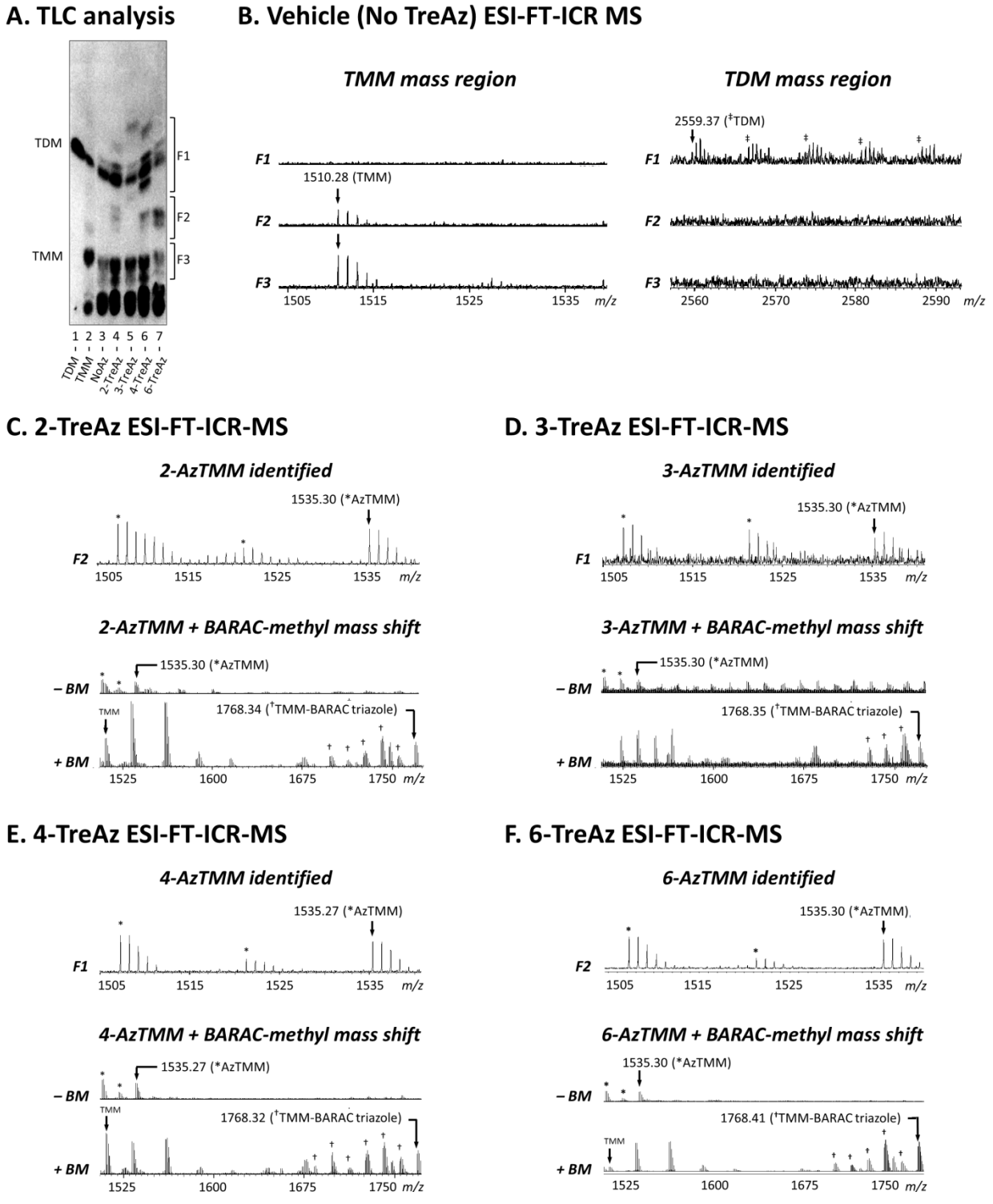


Figure S4. TLC and ESI-FT-ICR MS analysis of azide-modified glycolipids. As described in the methods section (page S28), total lipid extracts were collected from *Msmeg*, partially purified, and analyzed by TLC and high resolution mass spectrometry. (A) TLC analysis of solvent-fractionated lipids (TLC stain, 5% H_2SO_4 in EtOH; TLC plate image was contrast-adjusted to improve visualization of spots) revealed that each TreAz-treated sample (lanes 4–7) contained

new spots that were not present in the untreated sample (lane 3). Compared to TDM and TMM standards (lanes 1 and 2), new spots of intermediate polarity were observed for all four TreAz-treated samples, putatively representing azide-labeled TMM (AzTMM). New compounds with lower polarity than TDM – potentially azide-labeled TDM (AzTDM) – were observed for 2-, 3-, and 4-TreAz but not 6-TreAz, which is unable to form AzTDM due to its blocked 6-position. Samples were further purified over silica gel into three fractions (F1–F3, shown on the right TLC border) and analyzed by mass spectrometry. (B) ESI-FT-ICR MS of fractions F1–F3 collected from untreated *Msmeg* showed native TMM and TDM in the expected regions but no ions corresponding to AzTMM. (C–F) ESI-FT-ICR MS of fractions collected from TreAz-treated *Msmeg* confirmed the presence of AzTMM (represented by a series of ions separated by 14 mass units, deriving from heterogeneity in lipid chain length) in F1 or F2 of all four samples (top). To verify azide incorporation, AzTMM-containing fractions were treated with BARAC-methyl[3] (BM), which generated mass-shifted TMM-BARAC triazole conjugates (bottom). TDM is large, apolar, and thus ionizes poorly via ESI, and this hindered our ability to identify putative AzTDM TLC spots in F1 by MS. However, we speculate that the most nonpolar spots observed by TLC for 2-, 3-, and 4-TreAz represent AzTDM. * = AzTMM peaks; † = TMM-BARAC triazole peaks; ‡ = TDM.

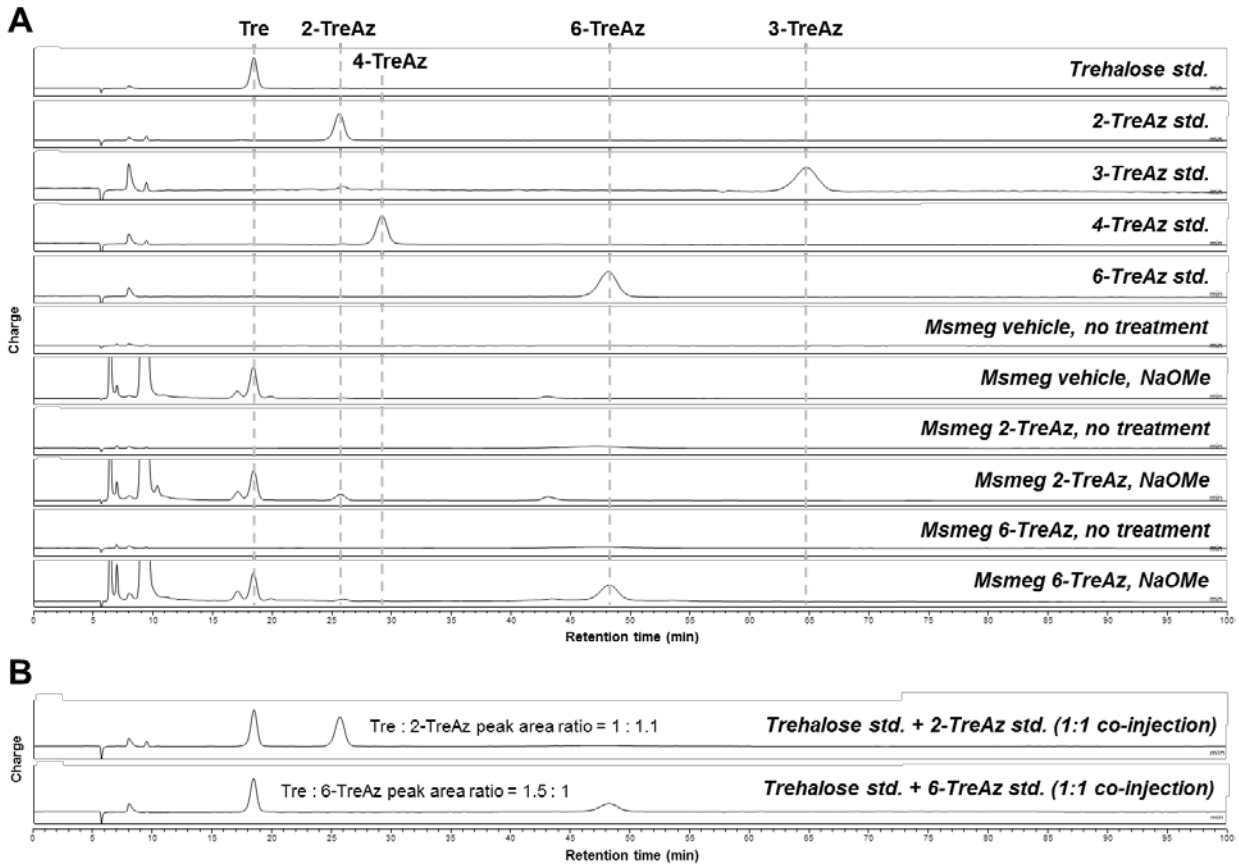


Figure S5. HPAEC-PAD analysis of free sugars cleaved from glycolipids. As described in the methods section (page S29), *Msmeg* wild type was cultured in the presence of 2- or 6-TreAz (or vehicle control) followed by lipid extraction, NaOMe treatment (or no treatment control), and analysis of sugars by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). (A) Both 2- and 6-TreAz were detected in *Msmeg* samples labeled with these analogues, but not in the vehicle control or control samples that were not subjected to NaOMe treatment. Relative to natural trehalose, 2- and 6-TreAz peaks were quantified at approximately 20% and 70%, respectively, after adjustment based on relative peak areas of standards shown in Figure S5B. Chromatogram signals are scaled based on the trehalose peak.

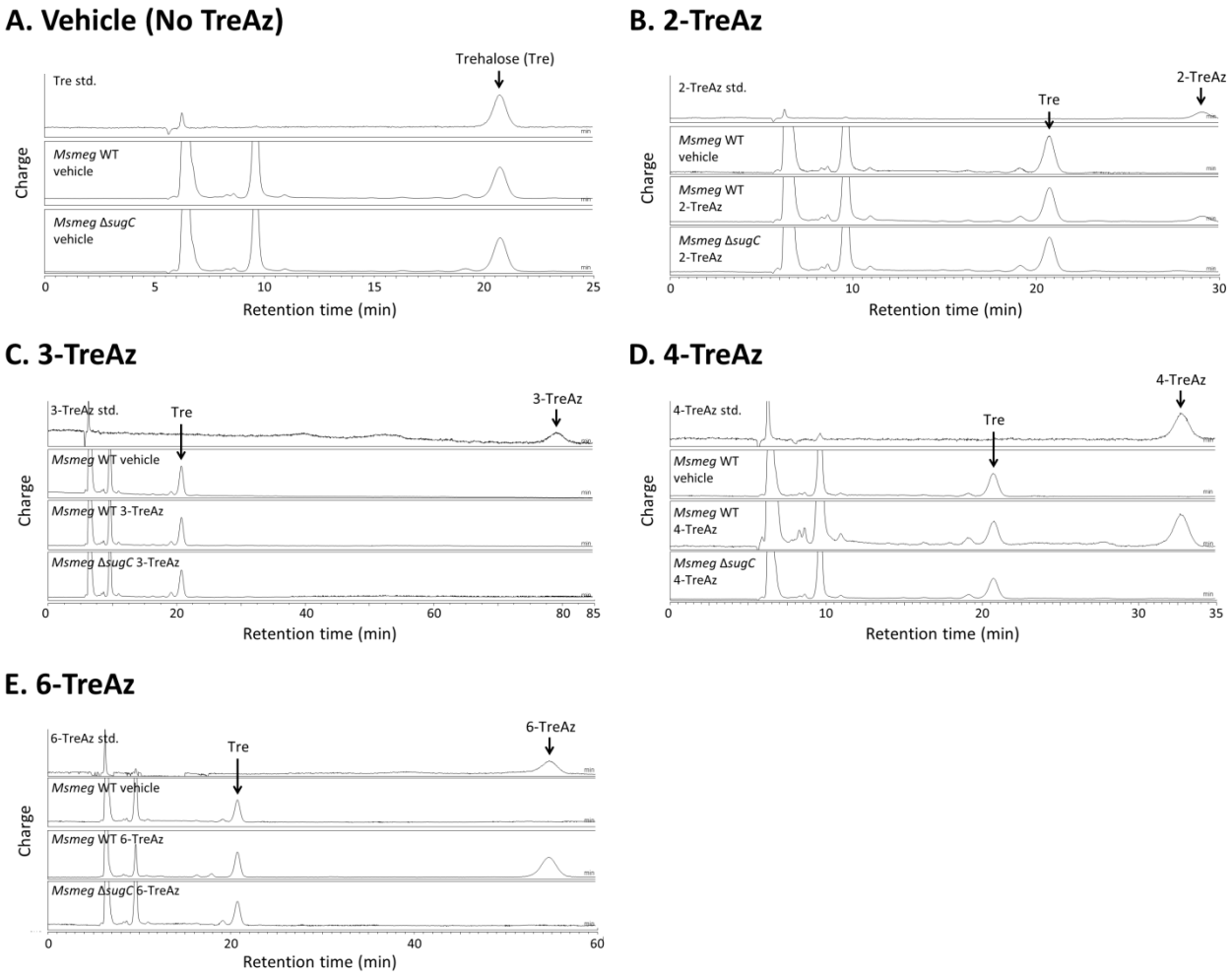


Figure S6. HPAEC-PAD analysis of TreAz uptake in *Msmeg*. As described in the methods section (page S30), *Msmeg* wild type (WT) or the Δ*sugC* mutant were cultured in the presence or absence of TreAz analogues, washed, and then lysed to collect cytosolic metabolites that were analyzed by HPAEC-PAD. Authentic trehalose and TreAz standards were used to identify peaks of interest. (A) Trehalose was observed in extracts from untreated *Msmeg* WT and the Δ*sugC* mutant. (B–E) Peaks for 2-, 4-, and 6-TreAz were observed in extracts from TreAz-treated *Msmeg* WT but not in the vehicle control or in the Δ*sugC* mutant, while 3-TreAz was not observed in any extracts. These data indicated that 2-, 4-, and 6-TreAz accessed the cytoplasm through the trehalose-specific transporter SugABC-LpqY, while 3-TreAz did not. Chromatograms for cytosolic extracts are scaled for signal based on the trehalose peak.

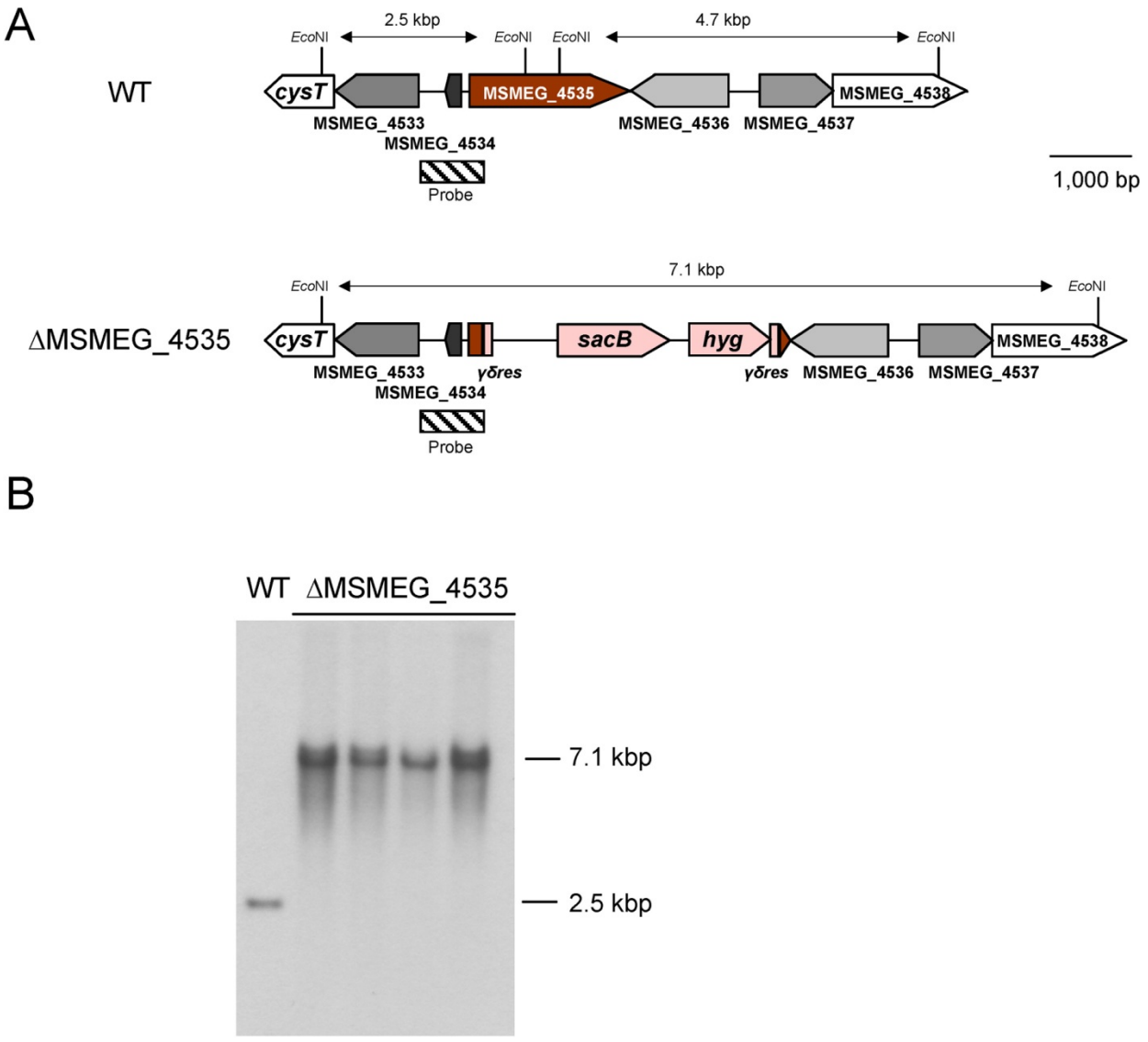


Figure S7. Construction of the *Msmeg* ΔMSMEG_4535 (trehalase) mutant. (A) Organization of the MSMEG_4535 locus in *Msmeg* wild type and the ΔMSMEG_4535 mutant. The sizes of relevant fragments as well as the location of the probe used for Southern analysis are indicated. WT, wild type; γδ*res*, *res*-sites of the γδ-resolvase; *hyg*, hygromycin resistance gene. (B) Southern analysis of *Eco*NI-digested genomic DNA using a probe hybridizing to the position indicated in (A), showing gene deletion in the ΔMSMEG_4535 mutant.

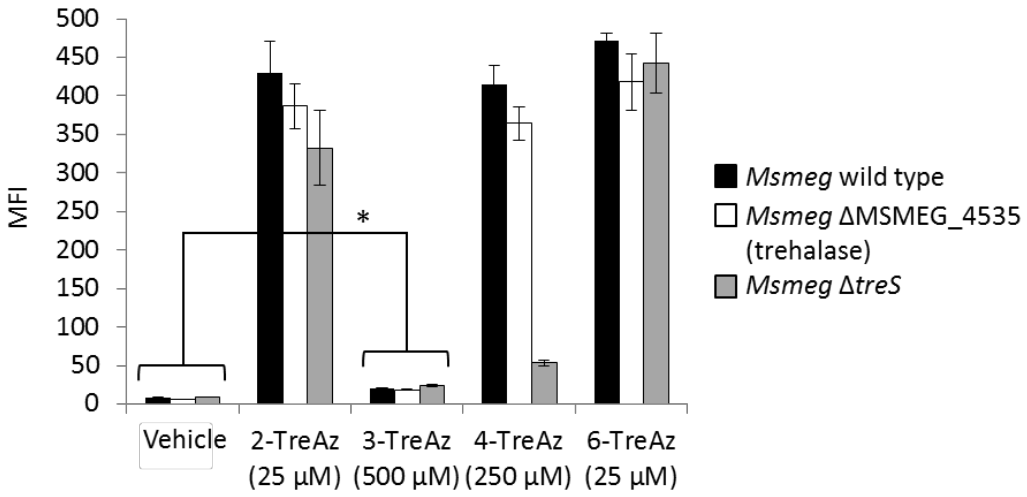


Figure S8. Trehalase and TreS dependence of TreAz labeling in *Msmeg*. According to the general procedure in the methods section (page S27), *Msmeg* wild type, ΔMSMEG_4535 (trehalase), or Δ*treS* strains were incubated in the presence or absence of TreAz, reacted with BARAC-Fluor (1 µM, 30 min), fixed, and analyzed by flow cytometry. No labeling phenotypes were observed in the ΔMSMEG_4535 (trehalase) mutant, and labeling with 2-, 3-, and 6-TreAz was unaffected in the Δ*treS* mutant. However, a reduction in 4-TreAz labeling was seen in the Δ*treS* mutant, which could imply that this analogue selectively labeled downstream products of the TreS pathways such as capsular α-glucan or methylglucose lipopolysaccharide.[4] Alternatively, the observed decrease in labeling could instead simply result from competition by intracellular trehalose, which is elevated in the *Msmeg* Δ*treS* mutant (Figure S9). 4-TreAz is the most sensitive of the four analogues to competition by exogenous trehalose in *Msmeg* (Figure S3), which would explain why 2- and 6-TreAz were not similarly affected in the experiment. An *M. bovis* BCG-Pasteur Δ*treS* mutant was also generated (Figure S10) but showed no phenotype for 4-TreAz (Figure S11), which is explainable by the different 4-TreAz incorporation routes in *Msmeg* and BCG (Figures 4 and 5, respectively). Data represents means of triplicates ± standard deviation, and are representative of two independent experiments. * $p < 0.05$.

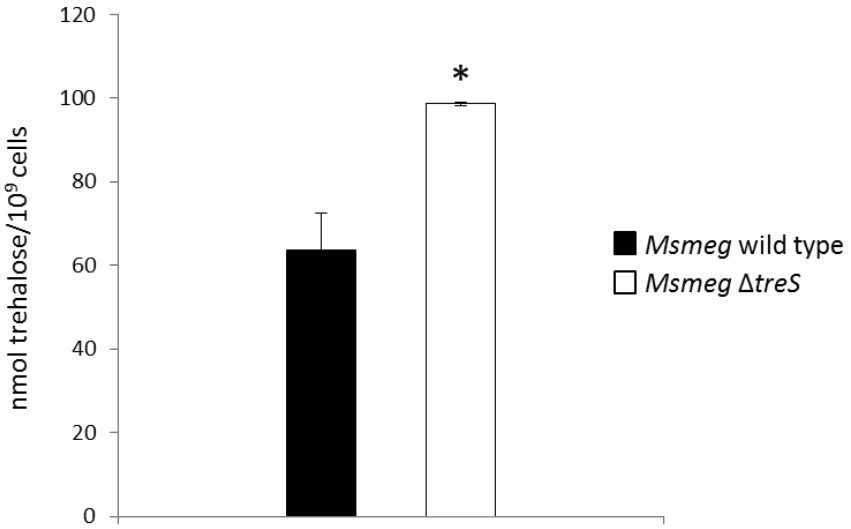


Figure S9. Trehalose quantification in *Msmeg* wild type and Δ*treS* as determined by a previously-described trehalase–glucose oxidase assay.[5] Error bars denote the standard deviation of three replicate experiments. * $p < 0.05$.

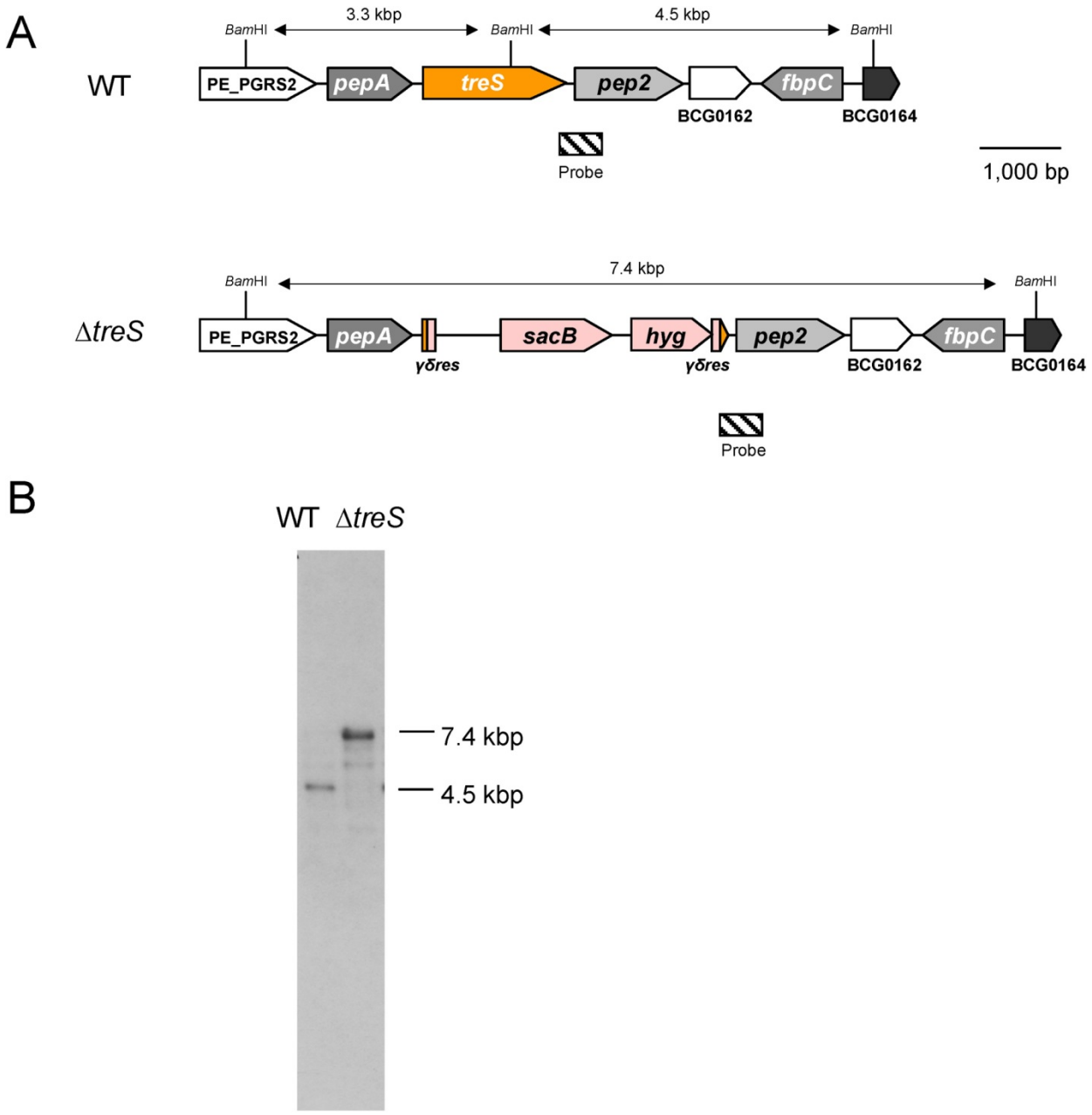


Figure S10. Construction of the *M. bovis* BCG-Pasteur Δ*treS* mutant. (A) Organization of the *treS* locus in *M. bovis* BCG-Pasteur wild type and the Δ*treS* mutant. The sizes of relevant fragments as well as the location of the probe used for Southern analysis are indicated. WT, wild type; γδ*res*, *res*-sites of the γδ-resolvase; *hyg*, hygromycin resistance gene. (B) Southern analysis of *Bam*HI-digested genomic DNA using a probe hybridizing to the position indicated in (A), showing gene deletion in the Δ*treS* mutant.

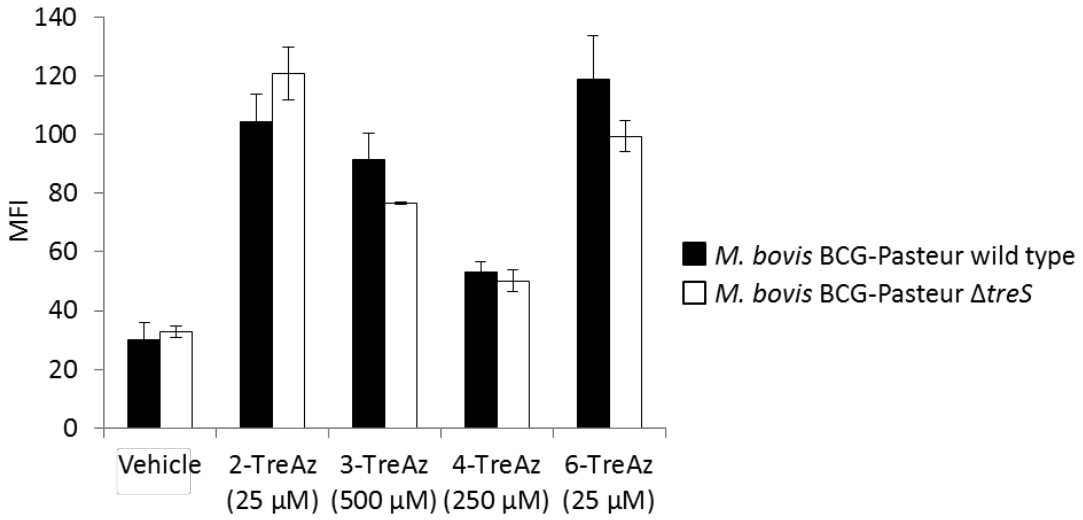


Figure S11. TreS does not affect TreAz labeling in *M. bovis* BCG-Pasteur. According to the general procedure in the methods section (page S27), *M. bovis* BCG-Pasteur wild type or Δ*treS* strains were incubated in the presence or absence of TreAz for 72 h, reacted with BARAC-Fluor (1 µM, 30 min), fixed, and analyzed by flow cytometry. No substantial decreases in labeling were observed for any TreAz analogues. Notably, the reduction in 4-TreAz labeling in the *Msmeg* Δ*treS* mutant (Figure S8) was not recapitulated in the *M. bovis* BCG-Pasteur Δ*treS* mutant. This is consistent with the different 4-TreAz incorporation routes in the two species, with 4-TreAz labeling via the recycling pathway in *Msmeg* but not BCG (Figures 4 and 5). Data represents means of triplicates ± standard deviation, and are representative of two independent experiments.

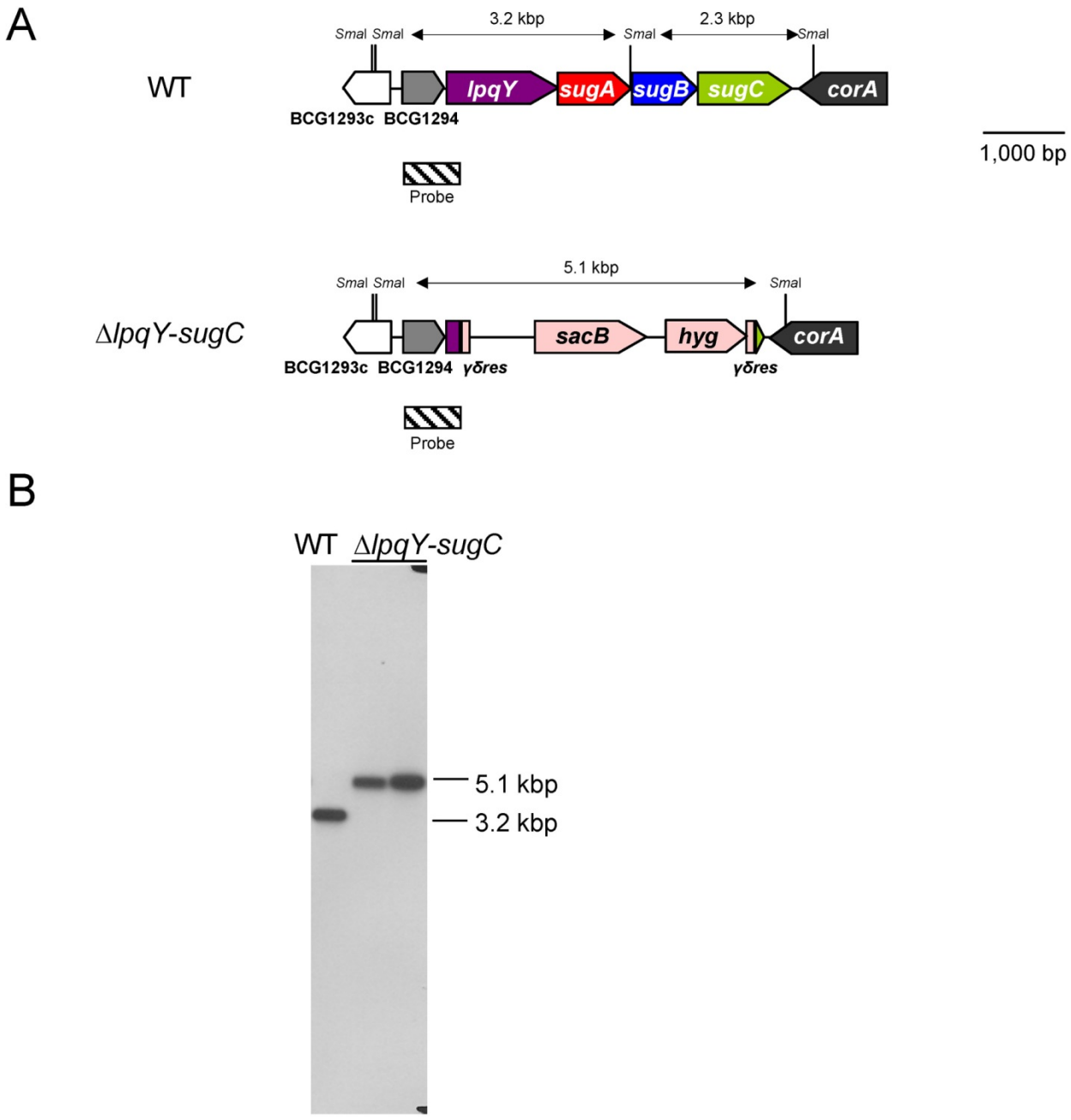


Figure S11. Construction of the *M. bovis* BCG-Pasteur Δ*lpqY-sugC* mutant. (A) Organization of the *lpqY-sugA-sugB-sugC* locus in *M. bovis* BCG-Pasteur wild type and the Δ*lpqY-sugC* mutant. The sizes of relevant fragments as well as the location of the probe used for Southern analysis are indicated. WT, wild type; γδ*res*, *res*-sites of the γδ-resolvase; *hyg*, hygromycin resistance gene. (B) Southern analysis of *Sma*I-digested genomic DNA using a probe hybridizing to the position indicated in (A), showing gene deletion in the Δ*lpqY-sugC* mutant.

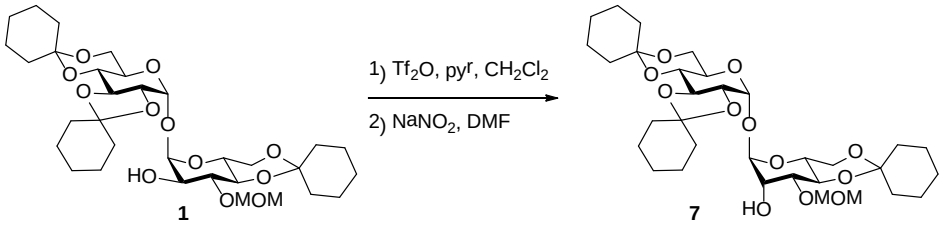
II. Supplementary Methods

Synthesis of TreAz analogues

General methods for synthesis. Materials and reagents were obtained from commercial sources without further purification unless otherwise noted. Anhydrous solvents were obtained either commercially or from an alumina column solvent purification system. Water content of alumina-purified solvents was < 10 ppm as measured by Karl-Fischer coulometric analysis. All reactions were carried out in oven-dried glassware under nitrogen unless otherwise noted. Analytical TLC was performed on SiliCycle® glass-backed silica gel 60 Å plates (thickness 250 μm) and detected by UV lamp or charring with 5% H_2SO_4 in EtOH. Column chromatography was performed using SiliCycle® Silia*Flash*® P60 silica gel (40-63 μm). ^{1}H NMR spectra were recorded at 400 or 600 MHz with chemical shifts in ppm (δ) referenced to solvent peaks. ^{13}C NMR spectra were recorded at 100 or 150 MHz with chemical shifts referenced to solvent peaks. NMR spectra were obtained on Bruker AVB-400 or AV-600 instruments. Coupling constants (*J*) are reported in hertz (Hz). High resolution electrospray ionization mass spectrometry (HR ESI MS) was performed at the UC Berkeley Mass Spectrometry Facility.

Note: Compound numbering of synthetic intermediates continues from Scheme S1.

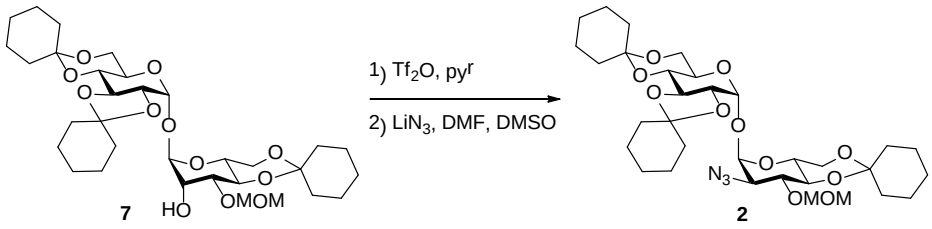
4,6-*O*-Cyclohexylidene-3-*O*-methoxymethyl-α-D-mannopyranosyl-(1→1)-2,3;4,6-di-*O*-cyclohexylidene-α-D-glucopyranoside (7)



To a stirring solution of known compound **1**[6] (626 mg, 1.00 mmol) and anhydrous pyridine (0.240 mL, 3.00 mmol) in anhydrous CH_2Cl_2 (10.0 mL) at 0 °C under a nitrogen atmosphere was added Tf_2O (0.336 mL, 2.00 mmol) dropwise. After 15 min, TLC showed complete conversion to the intermediate triflate [TLC (hexanes/EtOAc, 4:1): R_f = 0.55]. While still at 0 °C, the reaction was quenched by addition of ice-cold saturated aqueous $NaHCO_3$. The resulting mixture

was extracted three times with CH_2Cl_2, and the combined organic layer was dried over anhydrous Na_2SO_4 and co-evaporated with toluene three times under reduced pressure. The crude material was dissolved in anhydrous DMF under a nitrogen atmosphere and treated with $NaNO_2$ (690 mg, 10.0 mmol). The reaction mixture was heated to 65 °C and stirred overnight. After cooling to rt, the reaction was diluted with EtOAc and washed with brine three times, dried over anhydrous Na_2SO_4, and concentrated in vacuum. The crude residue was chromatographed on silica gel (hexanes/EtOAc, 2.5:1) to give **7** (260 mg, 42% over two steps) as a white solid. A less polar elimination product [TLC (hexanes/EtOAc, 4:1): R_f = 0.30] was also obtained (122 mg, 20% over two steps) from the reaction mixture. TLC (hexanes/EtOAc, 4:1): R_f = 0.07. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.44 (d, *J* = 2.8 Hz, 1 H), 5.16 (s, 1 H), 4.90 (d, *J* = 6.8 Hz, 1 H), 4.78 (d, *J* = 6.4 Hz, 1 H), 4.10-4.04 (m, 3 H), 4.00-3.91 (m, 3 H), 3.89-3.80 (m, 5 H), 3.62-3.56 (m, 1 H), 3.51 (dd, *J* = 3.2, 9.2 Hz, 1 H), 3.44 (s, 3 H), 2.73 (d, *J* = 1.2 Hz, 1 H), 2.20-2.10 (m, 2 H), 1.87-1.30 (m, 28 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 112.63, 100.15, 99.94, 96.89, 96.48, 93.61, 76.23, 73.81, 73.34, 73.21, 70.55, 69.75, 66.54, 64.84, 61.78, 61.64, 55.96, 38.17, 37.98, 36.36, 36.19, 28.03, 27.99, 25.78, 25.70, 25.16, 24.01, 23.66, 23.02, 22.84, 22.64. HR ESI MS: calcd. for $C_{32}H_{50}O_{12}Na$ $[M+Na]^+$ *m/z*, 649.319; found, 649.320.

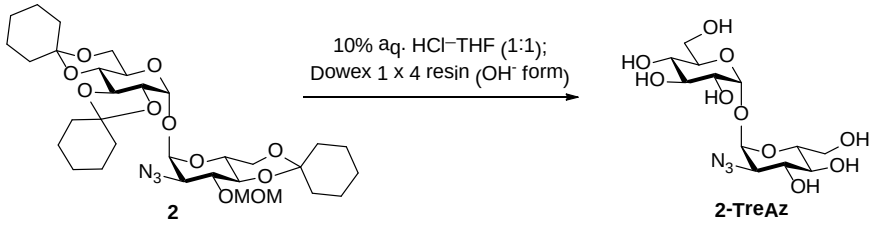
2-Azido-2-deoxy-4,6-*O*-cyclohexylidene-3-*O*-methoxymethyl-α-D-glucopyranosyl-(1→1)-2,3;4,6-di-*O*-cyclohexylidene-α-D-glucopyranoside (2)



To a stirring solution of **7** (176 mg, 0.281 mmol) in anhydrous pyridine (5.0 mL) at 0 °C under a nitrogen atmosphere was added Tf_2O (0.120 mL, 0.715 mmol) dropwise. The reaction gradually warmed to rt while stirring overnight, after which TLC showed complete conversion to the intermediate triflate [TLC (hexanes/EtOAc, 3:1): R_f = 0.78]. The reaction mixture was cooled to 0 °C and quenched by addition of ice-cold saturated aqueous $NaHCO_3$, then extracted three times with CH_2Cl_2. The combined organic layer was dried over anhydrous Na_2SO_4 and co-evaporated with toluene three times under reduced pressure. The resulting residue was dissolved in

anhydrous DMF (5.0 mL) at rt under a nitrogen atmosphere and treated with a freshly prepared solution of LiN_3 [2.81 mL of a 1.00 M solution of LiN_3 in DMF–DMSO (10:1)]. After heating the reaction to 60 °C and stirring at rt for 4 h, TLC showed conversion of the starting material to an approximate 2:1 mixture of spots. The reaction was diluted with EtOAc, washed with brine three times, dried over anhydrous Na_2SO_4, and co-evaporated with toluene under reduced pressure. The crude residue was chromatographed on silica gel (toluene/EtOAc, 16:1) to give the desired product **2** (80 mg, 44% over two steps) as a white solid. A more polar elimination product [TLC (toluene/EtOAc, 8:1): R_f = 0.40] was also obtained (40 mg, 24%) from the reaction mixture. TLC (toluene/EtOAc, 8:1): R_f = 0.45. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.38 (d, *J* = 2.0 Hz, 1 H), 5.16 (d, *J* = 2.4 Hz, 1 H), 5.01 (d, *J* = 4.4 Hz, 1 H), 4.77 (d, *J* = 4.4 Hz, 1 H), 4.13 (t, *J* = 6.4 Hz, 1 H), 4.03-3.94 (m, 3 H), 3.90 (dd, *J* = 2.8, 6.4 Hz, 1 H), 3.83-3.76 (m, 3 H), 3.73 (t, *J* = 7.2 Hz, 1 H), 3.64 (t, *J* = 6.4 Hz, 1 H), 3.41-3.36 (m, 4 H), 3.36 (dd, *J* = 2.4, 6.8 Hz, 1 H), 2.21-2.13 (m, 2 H), 1.81-1.75 (m, 2 H), 1.70-1.24 (m, 26 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 112.71, 100.06, 99.90, 97.52, 95.52, 94.17, 76.26, 74.25, 73.52, 73.35, 73.16, 66.80, 64.12, 62.86, 61.81, 61.69, 56.29, 38.04, 37.93, 36.42, 36.17, 28.08, 27.93, 25.78, 25.73, 25.21, 24.05, 23.71, 23.03, 22.91, 22.73, 22.67. HR ESI MS: calcd. for $C_{32}H_{49}N_3O_{11}Na$ $[M+Na]^+$ *m/z*, 674.326; found, 674.327.

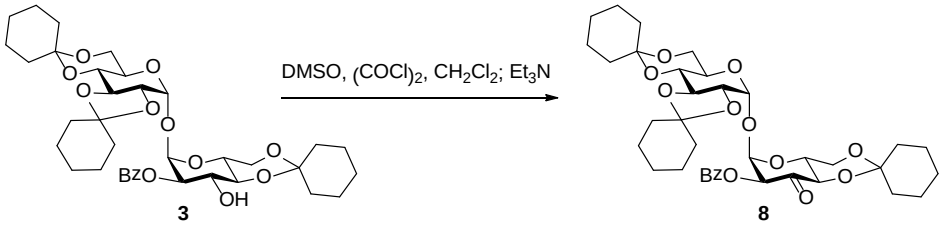
2-Azido-2-deoxy-α,α-D-trehalose (2-TreAz)



To a stirring solution of **2** (75 mg, 0.115 mmol) in THF (1.5 mL) at 0 °C was added 10% aqueous HCl (1.5 mL) dropwise. The reaction was warmed to rt and stirred for 6 h, then neutralized to pH 7 with Dowex 1 × 4 (Cl^- form, exchanged to OH^- form). After filtration and concentration under vacuum, the crude residue was chromatographed on silica gel ($CHCl_3$/MeOH, 1.5:1) to give **2-TreAz** (61 mg, 69%) as a white solid. TLC ($CHCl_3$/MeOH, 1.5:1): R_f = 0.34. ^{1}H NMR (600 MHz, D_2O): δ 5.31 (d, *J* = 3.6 Hz, 1 H), 5.20 (d, *J* = 3.6 Hz, 1 H), 4.05 (t, *J* = 10.2 Hz, 1 H), 3.89-3.76 (m, 7 H), 3.65 (dd, *J* = 4.2, 10.2 Hz, 1 H), 3.53 (t, *J* =

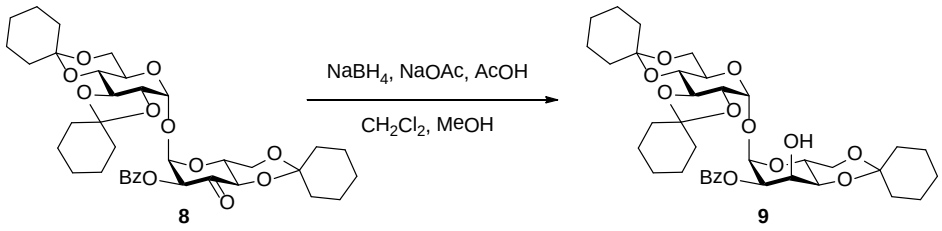
9.6 Hz, 1 H), 3.47 (t, *J* = 9.6 Hz, 1 H), 3.42 (dd, *J* = 3.6, 10.8 Hz, 1 H). ^{13}C NMR (150 MHz, D_2O): δ 93.35, 92.60, 72.63, 72.60, 72.35, 70.78, 70.44, 69.75, 69.47, 62.39, 60.46, 60.33. HR ESI MS: calcd. for $C_{12}H_{21}N_3O_{10}Na$ $[M+Na]^+$ *m/z*, 390.112; found, 390.112.

2-*O*-Benzoyl-4,6-*O*-cyclohexylidene-α-D-*ribo*-hexapyranosid-3-ulosyl-(1→1)-2,3;4,6-di-*O*-cyclohexylidene-α-D-glucopyranoside (8)



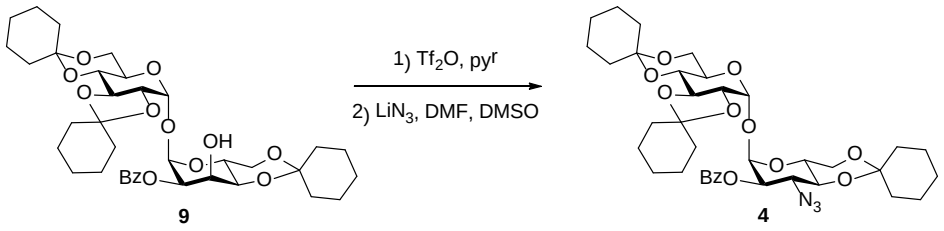
To a stirring solution of oxalyl chloride (0.362 mL, 4.22 mmol) in anhydrous CH_2Cl_2 (25.0 mL) at –78 °C under a nitrogen atmosphere was added anhydrous DMSO (0.450 mL, 6.33 mmol). After 15 min, a freshly prepared solution of reported compound **3**[6] in anhydrous CH_2Cl_2 (5.0 mL of 0.422 M solution, 2.11 mmol) was added dropwise. The reaction was stirred at –78 °C for 30 min, after which Et_3N (1.77 mL, 12.6 mmol) was added and the reaction gradually warmed to rt over 1 h. The reaction mixture was diluted with CH_2Cl_2, washed with H_2O three times, dried over anhydrous Na_2SO_4, and concentrated in vacuum. The crude residue was chromatographed on silica gel (toluene/EtOAc, 10:1) to give **8** (1.16 g, 80%) as a white solid. TLC (toluene/EtOAc, 6:1): R_f = 0.54. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.12 (d, *J* = 7.6 Hz, 2 H), 7.59 (t, *J* = 7.6 Hz, 1 H), 7.48 (t, *J* = 7.6 Hz, 2 H), 5.67 (d, *J* = 4.0 Hz, 1 H), 5.62 (d, *J* = 4.0 Hz, 1 H), 5.42 (d, *J* = 2.8 Hz, 1 H), 4.52 (d, *J* = 10.0 Hz, 1 H), 4.37 (dt, *J* = 5.6, 10.0 Hz, 1 H), 4.13 (t, *J* = 9.2 Hz, 1 H), 4.03-3.93 (m, 3 H), 3.81 (dt, *J* = 4.8, 10.0 Hz, 1 H), 3.73 (t, *J* = 10.4 Hz, 1 H), 3.60 (dd, *J* = 4.8, 10.0 Hz, 1 H), 3.50 (dd, *J* = 2.8, 8.8 Hz, 1 H), 2.15-2.04 (m, 2 H), 1.85-1.25 (m, 28 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 192.77, 165.34, 133.93, 130.25, 128.89, 112.96, 100.74, 99.95, 97.75, 94.91, 76.09, 75.06, 74.50, 73.42, 72.91, 67.31, 66.85, 62.02, 61.38, 38.02, 37.64, 36.42, 35.89, 28.05, 27.73, 25.74, 25.64, 25.21, 24.07, 23.74, 22.86, 22.69. HR ESI MS: calcd. for $C_{37}H_{48}O_{12}Na$ $[M+Na]^+$ *m/z*, 707.304; found, 707.304.

2-*O*-Benzoyl-4,6-*O*-cyclohexylidene-α-D-allopyranosyl-(1→1)-2,3;4,6-di-*O*-cyclohexylidene-α-D-glucopyranoside (9)



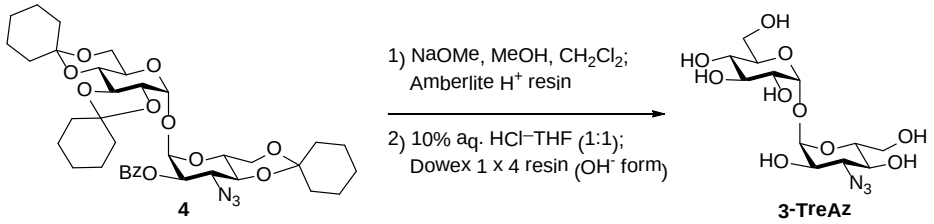
To a solution of **8** (722 mg, 1.05 mmol) stirring in anhydrous CH_2Cl_2 (5.0 mL) at room temperature under a nitrogen atmosphere was added a freshly prepared solution (5.0 mL) of anhydrous NaOAc in MeOH, which had been adjusted to pH 6.5 by addition of acetic acid. The mixture was cooled to –60 °C and $NaBH_4$ (157 mg, 4.20 mmol) was added in portions over 2 h while maintaining cooling (–60 °C) and a pH of approximately 7 by dropwise addition of 20% acetic acid in CH_2Cl_2. After TLC showed completion, the reaction was diluted with EtOAc, washed with brine three times, dried over anhydrous Na_2SO_4, and concentrated in vacuum. The crude residue was chromatographed on silica gel (hexanes/EtOAc, 4:1) to give **9** (533 mg, 74%) as a white solid. TLC (hexanes/EtOAc, 3:1): R_f = 0.46. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.12 (d, *J* = 7.2 Hz, 2 H), 7.59 (t, *J* = 7.6 Hz, 1 H), 7.48 (t, *J* = 7.6 Hz, 2 H), 5.43 (d, *J* = 3.2 Hz, 1 H), 5.39 (d, *J* = 3.6 Hz, 1 H), 5.10 (t, *J* = 3.6 Hz, 1 H), 4.39 (d, *J* = 7.6 Hz, 1 H), 4.26 (dt, *J* = 5.2, 10.0 Hz, 1 H), 4.10 (t, *J* = 9.2 Hz, 1 H), 3.98-3.91 (m, 2 H), 3.80 (t, *J* = 10.8 Hz, 1 H), 3.76 (dd, *J* = 2.4, 10.4 Hz, 1 H), 3.67 (t, *J* = 10.0 Hz, 1 H), 3.65-3.57 (m, 1 H), 3.54-3.45 (m, 2 H), 2.87 (d, *J* = 8.4 Hz, 1 H), 2.06-1.80 (m, 4 H), 1.72-1.27 (m, 26 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.78, 133.80, 130.09, 128.90, 112.86, 99.94, 99.85, 94.31, 93.81, 75.86, 73.89, 72.77, 70.15, 69.80, 68.47, 66.73, 61.75, 61.27, 59.58, 37.94, 37.89, 36.45, 36.10, 28.05, 27.96, 25.79, 25.70, 25.18, 23.99, 23.67, 22.97, 22.80, 22.64. HR ESI MS: calcd. for $C_{37}H_{50}O_{12}Na$ $[M+Na]^+$ *m/z*, 709.319; found, 709.320.

3-Azido-2-*O*-benzoyl-4,6-*O*-cyclohexylidene-3-deoxy-α-D-glucopyranosyl-(1→1)-2,3;4,6-di-*O*-cyclohexylidene-α-D-glucopyranoside (4)



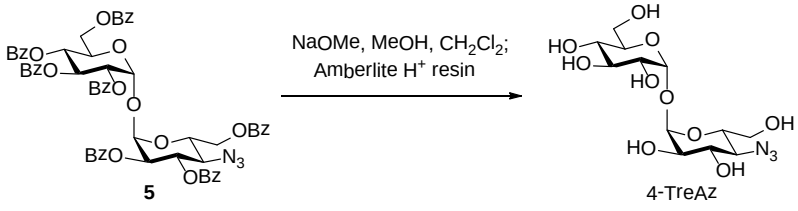
To a solution of **9** (567 mg, 0.826 mmol) stirring in anhydrous pyridine (10.0 mL) at 0 °C under a nitrogen atmosphere was added Tf_2O (0.554 mL, 3.30 mmol) dropwise. The reaction gradually warmed to rt while stirring overnight, after which TLC showed complete conversion to the intermediate triflate [TLC (toluene/EtOAc, 10:1): R_f = 0.54]. The reaction mixture was diluted with EtOAc, washed with brine three times, dried over anhydrous Na_2SO_4, and co-evaporated with toluene three times to remove residual pyridine. The resulting residue was dissolved in anhydrous DMF (20.0 mL) at rt under a nitrogen atmosphere and treated with a freshly prepared solution of LiN_3 [4.20 mL of a 0.99 M solution of LiN_3 in DMF–DMSO (10:1)]. After stirring at rt for 4 h, an additional 2.10 mL of the LiN_3 solution was added and stirring continued for an additional 1 h, leading to complete conversion. The reaction was then diluted with EtOAc, washed with brine three times, dried over anhydrous Na_2SO_4, and co-evaporated with toluene under reduced pressure. The crude residue was chromatographed on silica gel (toluene/EtOAc, 20:1) to give **4** (304 mg, 52% over two steps) as a white solid. Note: Alternatively, compound **3** could also be carried through four steps to compound **4** with a single chromatographic step at the end, yielding 51% over the sequence. TLC (toluene/EtOAc, 10:1): R_f = 0.60. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10 (d, *J* = 7.2 Hz, 2 H), 7.59 (t, *J* = 7.2 Hz, 1 H), 7.48 (t, *J* = 7.2 Hz, 2 H), 5. 38 (d, *J* = 3.6 Hz, 1 H), 5.34 (d, *J* = 2.8 Hz, 1 H), 4.98 (dd, *J* = 3.6, 10.4 Hz, 1 H), 4.16-4.10 (m, 2 H), 4.04 (t, *J* = 10.0 Hz, 1 H), 3.91-3.85 (m, 2 H), 3.79 (t, *J* = 10.8 Hz, 1 H), 3.57-3.47 (m, 3 H), 3.17 (dd, *J* = 3.2, 8.8 Hz, 1 H), 2.27-2.17 (m, 1 H), 2.06-1.97 (m, 1 H), 1.90-1.82 (m, 1 H), 1.71-1.24 (m, 27 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.68, 133.87, 129.97, 128.95, 112.71, 100.48, 99.72, 95.04, 93.99, 76.15, 73.69, 72.84, 72.46, 71.84, 66.57, 64.43, 61.75, 61.13, 60.80, 38.06, 37.85, 36.38, 36.30, 28.05, 27.94, 25.73, 25.68, 25.19, 24.01, 23.75, 22.90, 22.77, 22.62, 22.53. HR ESI MS: calcd. for $C_{37}H_{49}N_3O_{11}Na$ $[M+Na]^+$ *m/z*, 734.326; found, 734.327.

3-Azido-3-deoxy-α,α-D-trehalose (3-TreAz)



To a stirring solution of **4** (278 mg, 0.391 mmol) in anhydrous MeOH-CH_2Cl_2 (4:1, 10.0 mL) at rt under a nitrogen atmosphere was added a freshly prepared solution of NaOMe to a final concentration of 0.05 M. After stirring overnight at rt, the reaction was neutralized to pH 7 with Amberlite H^+ resin, filtered, concentrated in vacuum, and chromatographed on silica gel (hexanes/EtOAc, 3:1) to give the debenzoylated intermediate [200 mg, 84%; TLC (toluene/EtOAc, 10:1): R_f = 0.27], which was taken to the next step. The intermediate (156 mg, 0.257 mmol) was dissolved in THF (5.0 mL), cooled to 0 °C, and treated with 10% aqueous HCl (5.0 mL) by dropwise addition. After warming to rt and stirring for 4 h, the reaction was neutralized to pH 7 with Dowex 1 × 4 (Cl^- form, exchanged to OH^- form). After filtration and concentration under vacuum, the crude residue was chromatographed on silica gel ($CHCl_3$/MeOH, 2:1) to give **3-TreAz** (61 mg, 65%) as a white solid. TLC ($CHCl_3$/MeOH, 1.5:1): R_f = 0.46. ^{1}H NMR (600 MHz, CD_3OD): δ 5.20 (d, J = 4.2 Hz, 1 H), 5.19 (d, J = 4.2 Hz, 1 H), 3.86-3.81 (m, 6 H), 3.78-3.74 (m, 2 H), 3.67 (t, J = 9.6 Hz, 1 H), 3.66 (t, J = 9.6 Hz, 1 H), 3.50 (t, J = 9.6 Hz, 1 H), 3.45 (t, J = 9.6 Hz, 1 H), . ^{13}C NMR (150 MHz, CD_3OD): δ 93.22, 92.63, 72.47, 72.17, 71.95, 70.96, 69.77, 69.61, 68.44, 65.48, 60.45. HR ESI MS: calcd. for $C_{12}H_{21}N_3O_{10}Na$ $[M+Na]^+$ *m/z*, 390.112; found, 390.112.

4-Azido-4-deoxy-α,α-D-trehalose (4-TreAz)



To a stirring solution of known compound **5**[7] (490 mg, 0.458 mmol) in anhydrous MeOH-CH_2Cl_2 (4:1, 10.0 mL) at rt under a nitrogen atmosphere was added a freshly prepared solution

of NaOMe to a final concentration of 0.05 M. After stirring overnight at rt, the reaction was neutralized to pH 7 with Amberlite H^+ resin, filtered, and concentrated in vacuum. The residue was first triturated with hexanes to remove most of the methyl benzoate, and then chromatographed on silica gel ($CHCl_3$/MeOH, 2.5:1) to give **4-TreAz** (136 mg, 81%) as a white solid. TLC ($CHCl_3$/MeOH, 2.5:1): R_f = 0.26. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.21 (d, *J* = 3.6 Hz, 1 H), 5.16 (d, *J* = 3.6 Hz, 1 H), 3.99 (t, *J* = 9.6 Hz, 1 H), 3.86-3.74 (m, 7 H), 3.71 (dd, *J* = 4.2, 10.2 Hz, 1 H), 6.63 (dd, *J* = 3.6, 10.2 Hz, 1 H), 3.49 (t, *J* = 10.2 Hz, 1 H), 3.44 (t, *J* = 9.6 Hz, 1 H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 95.35 (includes both anomeric carbons), 74.63, 73.97, 73.76, 73.42, 73.27, 72.23, 71.89, 63.91, 62.73, 62.42. HR ESI MS: calcd. for $C_{12}H_{21}N_3O_{10}Na$ $[M+Na]^+$ *m/z*, 390.112; found, 390.111.

Metabolic labeling of mycobacteria with TreAz analogues

Preparation of TreAz stock solutions. Stock solutions of TreAz were prepared in 18 MΩ H_2O at concentrations of 1 mM and 25 mM, sterile-filtered (0.2 µm), and stored at –20 °C. Prior to usage in labeling experiments, stock solutions were diluted to the appropriate concentration with culture medium and temporarily stored at 4 °C.

General procedure for labeling mycobacteria. Starter cultures of *Msmeg* wild type or mutants were generated by inoculating a single colony from a freshly streaked plate (selective for antibiotic resistance, if necessary) into 3 mL Middlebrook 7H9 liquid medium supplemented with ADC (albumin, dextrose, and catalase), 0.5% glycerol, and 0.05% Tween-80 (and antibiotic, if necessary) in a culture tube. Starter cultures of *Mtb* wild type and *M. bovis* BCG-Pasteur wild type or mutants were generated by inoculating a 1 mL frozen stock (thawed) into 50 mL Middlebrook 7H9 liquid medium supplemented with OADC (oleic acid, albumin, dextrose, and catalase), 0.5% glycerol, and 0.05% Tween-80 (and antibiotic, if necessary) in a roller bottle. Starter cultures were incubated at 37 °C with shaking or rotation until reaching logarithmic phase and then diluted with 7H9 liquid medium to the desired density for initiating experiments. Most experiments were performed in a flat-bottom 96-well plate, in which bacteria were mixed with 7H9 liquid medium and TreAz solution or vehicle to achieve a final volume of 200 µL. To prevent evaporation, center wells were used and damp rags were placed in the container along with the plate. Plates were incubated at 37 °C with shaking until the desired end-point (for *Msmeg*, typical culture time 12-16 h, OD_{600} ~0.8-1.0; for *Mtb* and *M. bovis* BCG-Pasteur, typical culture time 72 h, with resuspension of cells every 24 h to prevent clumping, OD_{600} ~0.8-1.0). Next, all samples were transferred to a v-bottom 96 well plate, then centrifuged (3500 rpm, 3 minutes, 4 °C) and washed [PBS 1x with 0.5% bovine serum albumin (PBSB), volume 200 µL] three times. Subsequently, cells were incubated with BARAC-Fluor (1:1000 dilution of 1 mM stock solution in DMSO into PBSB; volume 150 µL) for 30 min at rt in the dark. After centrifugation (3500 rpm, 3 minutes, 4 °C) and washing (PBSB, 200 µL) three times, cells were fixed with 200 µL 4% paraformaldehyde in PBS 1x in the dark (for *Msmeg*, 10 min; for *Mtb* and *M. bovis* BCG-Pasteur, 1 h). Final washing with PBS 1x two times readied cells for analysis by flow cytometry or fluorescence microscopy. Alternatively, TreAz-labeled cells could be fixed as

just described and subjected to Cu(I)-catalyzed azide-alkyne cycloaddition for attachment of alkyne-functionalized Alexa Fluor 488 (alk-AF488, Invitrogen) as described previously.[8]

Flow cytometry. After fluorescent labeling of mycobacteria according to the above general procedure, cells were triturated through a hypodermic needle (23.5 gauge) three times to de-clump and diluted to 400 µL with PBS 1x. Flow cytometry was performed on a BD Biosciences FACSCalibur flow cytometer equipped with a 488-nm argon laser. Fluorescence data was collected for 100,000 cells for each sample and processed using FlowJo (Tree Star). All flow cytometry experiments were performed with three replicate samples, and data shown was representative of at least two independent experiments.

Fluorescence microscopy. After fluorescent labeling of mycobacteria according to the above general procedure, cells were spotted onto slides and air-dried. Vectashield without DAPI stain (8 µL, Vector Laboratories) mounting medium and was added, then cover slips were placed over the sample and immobilized with adhesive. Microscopy was performed on a Zeiss Axiovert 200 M inverted microscope equipped with a 100 × 1.3 numerical aperture Plan-Apochromat oil immersion lens and the channels imaged were bright-field differential interference contrast (DIC) and FITC (max. exciter at 487 nm, max. emitter at 535 nm). A 175-W xenon lamp housed in a Sutter DG4 illuminator linked to the microscope by an optical fiber assured shuttering and illumination. Images were acquired by using a CoolSnap HQ camera (Roper Scientific). Slidebook software (Intelligent Imaging Solutions) was used to control the microscope and the camera. All image acquisition and processing was performed under identical conditions for test and control samples.

Lipid analysis by TLC and mass spectrometry. *Msmeg* wild type was cultured in the presence or absence of TreAz (2-TreAz, 25 µM, 50 mL; 3-TreAz, 1000 µM, 25 mL; 4-TreAz, 250 µM, 25 mL; 6-TreAz, 25 µM, 50 mL) at the indicated volume in Tween-free 7H9 liquid medium containing ADC and 0.5% glycerol for 16 h to logarithmic phase. Cells were centrifuged (3500*g*, 10 min, 4 °C) and washed (dd-H_2O, 5 mL) three times, then extracted with $CHCl_3$/MeOH (2:1, 4.5 mL) in glass scintillation vials overnight at rt with shaking. Vials were centrifuged gently (1500*g*, 10 min) to remove debris, and the supernatants were collected and dried under a stream of nitrogen.

Solvent fractionation of samples was performed to yield acetone-insoluble, THF-soluble lipids[9] that were analyzed by TLC (eluent $CHCl_3$/MeOH/acetone, 90:15:10; stained for sugar with 5% H_2SO_4 in EtOH). For TLC standards, TDM from *Mtb* was purchased (Sigma Aldrich) and TMM was synthesized by partial saponification of the TDM standard with 0.05 M NaOMe/MeOH. Solvent-fractionated lipid extracts required additional enrichment for ESI-FT-ICR MS analysis, so samples were further purified by silica gel column chromatography. A silica gel-loaded Pasteur pipet was used as a micro-column, and lipids were separated using gradient elution ($CHCl_3$/MeOH/acetone, 90:10:10 → 90:15:10) into three fractions including TMM, TDM, and in-between regions (F1–F3, see Figure S4) that were analyzed by ESI-FT-ICR MS directly. AzTMM-containing fractions (as judged by MS) were split into two aliquots, one left untreated and one treated with 500 µM BARAC-methyl[10] in $CHCl_3$/MeOH (2:1) overnight at rt and analyzed by ESI-FT-ICR MS directly.

Mass spectra were obtained on an Apex II FT-ICR MS (Bruker Daltonics) equipped with a 7 T actively shielded superconducting magnet. Samples were introduced into the ion source via direct injection at a rate of 2 µL/min. Ions were generated with an Apollo pneumatically assisted electrospray ionization source (Bruker Daltonics) operating in the negative ion mode and were accumulated in an rf-only external hexapole for 0.5–2 s before being transferred to the ICR cell for mass analysis. Mass spectra consist of 256,000 to 1 million data points and are an average of 24–32 scans. The spectra were acquired using XMASS version 6.0.0 or 7.0.8 (Bruker Daltonics). For accurate mass measurements, spectra were internally calibrated with known compounds. DataAnalysis 3.4 (Bruker Daltonics) was used for data processing.

Analysis of TreAz incorporation into glycolipids by HPAEC-PAD. *Msmeg* wild type was cultured in the presence or absence of TreAz (2-TreAz, 25 µM; 6-TreAz, 25 µM) at the indicated concentration in 100 mL 7H9 liquid medium containing ADC and 0.5% glycerol for 16 h to logarithmic phase. Cells were centrifuged (3500*g*, 10 min, 4 °C) and washed (PBS 1x, 25 mL) three times. As previously described,[11] surface-exposed lipids were extracted from cells in MeOH–0.3% aqueous NaCl (10:1, 10 mL) with petroleum ether (5 mL) three times. The petroleum ether layers were combined and centrifuged (1500*g*, 15 min, 4 °C) to pellet debris, and the clarified organic phase was analyzed by TLC to confirm that trehalose glycolipids TMM and TDM were present. Next, the extracts were split into two equal aliquots and concentrated under a stream of nitrogen. One aliquot was left untreated, and the other was resuspended in anhydrous

dichloromethane (0.2 mL), treated with NaOMe/MeOH (2.0 M, 0.2 mL), and stirred vigorously for 16 h at 60 °C in a sealed vial. The reaction was neutralized with Amberlite H^+ resin to pH 7, filtered, and dried under a stream of nitrogen. The NaOMe-treated and untreated control samples were subsequently processed in parallel: material was resuspended in chloroform (1.0 mL) and extracted three times with 18 MΩ H_2O (0.5 mL). The combined aqueous layers were filtered (0.45 µm), dried in a speed-vac, resuspended in 300 µL 18 MΩ H_2O and analyzed by HPAEC-PAD

HPAEC-PAD was performed on a Dionex ICS3000 system with a CarboPac MA-1 analytical column (4 mm x 250 mm) and MA-1 guard column (4 mm x 50 mm) kept at 30 °C. Pulsed amperometry with standard quadrupole waveform was used for detection. The system was equipped with an autosampler kept at 10 °C that was set up to inject 10 µL sample volumes. Isocratic elution was performed using H_2O (76%) and 2 M NaOH (24%) for a final eluent concentration of 480 mM NaOH at a flow rate of 0.4 mL/min. For standards (prepared at 10 µg/mL), trehalose was purchased (Sigma Aldrich) and TreAz analogues were synthesized as described above. Chromeleon 7 software (Dionex) was used for data processing.

TreAz uptake analysis by HPAEC-PAD. *Msmeg* wild type or Δ*sugC* mutant were cultured in the presence or absence of TreAz (2-TreAz, 25 µM; 3-TreAz, 500 µM; 4-TreAz, 250 µM; 6-TreAz, 25 µM) at the indicated concentration in 2.0 mL 7H9 liquid medium containing ADC, 0.5% glycerol, and 0.05% Tween-80 for 16 h, at which point culture densities as measured by absorbance (OD_{600}) were all between 1.1-1.3. Cells were centrifuged (3500 rpm, 3 min) and washed (PBSB, 1.0 mL) three times, then resuspended in 2.0 mL 70% EtOH and lysed by mechanical disruption using 0.1 mm zirconia/silica beads (BioSpec Products) on a FastPrep (MP Biomedicals) bead-beating instrument. Supernatants were cleared by centrifugation (16,000*g*, 10 min, 4°C), dried in a speed-vac, and resuspended in 500 µL 18 MΩ H_2O. Small molecule metabolites were collected via filtration through a spin concentrator with a 10-kDa molecular weight cutoff (Amicon). HPAEC-PAD was performed as described above.

Generation of mycobacterial mutants

Table S1. Mutants of *Msmeg* mc^{2}155 and *M. bovis* BCG-Pasteur used in this study.

***Msmeg* mc^{2}155**	**MSMEG locus name**	**Reference**
Δ*treS*	MSMEG_6515	[4]
Δ*lpqY*	MSMEG_5061	[12]
Δ*sugC*	MSMEG_5058	[12]
ΔMSMEG_4535	MSMEG_4535	this study
***M. bovis* BCG**	**Rv gene number[a]**	
Δ*treS*	Rv0126	this study
Δ*lpqY-sugC*	Rv1235-Rv1238	this study

[a]Rv gene number refers to the corresponding gene in the *Mtb* H37Rv genome.

Generation of targeted gene deletion mutants. Targeted gene deletion mutants in mycobacteria were generated by specialized transduction employing temperature-sensitive mycobacteriophages essentially as described previously.[13] Briefly, for generation of allelic exchange constructs for gene replacement in *Msmeg* and *M. bovis* BCG-Pasteur with a γδ*res-sacB-hyg-*γδ*res* cassette comprising a *sacB* as well as a hygromycin resistance gene flanked by *res*-sites of the γδ-resolvase, upstream- and downstream-flanking DNA regions were amplified by PCR employing the oligonucleotides listed in Table S2. Subsequently, the upstream and downstream flanks were digested with the indicated restriction enzymes, and ligated with *Van*91I-digested p0004S vector arms (T. Hsu and W. R. Jacobs, Jr., unpublished results). The resulting knock-out plasmids were then linearized with *Pac*I and cloned and packaged into the temperature-sensitive phage phAE159 (J. Kriakov and W. R. Jacobs, Jr., unpublished results), yielding knock-out phages which were propagated in *Msmeg* at 30°C. Allelic exchange in *Msmeg* and *M. bovis* BCG-Pasteur using the knock-out phages was achieved by specialized transduction using hygromycin (50 mg/l) for selection, resulting in gene deletion and replacement by the γδ*res-sacB-hyg-*γδ*res* cassette. The obtained mutants were verified by

Southern analysis of digested genomic DNA using appropriate restriction enzymes and probes as shown in Figures S6, S8, and S10.

Genetic complementation of *M. bovis* BCG-Pasteur mutant strains. Mutant strains were complemented by heterologous expression of the corresponding genes from *Mtb* H37Rv. The *lpqY-sugA-sugB-sugC* operon comprising an additional 884-bp upstream fragment containing the putative native promoter region was amplified from genomic DNA by PCR using the oligonucleotide pair 5'-TTTTTTAAGCTTGCTGGTAAGCAGGCGGGTAAC-3' and 5'-TTTTTTATCGATTAGGCGCGGTGCGGAATGGTCAG-3' and cloned using the restriction enzymes *Hin*dIII and *Cla*I (underlined) into the promoter-less single-copy integrative plasmid pMV306(Kan),[14] resulting in plasmid pMV306::Rv1235-Rv1238. The *treS* gene was PCR amplified using the oligonucleotide pair TTTTTTCAGCTGCAATGAACGAGGCAGAACACAGCGTC-3' and 5'-TTTTTTAAGCTTCATAGGCGCCGCTCTCCCCCGC-3' and cloned using the restriction enzymes *Pvu*II and *Hin*dIII (underlined) into the single-copy integrative plasmid pMV361(Kan) providing constitutive gene expression from the HSP60 promoter,[14] resulting in plasmid pMV361::Rv0126. The plasmids were transformed by electroporation into the *M. bovis* BCG-Pasteur Δ*lpqY-sugC* or Δ*treS* mutant, respectively.

Table S2. Oligonucleotides used for generation of allelic exchange substrates.

	Upstream flanking sequence			Downstream flanking sequence		
***Msmeg* mc^{2}155**						
	5` primer	**3` primer**	**Restriction site**	**5` primer**	**3` primer**	**Restriction site**
MSMEG_4535	5´ TTTTTG-CATAAA-TTGCCAG-AGTTGAA-CCGTCAC-AGCGGA-AG 3´	5´ TTTTTG-CATTTCT-TGCTGAT-CAGGCA-CGTGGTC-TCGCAGT-C 3´	*Bst*API	5´ TTTTTCCAT-AGATTGGGGT-AGGCATCTGG GCAACTTCC 3´	5´ TTTTTCCAT-CTTTTGGAGG-CCGCCATCTG-GTTCTTCAC 3´	*Van*91I
***M. bovis* BCG-Pasteur**						
	Upstream flanking sequence			**Downstream flanking sequence**		
	5` primer	**3` primer**	**Restriction site**	**5` primer**	**3` primer**	**Restriction site**
Δ*lpqY-sugC*	5` TTTTTTTT-GCATAA-ATTGCGC-GGGTTGA-GTGGATA-GTGTG 3`	5` TTTTTTTT-GCATTTC-TTGCAA-CTGTTGG-TTGCAGC-GTTGG 3`	*Bst*API	5` TTTTTTTTCCAT AGATTGGTC-GGTCGAGTTGG CTTTCGATAC 3`	5` TTTTTTTTCCA TCTTTTGGTT-CGTCGGCAAG GATTTCGTG 3`	*Van*91I
Δ*treS*	5' TTTTTGC-ATAAATT-GCCCACC-GACATCA-ATGCGTT-CAG 3'	5' TTTTTGC-ATTTCTT-GCCACAC-CGCCTTC-GACGTG-ACT 3'	*Bst*API	5' TTTTTCCATAG-ATTGGCGCTG-CCAGGACAC-GGGTTCTACT 3'	5' TTTTTCCATCT-TTTGGCACAG-ACGGCGGACG-AGACGATTA 3'	*Van*91I

IV. References

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