Chemistry & Biology, Volume *20*

Supplemental Information

Flux through Trehalose Synthase Flows from Trehalose to the Alpha Anomer of Maltose in Mycobacteria

Farzana Miah, Hendrik Koliwer-Brandl, Martin Rejzek, Robert A. Field, Rainer Kalscheuer, and Stephen Bornemann

Inventory of Supplemental Information

Figure S1, related to Figure 2. Generation of *M. smegmatis* Gene Deletion Mutants Defective in Trehalose Biosynthesis Pathways

Figure S2, related to Figure 3. NMR Spectroscopy of Enzymic and Non-Enzymatic Reactions With Trehalose and Maltose

Figure S3, related to Figure 4. NMR Spectroscopy of Enzymic Reactions With Deoxyfluoromaltose

Supplemental Experimental Procedures

Supplemental References

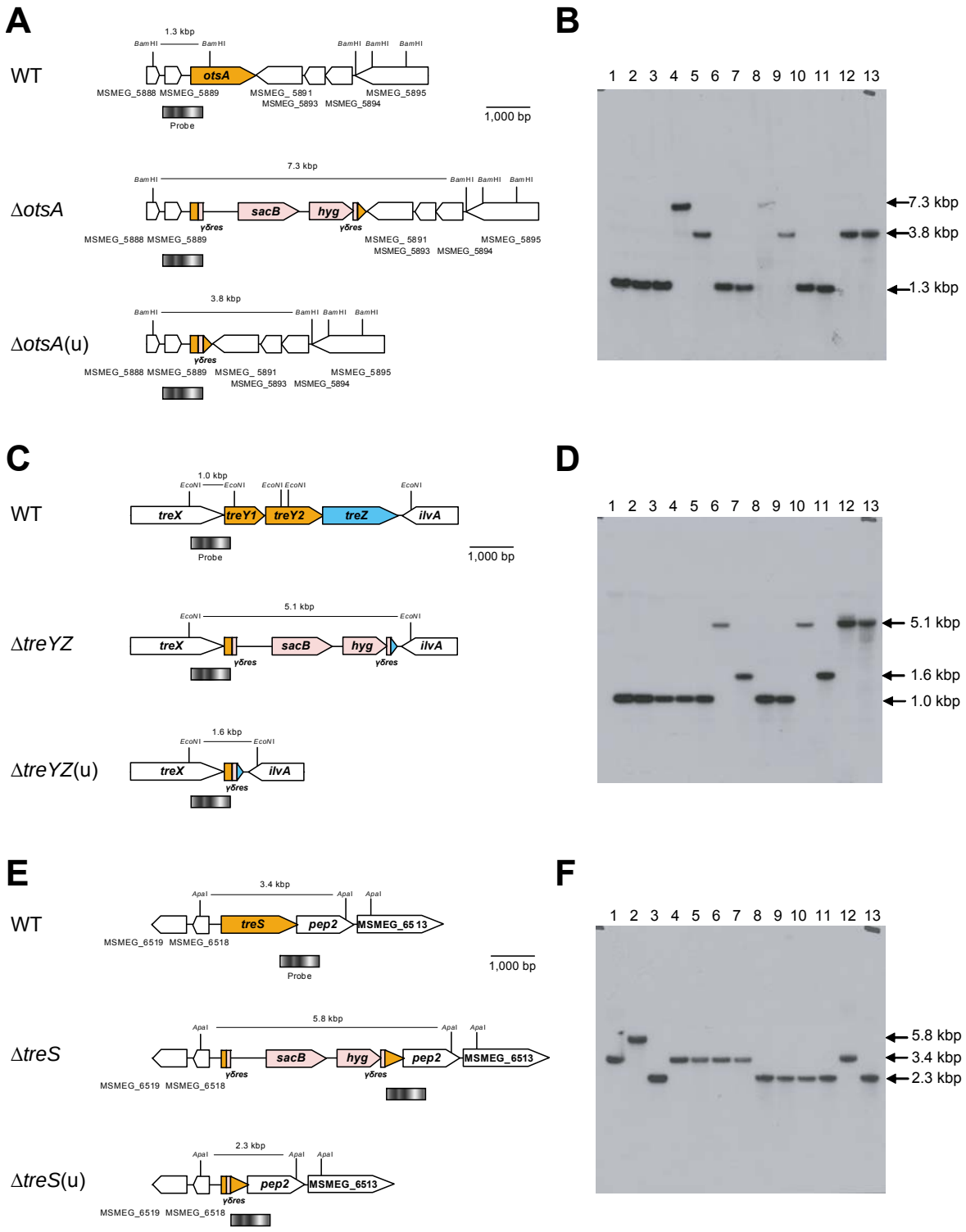

Figure S1, related to Figure 2. Generation of *M. smegmatis* Gene Deletion Mutants Defective in Trehalose Biosynthesis Pathways

(A) Organization of the *otsA* locus in *M. smegmatis* wild-type as well as in marked and unmarked gene deletion mutants. The sizes of relevant fragments as well as the location of the probes used for Southern analyses are indicated. WT, wild-type; (u), unmarked locus; γδ*res*, *res*-sites of the γδ-resolvase; *hyg*, hygromycin resistance gene. See Fig. 2 for phenotyping.

(B) Southern analysis of *Bam*HI-digested genomic DNA using a probe hybridizing to the position indicated in (A), showing *otsA* gene deletion in relevant marked and unmarked mutant strains. Strain designations: 1, wild-type; 2, Δ*treS*; 3, Δ*treS*(u); 4, Δ*otsA*; 5, Δ*otsA*(u); 6, Δ*treYZ*; 7, Δ*treYZ*(u); 8, Δ*treS*(u)Δ*otsA*; 9, Δ*treS*(u)Δ*otsA*(u); 10, Δ*treS*(u)Δ*treYZ*; 11, Δ*treS*(u)Δ*treYZ*(u); 12, Δ*otsA*(u)Δ*treYZ*; 13, Δ*treS*(u)Δ*otsA*(u)Δ*treYZ*.

(C) Organization of the *treXYZ* locus in *M. smegmatis* wild-type as well as in marked and unmarked gene deletion mutants. See (A) for more details. Please note that the *treY* gene in *M. smegmatis* strain mc^2155 is split into two open reading frames *treY1* and *treY2* by a frame shift mutation.

(D) Southern analysis of *Eco*NI-digested genomic DNA using a probe hybridizing to the position indicated in (C), showing *treY1-treY2-treZ* gene deletion in relevant marked and unmarked mutant strains. Strain designations are as in (B).

(E) Organization of the *treS* locus in *M. smegmatis* wild-type as well as in marked and unmarked gene deletion mutants. See (A) for more details.

(F) Southern analysis of *Apa*I-digested genomic DNA using a probe hybridizing to the position indicated in (E), showing *treS* gene deletion in relevant marked and unmarked mutant strains. Strain designations are as in (B).

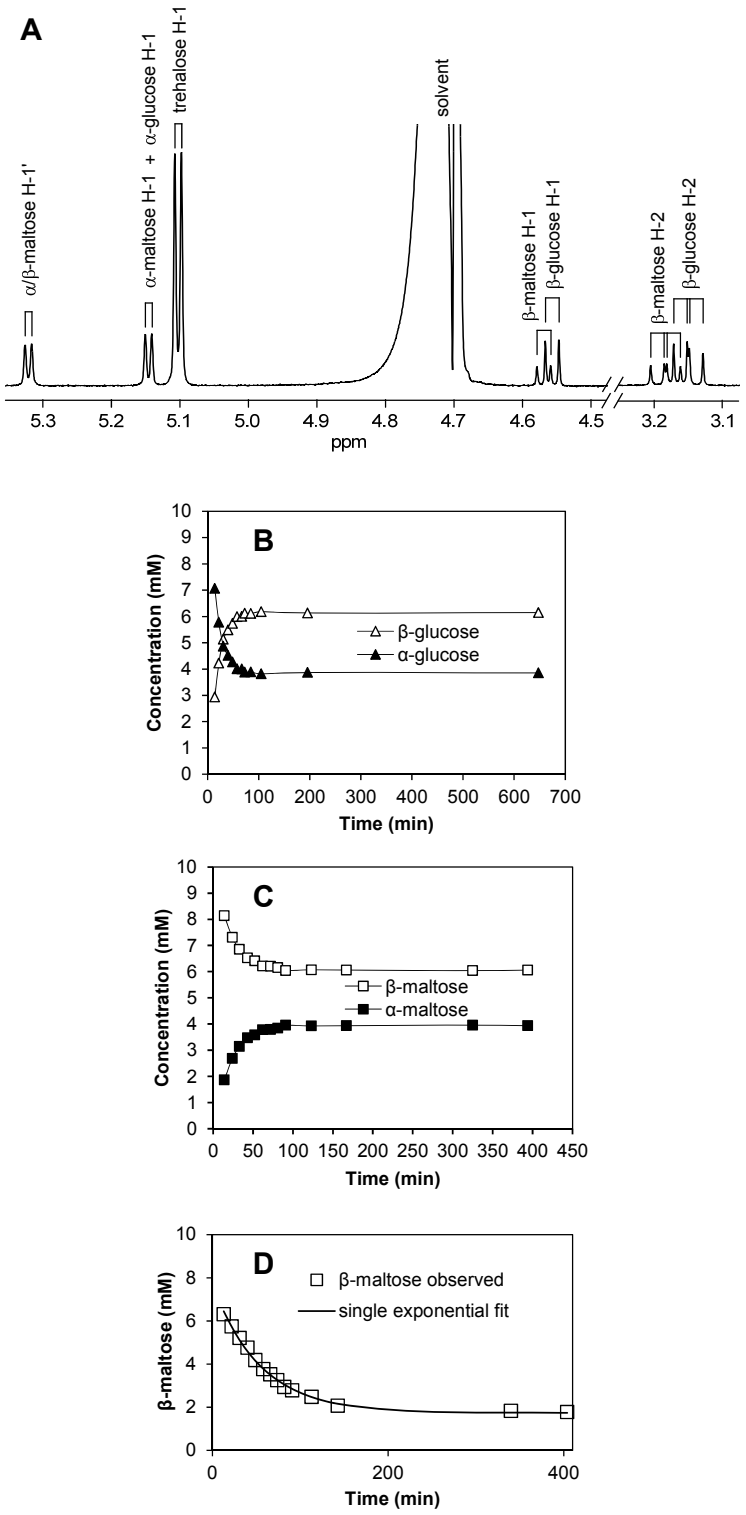

Figure S2, related to Figure 3. NMR Spectroscopy of Enzymic and Non-Enzymatic Reactions With Trehalose and Maltose

(A) Representative ^{1}H-NMR spectrum of the products generated from maltose with TreS allowing their quantification. The spectrum was recorded 919 min after the addition of TreS (2 μM) to trehalose (10 mM). Only the regions of the spectrum used to quantify each reaction component are shown for clarity. See Supplemental Experimental Procedures and Fig. 3D for assignments.

(B) Time course of the non-enzymatic mutarotation of glucose. The concentrations of each anomer of glucose were determined as a function of time after the dissolution of solid glucose (giving 10 mM in 90 mM citrate buffer, pH 6.7, containing 10% (v/v) D_2O at 25 °C) using ^{1}H-NMR spectroscopy. Fitting of the data gave rate constants of 0.0316 ± 0.0004 and 0.0192 ± 0.0004 min^{-1} for the mutarotation of the α to the β anomer and the reverse reaction, respectively. These values were consistent with not only the literature (0.027 min^{-1} in 5 mM EDTA, pH 7.4, at 25 °C (Bailey et al., 1967) and 0.018 min^{-1} in water at 21 °C (Stults et al., 1987), respectively) but also the expected dominance of the β anomer.

(C) Time course of the non-enzymatic mutarotation of maltose. The concentrations of each anomer of maltose were determined as a function of time after the dissolution of solid maltose (giving 10 mM in 90 mM citrate buffer, pH 6.7, containing 10% (v/v) D_2O at 25 °C) using ^{1}H-NMR spectroscopy. Fitting of the data gave rate constants of 0.0286 ± 0.0002 and 0.0187 ± 0.0001 min^{-1} for the mutarotation of the α to the β anomer and the reverse reaction, respectively, consistent with the literature (0.017 min^{-1} in 5 mM EDTA, pH 7.4, at 25 °C (Bailey et al., 1967) for the reverse reaction) and the expected dominance of the β anomer.

(D) Fitting of the time course of the depletion of β-maltose during a reaction with α/β-maltose and TreS. The conversion of α/β-maltose (10 mM) by TreS (2 μM) was monitored using ^{1}H-NMR spectroscopy (see Fig. 3B). Fitting of the data for β-maltose (open squares) to a single exponential (solid line) gave a rate of 0.019 min^{-1}. This was indistinguishable from the rate constant determined for the mutarotation of β-maltose to α-maltose (0.0187 ± 0.0001 min^{-1} shown above).

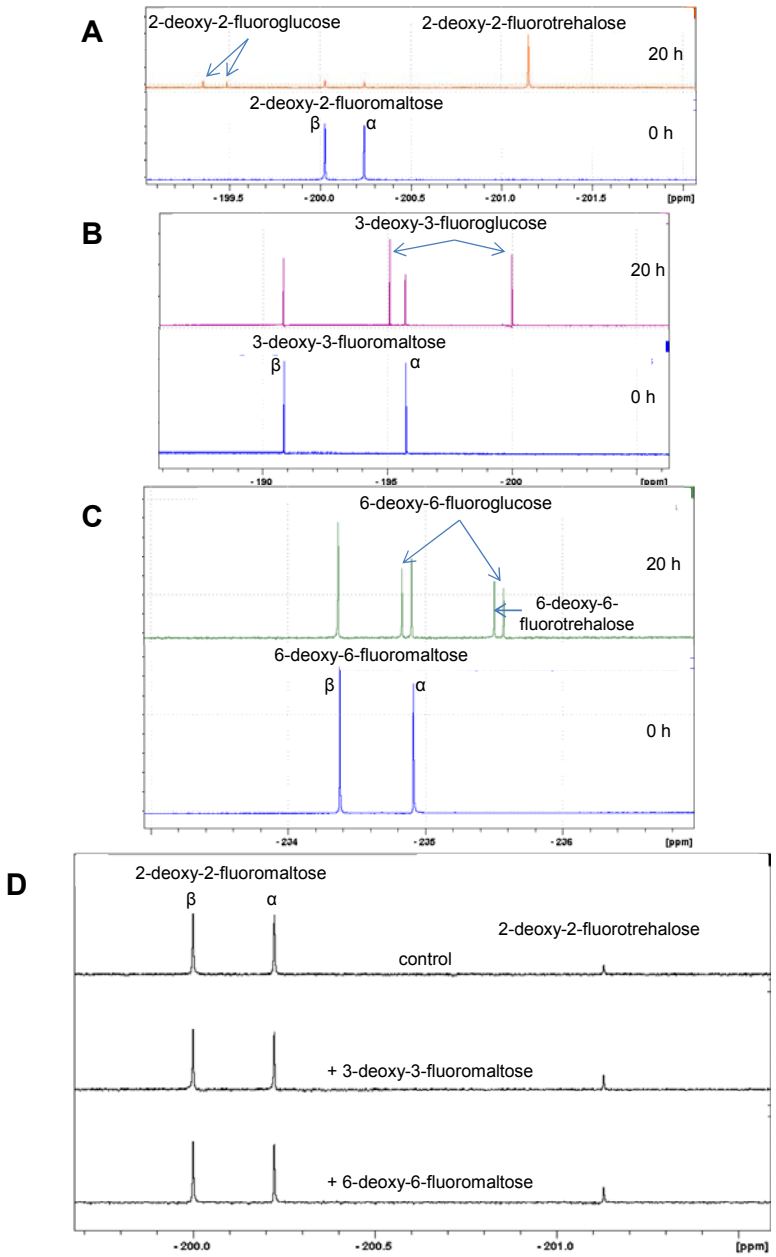

Figure S3, related to Figure 4. NMR Spectroscopy of Enzymic Reactions With Deoxyfluoromaltose

(A) ^{19}F-NMR spectra of products generated by TreS from 2-deoxy-2-fluoromaltose. The production of 2-deoxy-2-fluorotrehalose was observed with small quantities of hydrolysis products being detected at long incubation times. See Supplemental Experimental Procedures for assignments.

(B) ^{19}F-NMR spectra of products generated by TreS from 3-deoxy-3-fluoromaltose. The rate of reaction was slow with only hydrolysis products detected at long times.

(C) ^{19}F-NMR spectra of products generated by TreS from 6-deoxy-6-fluoromaltose.

The rate of reaction was very low with 6-deoxy-6-fluorotrehalose and hydrolysis products being detected at long times.

(D) Lack of inhibition of the TreS-catalysed conversion of 2-deoxy-2-fluoromaltose to trehalose by either 3-deoxy-3-fluoro or 6-deoxy-6-fluoromaltose according to ^{19}F-NMR spectroscopy. TreS (0.2 μM) was incubated with 2-deoxy-2-fluoromaltose (0.52 mM) for 30 min with or without pre-incubation with either 3-deoxy-3-fluoromaltose (2.1 mM) or 6-deoxy-6-fluoromaltose (2.1 mM). A relatively short incubation time was used to allow the estimation and comparison of the initial rates of the reactions.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Strains and Growth Conditions

All strains were derived from *M. smegmatis* mc^{2}155 and *M. tuberculosis* H37Rv and are listed below. Cells were grown aerobically at 37 °C in Middlebrook 7H9 media supplemented with 0.5% (*v/v*) glycerol and 0.05% (*v/v*) Tyloxapol and containing either 10% (*v/v*) ADS enrichment (5% *w/v* bovine serum albumin fraction V, 2% *w/v* glucose and 0.85% *w/v* sodium chloride) or OADC enrichment (Becton Dickinson Microbiology Systems, Spark, MD) for cultivation of *M. smegmatis* or *M. tuberculosis*, respectively. Hygromycin (50 mg l^{-1}) and kanamycin (20 mg l^{-1}) were added for selection for appropriate strains.

Generation of Site-Specific Gene Deletion Mutants

Site-specific gene deletion mutants in *M. smegmatis* mc^{2}155 were generated by specialized transduction employing temperature-sensitive mycobacteriophages essentially as described previously (Bardarov et al., 2002). Briefly, for generation of allelic exchange constructs for gene replacement 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 below. 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, 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 *M. smegmatis* at 30 °C. Allelic exchange in *M. smegmatis* using the knock-out phages was achieved by specialized transduction using hygromycin (50 mg l^{-1}) for selection, resulting in gene deletion and replacement by the γδ*res*-*sacB*-*hyg*-γδ*res* cassette. For generation of unmarked mutants, the γδ*res*-*sacB*-*hyg*-γδ*res* cassette was removed employing specialized transduction using the phage phAE7-1 expressing the γδ-resolvase (J. Kriakov and W.R. Jacobs, Jr., unpublished results) using sucrose (3% *w/v*) for counterselection. The obtained mutants were verified by Southern analysis of digested genomic DNA using appropriate restriction enzymes and probes as shown in Fig. S1.

Site-specific gene deletion mutants of *M. smegmatis* mc^{2}155 and *M. tuberculosis* H37Rv used in this study. (u), unmarked locus.

M. smegmatis	**MSMEG locus name**	**Relevant genotype**	**Relevant phenotype**	**Reference**
Δ*treS*	MSMEG_6515	Δ*treS*::γδ*res-sacB-hyg*-γδ*res*	trehalose prototroph	(Kalscheuer et al., 2010)
Δ*treS*(u)	MSMEG_6515	Δ*treS*::γδ*res*	trehalose prototroph	this study
Δ*otsA*	MSMEG_5892	Δ*otsA*::γδ*res-sacB-hyg*-γδ*res*	trehalose prototroph	this study
Δ*otsA*(u)	MSMEG_5892	Δ*otsA*::γδ*res*	trehalose prototroph	this study
Δ*treYZ*	MSMEG_3184-3186	Δ*treYZ*::γδ*res-sacB-hyg*-γδ*res*	trehalose prototroph	this study
Δ*treS*(u)Δ*otsA*		Δ*treS*::γδ*res* Δ*otsA*::γδ*res-sacB-hyg*-γδ*res*	trehalose prototroph	this study
Δ*treS*(u)Δ*otsA*(u)		Δ*treS*::γδ*res* Δ*otsA*::γδ*res*	trehalose prototroph	this study
Δ*treS*(u)Δ*treYZ*		Δ*treS*::γδ*res* Δ*treYZ*::γδ*res-sacB-hyg*-γδ*res*	trehalose prototroph	this study
Δ*otsA*(u)Δ*treYZ*		Δ*otsA*::γδ*res* Δ*treYZ*::γδ*res-sacB-hyg*-γδ*res*	trehalose auxotroph	this study
Δ*treS*(u)Δ*otsA*(u)Δ*treYZ*		Δ*treS*::γδ*res* Δ*otsA*::γδ*res* Δ*treYZ*::γδ*res-sacB-hyg*-γδ*res*	trehalose auxotroph	this study
M. tuberculosis	**Rv locus name**	**Relevant genotype**	**Relevant phenotype**	**Reference**
Δ*treS*	Rv0126	Δ*treS*::γδ*res-sacB-hyg*-γδ*res*	trehalose prototroph	(Kalscheuer et al., 2010)

Oligonucleotides used for generation of allelic exchange substrates.

Locus	**Upstream flanking sequence**			**Downstream flanking sequence**		
	5` primer	**3` primer**	**Restriction site**	**5` primer**	**3` primer**	**Restriction site**
otsA	5´ TTTTTCAGAAACTGTCCC-GACGAAACAGTCAGACAAC	5´ TTTTTCAGTTCCTGACCTTCG-TAGTACTTCGCGACATC	*Alw*NI	5´ TTTTTCCATAGATTGGCGGTC-AACCAGAACCCAGAAGAG	5´ TTTTTCCATCTTTTGGTGGAAA-TCGCAACCGGCAAAG	*Van*91I
treYZ	5´ TTTTTCCATAAATTGGGTT-CACCCTCAACGATCTGGTCTC	5´ TTTTTCCATTTCTTGGTGGGA-TCGGTGACGTCGTAAC	*Van*91I	5´ TTTTTCCATAGATTGGAACCG-GTCACCATAGGCATCTC	5´ TTTTTCCATCTTTTGGTTGGAC-ACCGAACCCGATCTG	*Van*91I

Overexpression of *treS* in *M. tuberculosis* H37Rv

The *treS* gene was amplified from genomic DNA of *M. tuberculosis* H37Rv by PCR using the oligonucleotide pair 5'-TTTTTTCAGCTGCAATGAACGAGGCAGAACACAGCGTC-3' and 5'-TTTTTTAAGCTTCATAGGCGCCGCTCTCCCCCGC-3' and cloned using the underlined restriction sites *Pvu*II and *Hin*dIII into the integrative single-copy plasmid pMV361^{Kan} providing constitutive high-level gene expression in mycobacteria (Stover et al., 1991). The resulting plasmid pMV361::*treS* was transformed into *M. tuberculosis* H37Rv by electroporation.

Trehalose Quantification

Cells of *M. tuberculosis* were cultivated for 14 days, harvested and washed twice with phosphate-buffered saline containing 0.05% (*v/v*) tyloxapol. Washed cells were subsequently extracted with hot water (95 °C) for 4 h. Extract (25 µl) was mixed with 15 µl acetic acid (1 M), 60 µl sodium acetate (0.2 M, pH 5.2) and 5 µl trehalase solution (3.7 U ml^{-1}; Sigma, St. Louis, MO) and incubated at 37 °C for 15 h. For quantification of the liberated glucose, 10 µl of this trehalase-treated extract was mixed with 100 µl glucose oxidase/peroxidase/*o*-dianisidine solution (Sigma, St. Louis, MO) and incubated at 37 °C for 30 min. Reactions were stopped by addition of 75 µl sulfuric acid (6 M), and absorbance was measured at 531 nm in a VICTOR^3 V Multilabel Counter model 1420 (PerkinElmer, Waltham, MA). Controls without trehalase addition were carried out to correct for the presence of free glucose in the extracts. Trehalose concentrations were estimated using a standard curve.

Testing of Trehalose and Maltose Growth Requirements

Precultures grown in the presence of 50 µM trehalose were washed and resuspended in phosphate-buffered saline containing 0.05% (*v/v*) tyloxapol and incubated for 24 h to deplete for endogenous trehalose. Test cultures containing increasing trehalose concentrations (0-50 µM; 1 ml per well in 24-well microtiter plates) were inoculated at a density of 10^6 CFU ml^{-1} from starved precultures and incubated with shaking for 48 h. Growth was determined by measuring optical densities of cultures at 600 nm. For testing growth supplementation of trehalose-auxotrophic *M. smegmatis* mutants by maltose, culture conditions were essentially as described above except that test cultures contained 0-10 mM maltose but no trehalose.

Expression and Purification of TreS

The DNA sequence of the *Mycobacterium tuberculosis* H37Rv *treS* gene synthesised with optimum codon usage for expression in *Escherichia coli* (Genscript) is shown 5′-3′ with the start codon in bold and the first stop codon underlined. It is flanked at the 5′ end by an NdeI restriction site and a sequence encoding a His_6 tag and a TEV protease cleavage site, and at the 3′ end by a BamHI restriction site.

CAT**ATG**CATCATCATCATCATCATGAAAACCTGTATTTTCAGGGCAACGAAGCGGAACAT
AGCGTGGAACATCCGCCGGTGCAGGGCAGCCATGTGGAAGGCGGCGTGGTGGAACATCCG
GATGCGAAAGATTTTGGCAGCGCGGCGGCGCTGCCGGCAGATCCAACCTGGTTTAAACAT
GCGGTGTTTTATGAAGTGCTGGTGCGTGCATTTTTCGATGCGAGCGCGGATGGCAGCGGC
GATCTGCGTGGCCTGATTGATCGTCTGGATTATCTGCAGTGGCTGGGCATTGATTGCATT
TGGCTGCCGCCGTTTTATGATAGCCCGCTGCGTGATGGTGGATATGATATTCGTGATTTT
TATAAAGTGCTGCCGGAATTTGGCACCGTGGATGATTTTGTGGCGCTGGTGGATGCGGCG
CATCGTCGTGGCATTCGTATTATTACCGATCTGGTGATGAACCATACCAGCGAAAGCCAT
CCGTGGTTTCAGGAAAGCCGTCGTGATCCGGATGGCCCGTATGGCGATTATTATGTGTGG
AGCGATACCAGCGAACGTTATACCGATGCGCGTATTATTTTTGTGGATACCGAAGAAAGC
AACTGGAGCTTTGATCCGGTGCGTCGTCAGTTTTATTGGCATCGTTTCTTTAGCCATCAG
CCGGATCTGAACTATGATAACCCGGCGGTGCAGGAAGCGATGATTGATGTGATTCGTTTT
TGGCTGGGCCTGGGCATTGATGGCTTTCGTCTGGATGCGGTGCCGTATCTGTTTGAACGT
GAAGGCACCAACTGCGAAAACCTGCCGGAAACCCATGCGTTTCTGAAACGTGTGCGTAAA
GTGGTGGATGATGAATTTCCGGGCCGTGTGCTGCTGGCGGAAGCGAACCAGTGGCCGGGC
GATGTGGTGGAATATTTTGGCGATCCGAACACCGGCGGCGATGAATGCCACATGGCGTTT
CATTTTCCGCTGATGCCGCGTATTTTTATGGCGGTGCGTCGTGAAAGCCGTTTTCCGATT
AGCGAAATTATTGCGCAGACCCCGCCGATTCCGGATATGGCGCAGTGGGGCATTTTTCTG
CGTAACCATGATGAACTGACCCTGGAAATGGTGACCGATGAAGAACGTGATTATATGTAT
GCGGAATATGCGAAAGATCCGCGTATGAAAGCGAACGTGGGTATCCGTCGAAGACTGGCG
CCGCTGCTGGATAACGATCGTAACCAGATTGAACTGTTTACCGCGCTGCTGCTGAGCCTG
CCGGGCAGCCCGGTGCTGTATTATGGCGATGAAATTGGCATGGGCGATGTGATTTGGCTG
GGCGATCGTGATGGCGTGCGTATTCCGATGCAGTGGACCCCGGATCGTAACGCGGGCTTT
AGCACCGCGAACCCGGGCCGTCTGTATCTGCCGCCGAGCCAGGACCCAGTGTATGGCTAT
CAGGCGGTGAACGTGGAAGCGCAGCGTGATACCAGCACCAGCCTGCTGAACTTTACCCGT
ACCATGCTGGCGGTGCGTCGAAGACATCCGGCCTTTGCCGTGGGTGCCTTTCAGGAACTG
GGCGGCAGCAACCCGAGCGTGCTGGCGTATGTGCGTCAGGTGGCGGGCGATGATGGCGAT
ACCGTGCTGTGCGTGAACAACCTGAGCCGTTTTCCGCAGCCGATTGAACTGGATCTGCAG
CAGTGGACCAACTATACCCCGGTGGAACTGACCGGCCATGTGGAATTTCCGCGTATTGGC
CAGGTGCCGTATCTGCTGACCCTGCCGGGCCATGGCTTTTATTGGTTTCAGCTGACCACC
CATGAAGTGGGCGCACCTCCAACCTGCGGCGGCGAACGTCGTCTG<u>TAA</u>TAAGGATCC

The gene was sub-cloned in to a pET-21a(+) (Novagen) vector and heterologously expressed in *E. coli* BL21(DE3)pLysS. Cells containing the resulting expression plasmid were cultured in 2 l of selective Lennox broth at 37 °C (Lennox, 1955). When the cells reached an OD_{600nm} of 0.6, TreS expression was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside. After 20 h further incubation, the cells were harvested by centrifugation at 1700 × *g* for 10 min at 4 °C and re-suspended in 30 ml of 50 mM Tris-HCl, pH 7.4, containing 20 mM imidazole, 500 mM NaCl, 6 mg

ml^{-1} DNase 1 and a cOmplete™ protease inhibitor cocktail tablet (Roche). The cells were disrupted with a TS Series Benchtop 1.1 kW cell disruptor (Constant Systems Ltd) at 25 kPSI. The resulting cell lysate was separated from the cell debris by centrifugation at 20,200 × *g* for 25 min at 4 °C. TreS was purified from the cell lysate by application onto a 1 ml FF HisTrap column (GE Healthcare). After subsequent application of 10 column volumes of the re-suspension buffer, TreS was eluted with 50 mM Tris-HCl, pH 7.4, containing 500 mM NaCl and 500 mM imadazole. TreS was further purified using a HiLoad 16/600 Superdex 200 gel filtration column (GE healthcare) equilibrated with 10 mM sodium phosphate buffer, pH 6.8. Fractions that contained TreS were combined, concentrated and solvent exchanged into a buffer containing 100 mM citric acid, pH 6.7, using an Amicon Ultra-15 centrifugal filter unit with a 30 kDa cut-off (MerckMillipore). Aliquots of TreS were stored at -20 °C. TreS concentration was determined using the Bradford assays (Bradford, 1976).

NMR Spectroscopy

Spectra were recorded using a Bruker Avance III 400 spectrometer using standard pulse sequences and a probe temperature of 25 °C at 400 and 376 MHz for ^{1}H solvent-suppressed and ^{19}F proton-decoupled spectra, respectively. Chemical shifts are expressed in parts per million (ppm) relative to internal H_2O (4.70 ppm) or $CFCl_3$ (0.00 ppm). Spectra were analysed using Topspin 3.0 (Bruker) and resonances were integrated manually to ensure consistency.

NMR spectra associated with trehalose, maltose and glucose were assigned using COSY and HSQC experiments (data not shown), which were consistent with the literature (Usui et al., 1974). For ^{1}H-NMR spectra, the co-suppression of resonances within 1 ppm of that of solvent was determined with control samples using citrate as an internal standard. A spectrum with solvent-suppression of trehalose (10 mM), maltose (10 mM) in 90 mM citrate buffer, pH 6.7, and 10% (v/v) D_2O was compared with that of an identical sample without solvent-suppression after freeze drying and reconstitution in 100% D_2O. The four resonances from the citrate buffer arise from a second order spin system centred on 2.52 ppm that is remote from those of solvent (4.70 ppm) and carbohydrates. Doublet signals associated with α/β-maltose H-1′ (5.32 ppm) and trehalose H-1 (5.10 ppm) gave 89 and 87% of the expected integrals, respectively. Since the ratio of maltose anomers changed as a function of D_2O concentration, it was not possible to directly determine the degree of suppression of the maltose H-1 resonance (5.14 ppm) with confidence. However, since suppression was a function of how close the chemical shift was to that of solvent and the maltose H-1 resonance was between those of maltose H-1′ and trehalose H-1, the maltose H-1 signal was estimated to be 88% of that expected. The double doublet H-2 resonances associated with the β anomers of maltose and glucose (3.19 and 3.15 ppm, respectively) were distant enough from solvent (4.70 ppm) not to be suppressed. The corresponding doublet H-1 resonances of the β anomers (4.57 and 4.56 ppm, respectively) were too significantly supressed, so were not used to quantify reaction products. Thus, resonances associated with trehalose H-1, α/β-maltose H-1′, β-

maltose H-2, and β-glucose H-2 were used to quantify these species. The amount of α-maltose could then be calculated by subtracting the amount of β-maltose from that of α/β-maltose. Although the resonances of the α anomers of maltose and glucose overlapped completely, the concentration of α-glucose could be calculated by subtracting the amount of α-maltose from the combined amounts of the α anomers.

Singlet ^{19}F resonances were detected for 2-deoxy-2-fluorotrehalose (-201.1 ppm), 6-deoxy-6-fluorotrehalose (-235.5 ppm) (Backus et al., 2011), and for the α and β anomers of 2-deoxy-2-fluoromaltose (-200.2 and -200.0 ppm), 3-deoxy-3-fluoromaltose (-195.7 and -190.8 ppm), 6-deoxy-6-fluoromaltose (-234.9 and -234.4 ppm), 2-deoxy-2-fluoroglucose (-199.5 and -199.3 ppm), 3-deoxy-3-fluoroglucose (-200.0 and -195.2 ppm) and 6-deoxy-6-fluoroglucose (-235.5 and -234.8 ppm) (Tantanarat et al., 2012).

Mutarotation of Maltose and Glucose

Crystalline maltose and glucose were separately dissolved in buffer and each anomer was quantified as a function of time using ^{1}H-NMR spectroscopy as described above. A number of buffer conditions were tested in order to avoid artificially increasing the rates of mutarotation (Isbell and Pigman, 1969; Pigman and Isbell, 1968), avoid ^{1}H-NMR signals that interfere with the spectral analysis described above and readily support TreS activity, which is maximal at or near neutral pH (Pan et al., 2004). Phosphate buffer increased the rate of mutarotation as previously described for glucose (Pigman and Isbell, 1968; Stults et al., 1987), TRIS has been reported to inhibit *M. smegmatis* TreS (Pan et al., 2004), and MOPS, PIPES and HEPES interfered with the spectral analysis. The first order rate constants for the non-enzymatic mutarotation of each compound were therefore determined in 90 mM citrate buffer, pH 6.7, using COPASI (Hoops et al., 2006) with a minimal kinetic model comprising the reversible inter-conversion of each anomer.

TreS-Catalysed Reactions

Samples typically contained TreS, D_2O (10% v/v), 90 mM citrate buffer, pH 6.7, and either trehalose or maltose (10 mM). Maltose solutions were pre-equilibrated in the buffer at 25 °C until the α/β ratio remained constant. Reactions were initiated by the addition of the enzyme and were maintained at 25 °C. ^{1}H-NMR spectra were acquired and the integrals of signals were corrected for solvent suppression as described above, noting that all relevant resonances were associated with 1 proton except for that of trehalose, which had 2 × H-1. The contributions of trehalose and α/β-maltose were directly evident from their H-1 and H-1′ resonances, respectively. Similarly, the individual contributions of the β anomers of maltose and glucose were also directly evident from their H-2 resonances. The contribution of α-maltose could be determined knowing those from α/β-maltose and β-maltose by subtraction. Similarly, the contribution of α-glucose could subsequently be determined from the overlapping signals of the α anomers of glucose and maltose knowing the contribution of α-maltose. The initial concentration of the trehalose or maltose substrate was fixed

at 10 mM so the total concentration of disaccharides plus 2 × monosaccharides remained constant, allowing the concentrations of each species to be calculated. The rate of depletion of β-maltose was estimated by fitting data to a single exponential function using the Solver analysis tool of Microsoft Excel to minimise the RMS deviation of the fit to the data.

The 2-deoxy2-fluoro, 3-deoxy-3fluoro or 6-deoxy-6-fluoro analogues of maltose were prepared as described elsewhere (Tantanarat et al., 2012). Reaction mixtures with these analogues (10 mM) contained D_2O (100% v/v) and 10 mM deuteriated sodium phosphate buffer, pD 6.8, and were analysed directly by ^{19}F-NMR spectroscopy as described above. The deuteriated buffer was prepared by reconstituting a freeze dried aqueous buffer with D_2O.

SUPPLMENTAL REFERENCES

Bardarov, S., Bardarov, S., Pavelka, M.S., Sambandamurthy, V., Larsen, M., Tufariello, J., Chan, J., Hatfull, G., and Jacobs, W.R. (2002). Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. Microbiology-(UK) *148*, 3007-3017.

Bradford, M.M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem *72*, 248-254.

Isbell, H.S., and Pigman, W. (1969). Mutarotation of sugars in solution. Part II. Catalytic processes, isotope effects, reaction mechanisms, and biochemical aspects. Adv Carbohydr Chem Biochem *24*, 13-65.

Lennox, E.S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. Virology *1*, 190-206.

Pigman, W., and Isbell, H.S. (1968). Mutarotation of sugars in solution. Part 1. History, basic kinetics, and composition of sugar solutions. Adv Carbohydr Chem Biochem *23*, 11-57.

Stover, C.K., Delacruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., Bansal, G.P., Young, J.F., Lee, M.H., Hatfull, G.F.*, et al.* (1991). New use of BCG for recombinant vaccines. Nature *351*, 456-460.

Usui, T., Yokoyama, M., Yamaoka, N., Matsuda, K., Tuzimura, K., Sugiyama, H., and Seto, S. (1974). Proton magnetic resonance spectra of D-gluco-oligosaccharides and D-glucans. Carbohydr Res *33*, 105-116.