The OtsAB pathway is essential for trehalose biosynthesis in *Mycobacterium tuberculosis*

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\section*{Summary}

The disaccharide trehalose is the major free sugar in the cytoplasm of mycobacteria, it is a constituent of cell wall glycolipids, and it plays a role in mycolic acid transport during cell wall biogenesis. The pleiotropic role of trehalose in the biology of *Mycobacterium tuberculosis*, and its absence from mammalian cells, suggests that its biosynthesis may provide a useful target for novel drugs. However, there are three potential pathways for trehalose biosynthesis in *M. tuberculosis*, and the aim of the present study was to introduce mutations into each of the pathways in order to determine whether or not they are functionally redundant. The results show that the OtsAB pathway, which generates trehalose from glucose and glucose-6-phosphate, is the dominant pathway required for *M. tuberculosis* growth in laboratory culture and for virulence in a mouse model. Of the two \textit{otsB} homologues annotated in the genome sequence of *M. tuberculosis*, only OtsB2 (Rv3372) has a functional role in the pathway. OtsB2, trehalose-6-phosphate phosphatase, is strictly essential for growth and provides a tractable target for high-throughput screening. Inactivation of the TreYZ pathway, which can generate trehalose from \(\alpha\)-1,4-linked glucose polymers, had no effect on growth of *M. tuberculosis* \textit{in vitro} or in mice. Deletion of the \textit{treS} gene altered the late stages of pathogenesis of *M. tuberculosis* in mice, significantly increasing the time to death in a chronic infection model. Since the TreS enzyme catalyses the interconversion of trehalose and maltose, the mouse phenotype could reflect either a requirement for synthesis of additional trehalose or, conversely, a requirement for breakdown of stored trehalose to liberate free glucose.

\section*{Introduction}

The non-reducing disaccharide trehalose (\(\alpha\)-D-glucopyranosyl-(1,1)-\(\alpha\)-D-glucopyranoside) is found in bacteria, yeast, fungi, plants and invertebrates, but not in mammalian cells (reviewed in (1)). It can serve as a carbon source, as a storage carbohydrate, and as a stress protectant. Trehalose can function as a compatible solute to stabilise cells during osmotic stress, and its accumulation has been widely implicated in preserving cell viability during exposure to a range of environmental stresses including heat shock, dehydration and hypoxia (2-4). In mycobacteria and related actinomycetes, trehalose is also a major structural constituent of cell wall glycolipids, and acts as a carrier for mycolic acids during biosynthesis of the cell wall (5,6).

We are interested in analysing the role of trehalose in the pathogenesis of *Mycobacterium tuberculosis*. Current models of tuberculosis envisage the contribution of at least two phenotypic forms of the bacteria: an actively replicating form involved in the initial establishment of infection and during active disease, and a non-replicating form involved in
latent infection and in persistence during chemotherapy. Current drugs act primarily on replicating bacteria, and a key challenge for improved tuberculosis control is to develop novel compounds that are equally active against the non-replicating populations. It is anticipated that these would allow shortening of the conventional 6-month treatment regimen, as well as providing preventive therapy for individuals who are carrying a latent infection. We hypothesised that the dual role of trehalose – in biogenesis of the cell wall of replicating bacteria, and as a stress protectant in non-replicating bacteria – would make its biosynthesis an appropriate target for this new class of drug.

However, we have previously reported that three independent pathways are available for trehalose biosynthesis in \textit{M. tuberculosis}: the OtsAB pathway which utilises glucose and glucose-6-phosphate, the TreYZ pathway which makes trehalose from glycogen, and the TreS enzyme which can convert maltose to trehalose (7). If each of these pathways is able to generate the trehalose pool required for growth and survival of \textit{M. tuberculosis}, this would obviously compromise the activity of a drug targeting any single enzyme. In addition, genome sequence analysis has identified two open reading frames in \textit{M. tuberculosis} with homology to the \textit{otsB} gene that encodes the trehalose-6-phosphate phosphatase required for the OtsAB pathway (8). Interestingly, one of the homologues (\textit{otsB1}, \textit{Rv2006}) is a member of a regulon that is induced by exposure to hypoxia or to low concentrations of nitric oxide and is associated with non-replicating survival of \textit{M. tuberculosis} (9). Again, the existence of alternative branching pathways is an important consideration in assessment of potential drug targeting.

Recent reports have analysed the role of these three pathways for trehalose biosynthesis in the fast-growing saprophytic mycobacterium \textit{M. smegmatis} (10-13), and in the taxonomically-related \textit{Corynebacterium glutamicum} (14,15). Mutagenesis studies indicated that the three pathways are functionally redundant in \textit{M. smegmatis} (13). Single mutations targeting each of the pathways generated no apparent phenotypic defects in the resulting clones as assessed by growth or by glycolipid content. A triple mutant, with all three pathways inactivated, was dependent on provision of exogenous trehalose for growth, and was defective in stationary phase survival and in exposure to elevated temperature (13). A different hierarchy of trehalose biosynthesis pathways was found in \textit{C. glutamicum}. Again, mutation of all three pathways was accompanied by a marked growth defect, but in \textit{C. glutamicum} this was also observed in the case of an \textit{otsA-treY} double knockout. Analysis of the effect of different combinations of mutations on trehalose content and bacterial survival following osmotic stress, led to the conclusion that TreYZ represents the major pathway for trehalose biosynthesis in \textit{C. glutamicum}, with OtsAB playing a minor accessory role, and TreS contributing to trehalose degradation through its ability to catalyse the interchange of trehalose and maltose (14,15). To date the only information on the contribution of the various pathways to trehalose anabolism in \textit{M. tuberculosis} comes from a transposon site hybridisation mutagenesis study carried out by Sassetti, Boyd and Rubin (16). This showed that insertions into the \textit{otsA} and \textit{otsB2} genes were associated with severe growth defects \textit{in vitro}, and that insertions into \textit{treS} showed significantly reduced growth.

The aim of the present study was to assess the relative role of each of the three trehalose biosynthesis pathways in \textit{M. tuberculosis} during growth in laboratory media and during infection in a mouse model. In contrast to the results with \textit{M. smegmatis} and with \textit{C. glutamicum}, our studies demonstrate that OtsAB is the predominant pathway in \textit{M. tuberculosis}. The mutagenesis results are consistent with biochemical studies demonstrating that trehalose-6-phosphate phosphatase activity is associated with only one of the OtsB homologues in \textit{M. tuberculosis} (17), and highlight this enzyme as an attractive target for drug development.

**Experimental procedures**

**Construction of knock-out mutants**

Approximately 1.5kb flanking \textit{otsA}, \textit{otsB1}, \textit{otsB2}, \textit{treS} and \textit{treY} was amplified using the upstream and downstream primers listed in Table 1 and the Roche Expand High Fidelity PCR system. Constructs were designed to remove the entire coding region of the gene. Fragments were cleaved with the enzymes indicated in the table and cloned either side of the hygromycin resistance cassette in the vector.

[Table 1]

| Gene | Upstream Primer | Downstream Primer |
|------|----------------|------------------|
| otsA |                  |                  |
| otsB1|                  |                  |
| otsB2|                  |                  |
| treS |                  |                  |
| treY |                  |                  |
pSMT100, which contains sacB as counter-selectable marker (18). Ligations were transformed into E. coli DH5α and selected on LB plates containing 250 μg/ml hygromycin. Plasmids were purified and verified by restriction endonuclease mapping and sequencing. Approximately 1μg of DNA was exposed to 1000 μJoules cm−2 of UV irradiation using a UVCL-1000 cross-linker, before electroporation into M. tuberculosis H37Rv, M. bovis AF2122/97, and M. bovis BCG Pasteur as described (18). Double cross-overs were selected on Middlebrook 7H11 supplemented with OADC (Difco), 50 μg/ml hygromycin and 2% sucrose. Colonies were visible after 3 to 4 weeks. Recombination was confirmed by Southern blotting, PCR and sequencing (data not shown).

To facilitate inactivation of the otsB2 gene a diploid strain was constructed by integrating a second copy of the gene at the attB site (19). Briefly the otsB2 gene plus upstream flanking sequence, was amplified by PCR using primers B21F and B2R (Table 1). This 3kb fragment was cloned into pCRBlunt II (Invitrogen) and restricted with NheI to release the otsB2 gene plus a 400 bp upstream fragment that includes the promoter; this was cloned into the integrating vector pKINTA (20). Plasmid DNA was isolated and electroporated into M. tuberculosis H37Rv as described above, but without prior UV treatment. Deletion of the chromosomal copy of the otsB2 gene after transformation with the knock-out construct was monitored as described above.

In vivo analysis of knock-out mutants

Groups of 22 female C57BL/6 mice 10 to 14 weeks of age were infected intravenously in the tail vein with 10⁸ filtered single-cell mycobacterial suspension. The preparation of clump-free mid-log-phase mycobacteria suspensions has been described elsewhere (21,22), and the concentration of bacilli (>90% single cells) was directly estimated by microscopy using a conventional cytometer, and adjusted for all mycobacterial strains tested. The accuracy of estimations was confirmed by counting mycobacterial microcolonies as described (23). The variation between strains was less than 10%. Four mice from each group were culled on days 10, 35 and 56 post-infection and colony forming units counted in lungs and spleens. To assess mycobacterial loads in organs, 0.1 ml of serial 10-fold dilutions of whole-organ homogenates were plated onto Dubos agar, and colonies were counted after 18-20 days of incubation at 37 °C. The remaining 10 mice from each infection were assessed for survival over 10 months.

Expression and purification of trehalose biosynthesis enzymes

The genes encoding OtsB2 and TreS were amplified using the primers for expression indicated in Table 1, and restricted with NdeI and XhoI for cloning into pET15b (Novagen). These expression plasmids were transformed into the E. coli B strains BL21(DE3), BL21(DE3)pLysS, Origami(DE3) and Origami(DE3)pLysS and the E. coli K12 host HMS174(DE3) (all from Novagen). No significant differences in protein expression were found using these strains, and all subsequent experiments were carried out using BL21(DE3). Fifty millilitre log phase cultures (OD₆₀₀nm = 0.5-0.6) grown at 22 °C were induced overnight with 0.5 mM IPTG. The pellet was solubilised in 600 μl lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 1x Novagen Bugbuster) and the cleared lysate applied to an immobilised metal ion affinity chromatography spin column (Ni²⁺-NTA, Qiagen). The column was washed according to the manufacturer’s instruction before bound protein was eluted using 250 mM imidazole, dialysed against 50 mM NaH₂PO₄, 100 mM NaCl, pH 7.0, then stored at -70 °C. Samples were analysed on 10% SDS-PAGE gels and stained with Coomassie blue. Samples of TreS for NMR analysis were obtained from a pQE30-TreS clone (7) and purified using Ni²⁺-NTA and Q-sepharose anion exchange chromatography. Analytical gel filtration studies of His₆-TreS samples of between 1 & 10 mg ml⁻¹ protein concentration found that the protein eluted at a volume consistent with an apparent molecular weight of ~140k Da, suggesting that TreS was dimeric under these conditions.

Trehalose-6-phosphate phosphatase enzyme assay

Recombinant OtsB2 protein was dialyzed against 10mM Tris-Cl pH 7.0 to remove free phosphate, and protein concentration was determined using the BioRad protein assay with BSA as standard. Phosphatase activity was measured in a final volume of 50 μl containing 10 mM Tris-Cl pH 7.0, 2 mM MgCl₂, 1 mM...
trehalose-6-phosphate (Sigma) and 100 ng purified enzyme. This was incubated in a microtitre plate at 37 °C for 10 minutes, then 200 µl of filtered 1% ammonium molybdate, 0.15% malachite green, 12.5% v/v conc HCl was added and the absorbance at 630 nm measured to determine the amount of free phosphate produced, which was linear in the range of 0 to 0.1 mM (24).

Substrate specificity was determined by replacing the trehalose-6-phosphate with 1 mM of fructose-6-phosphate, galactose-6-phosphate, glucose-6-phosphate, mannose-6-phosphate, maltose-6-phosphate, or sucrose-6-phosphate (all from Sigma). Co-factor specificity was determined by replacing the 2 mM MgCl₂ in the above incubation buffer with 2 mM CaCl₂, CoCl₂, FeCl₃, or MnCl₂. A combination of 2 mM MgCl₂ and 2 mM CaCl₂ was tested, as was 2 mM MgCl₂ and 1 mM EDTA.

¹H NMR spectra were acquired at 298K on a Varian UNITYplus spectrometer (operating at a nominal frequency of 500 MHz) equipped with a triple resonance (¹H, ¹³C, ¹⁵N) probe including Z-axis pulse field gradients. One-dimensional ¹H spectra were obtained for 5mM maltose and trehalose (time 0), then ~10 µM TreS enzyme was added and spectra collected after 5, 120 and 240 minutes.

Results

Construction of M. tuberculosis mutants with deletions in trehalose biosynthesis pathways

Previous bioinformatic and biochemical analysis demonstrated the presence of three potential pathways for biosynthesis of trehalose in M. tuberculosis (7). To assess their relative contribution to mycobacterial biology, we targeted five genes from the three pathways for mutagenesis. Flanking regions for otsA, otsB1, otsB2, treS and treY were cloned on either side of the hygromycin resistance cassette in the pSMT100 vector and transformed into M. tuberculosis H37Rv. Clones in which a double cross-over had resulted in replacement of the targeted gene by the hygromycin cassette were identified by counter-selection against the sacB marker on the plasmid vector. Between 30 and 40 colonies were recovered per µg transforming DNA for treS, treY and otsB1; the majority of those screened corresponded to the anticipated knock-outs. Fewer than 10 colonies per µg transforming DNA were obtained for otsA; some of which were mutants. Only a few colonies were recovered on otsB2 plates; none were the required mutants. Recombination was confirmed by Southern blotting and PCR (data not shown).

The ΔotsB1, ΔtreY and ΔtreS clones showed no obvious growth impairment, but the ΔotsA clone grew slowly in liquid media and colonies on agar plates were visibly smaller than those formed by the other mutations. To explore the reason for the failure to obtain an otsB2 knockout, we generated a diploid strain of M. tuberculosis carrying a second copy of the otsB2 gene integrated into the attB site on the chromosome. Transformation of this strain with the pSMT100::otsB2 deletion construct resulted in multiple double cross-over mutants in which the hygromycin cassette had replaced the original chromosomal copy of the gene. This result confirms that failure to isolate the otsB2 mutant is due to fact that this gene has an essential role for in vitro replication of M. tuberculosis. This role cannot be performed by the otsB1 gene, and the lack of phenotype for the ΔotsB1 clone is consistent with the biochemical observation that the protein product of otsB1 lacks functional trehalose-6-phosphate phosphatase activity (17).

We repeated the deletion strategy with two other members of the M. tuberculosis complex; M. bovis AF2122/97, and M. bovis BCG Pasteur, the attenuated vaccine strain. M. bovis AF2122/97 belongs to a subset of M. bovis isolates which lack a functional treY gene as a result of a spontaneous chromosomal deletion (25). Again, we were unable to generate ΔotsB2 clones in M. bovis BCG (M. bovis AF2122/97 was not attempted) but, in contrast to M. tuberculosis, we were also unable to generate ΔotsA clones in the M. bovis strains, despite attempts to select for transformants on media containing 0.5% trehalose. The ΔtreS mutant in M. bovis AF2122/97 (i.e. the ΔtreSΔtreY double mutant) resembled the corresponding M. tuberculosis mutant in having no obvious growth phenotype in vitro.

Characterisation of trehalose biosynthesis mutants in a mouse model of tuberculosis

M. tuberculosis mutants representative of each of the three trehalose biosynthesis pathways – ΔotsA, ΔtreY, ΔtreS – were tested for their ability to cause progressive disease in C57BL/6 mice. Mice were infected by
intravenous inoculation of 10^6 bacteria and the number of colony forming units (CFUs) was measured in lungs and spleens and from animals sacrificed 10, 35 and 56 days after infection (Fig. 1). The bacterial load in animals receiving the 
\textit{OtsA} mutant was lower at all time points compared to controls, in both lungs and spleens (p<0.01 at days 35 and 56). Animals receiving the \textit{Atr} and \textit{Atr} mutants developed similar bacterial loads to the wild-type control, except at the late stage of infection (day 56), when the \textit{Atr} mutant showed a small but significant reduction in CFUs in the lungs (p=0.05). The same pattern for all mutants was observed in a second independent experiment.

Mutant phenotypes were further analysed by monitoring the time to death for animals in the different groups (Fig. 2). Again the \textit{OtsA} mutant had a marked phenotype, with >70% of the animals surviving longer than 300 days, compared to the animals infected with the wild-type strain, which had all died by day 168 (mean survival time of 132 ± 14 days). The \textit{Atr} mutant showed a significant attenuation in comparison to wild type, with a mean survival time of 235 ± 23 days. The \textit{Atr} mutant showed no significant difference from the wild-type control.

These results showed that the growth defect associated with the \textit{OtsA} mutation observed during \textit{in vitro} culture is also evident during murine infection, and uncovered a further \textit{in vivo} phenotype for the \textit{Atr} mutation during long-term infection in the mouse.

\textbf{Purification and characterisation of trehalose biosynthesis enzymes}

To further characterise trehalose biosynthesis in \textit{M. tuberculosis} and to explore options for drug targeting, genes encoding \textit{OtsB2} and \textit{TreS} were cloned into pET15b, and soluble protein products with an N-terminal hexa-histidine peptide tag were obtained. The corresponding protein products were purified by immobilised metal ion (Ni^{2+}) affinity chromatography.

The trehalose-6-phosphate phosphatase encoded by the \textit{otsB2} gene was analysed in a 96-well plate format suitable for high-throughput screening. The purified enzyme was incubated with a range of sugar-phosphates to determine its substrate specificity; no release of phosphate was detected from any substrate other than trehalose-6-phosphate (Table 2). Co-factor requirements were established by the addition of cations and EDTA to the incubation reaction with trehalose-6-phosphate as substrate (Table 3). The preferred cation was found to be Mg^{2+}; the reaction was inhibited by Ca^{2+} or by chelation of cations in the presence of EDTA. The K\textsubscript{m} was determined to be 0.6 mM (Figure 3).

We previously observed (7) the activity of TreS using either maltose or trehalose as substrate in an indirect biochemical assay of trehalose breakdown by trehalase. In order to extend and confirm that the trehalose synthase activity resides solely in the TreS protein product, we attempted to assay our recombinant TreS using one-dimensional 1H NMR to collect spectra that monitored the intensity of the alpha anomeric proton resonances of the substrate and product carbohydrates (Fig. 4). The NMR assay has the advantage over the biochemical assay in that the progress of the reaction can be monitored in real time, and from the identification of the anomeric proton chemical shifts we obtain simultaneous and unambiguous identification of all the reaction components (reactants and products). Upon addition of TreS to a solution of maltose NMR resonances indicating conversion to trehalose appear over a period of ca. 2 hours, with corresponding depletion of the maltose signal. In contrast, when TreS is added to a solution of trehalose signals corresponding to maltose reach a maximum on a much faster timescale (<5 minutes). In each case equilibrium was reached in which the trehalose signal is more intense than that of maltose. These spectra confirm that the enzyme is able to interconvert maltose and trehalose. In both cases NMR resonances corresponding to glucose appear during the time course of the reaction. This may reflect the presence of residual glycosidase activity in the TreS preparation.

\textbf{Discussion}

The presence of high concentrations of free trehalose in the cytoplasm of mycobacteria (26), together with its role in cell wall biogenesis (5) and its absence from mammalian cells, suggests that the biosynthesis of trehalose is an attractive target for novel anti-tuberculous agents. However, an obvious caveat to this approach is the fact that the \textit{M. tuberculosis} genome encodes the capacity for three independent pathways capable of generating the disaccharide (7). The
primary aim of the present study was to generate mutants in each of these pathways in order to determine whether or not they are functionally redundant for growth and survival in vitro and during infection. Our results demonstrate a clear dominance of the OtsAB pathway, which produces trehalose by condensation of glucose and glucose-6-phosphate. Deletion of the otsA gene resulted in marked defects in growth of *M. tuberculosis* in vitro and in vivo, and the functional OtsB homologue (encoded by otsB2) proved to be strictly essential for growth. The availability of exogenous trehalose did not reverse the phenotype. The absence of any apparent phenotypic consequences associated with treY deletion indicates that the TreYZ pathway, which has the potential of generating trehalose from α-1,4-linked glucose polymers, is not essential for *M. tuberculosis* under these conditions. Increased survival of mice infected with a Δ*treS* mutant suggests that interconversion between trehalose and maltose plays a role during prolonged *M. tuberculosis* infection. Our results with targeted deletion mutants are consistent with findings from high-throughput transposon mutagenesis experiments (16, 27). Transposon insertions into *treY*, *treZ* or *otsB1* had no significant consequences for growth in vitro or in a mouse model (27). Severe growth defects were associated with insertions into the *otsA* and *otsB2* genes, and significantly reduced growth was seen for insertions into *treS* (16). It is not clear whether the transposon insertions created null mutant phenotypes, or whether some residual enzyme activity remains that might explain the ability to detect the presence of the *otsB2* mutant in the pools of mutants examined; it may be that growth in a pool provides a degree of complementation sufficient to allow the mutants to survive, albeit with compromised growth. It is unlikely that lethality of the deletion construct is due to a polar effect since there is no evidence that *otsB2* is part of an operon. There is an intergenic region of 236 bp between *Rv3372 (otsB2)* and the gene immediately downstream *Rv3373 (echA18)*, which encodes a non-essential enoyl-CoA hydratase (26).

While the OtsAB pathway represents the most common mechanism for trehalose biosynthesis found in a wide range of bacteria, plants and invertebrates, its dominance in *M. tuberculosis* is in contrast to findings in closely related bacterial species. The TreYZ pathway predominates in *C. glutamicum* (14), and the three pathways are functionally redundant in *M. smegmatis* (13). In *Mycobacterium leprae* *treY* and *treS* are pseudogenes, leaving OtsAB the only intact pathway for trehalose biosynthesis (28). The reason for species-specific differences in the hierarchy of trehalose biosynthesis pathways is unknown, but may reflect differences in metabolic flux and in requirements for the free sugar in the cytoplasm as well as bound sugar in the cell wall. A difference was observed between *M. tuberculosis* and the closely-related *M. bovis*, in which we were unable to isolate Δ*otsA* mutants. This was also the case for an *M. bovis* field isolate with a spontaneous mutation in the TreYZ pathway, as well as for *M. bovis* BCG in which the *treY* and *treZ* genes are intact. Again, this may reflect some difference in carbohydrate metabolic flux between the two species; *M. bovis* has several lesions in carbohydrate catabolism compared to *M. tuberculosis*, including a mutation in *pykA* that prevents the entry of the glycolytic intermediate phosphoenolpyruvate into the TCA cycle (25).

Although they participate in a shared pathway, there was a difference in phenotypic consequences between deletion of the *otsA* and *otsB* genes in *M. tuberculosis*. The lethal phenotype of the Δ*otsB2* mutant demonstrates that the phosphatase function of OtsB cannot be arrived at by other enzymes, further reinforcing the conclusion from biochemical studies that open reading frame *Rv2006*, annotated as *otsB1* in the genome, is inactive in this respect (17). Trehalose-6-phosphate acts as an important signal for metabolic control in *Saccharomyces cerevisiae* where it regulates the first steps in glycolysis through the inhibition of hexokinase II, and its accumulation is detrimental to the organism (29). An analogous regulatory function in mycobacteria might explain the greater severity of the Δ*otsB2* mutation in comparison to the Δ*otsA* mutation observed in our study. Irrespective of the underlying mechanisms, our findings highlight OtsB2 as an attractive target for drug development, and we have described procedures for the overexpression, purification and assay of the enzyme, that are designed to provide a platform for such an endeavour. The assay is highly specific for trehalose-6-phosphate (Table 2) and does not act on any of the other sugar phosphates tested, suggesting that an inhibitor would not interfere with normal
mammalian metabolism. The ten minute assay reaction should facilitate high throughput rapid screening of potential inhibitors.

A key question in assessing the value of OtsB2 as a target is the role of trehalose biosynthesis in survival of non-replicating organisms during infection. While our results clearly show that the OtsAB pathway is important for initial replication of M. tuberculosis in mice, they do not allow us to determine whether prolonged survival of mice infected with the OtsAB mutant is simply a consequence of this difference in initial growth or reflects some additional impairment in bacterial persistence. To validate targets for drugs that are designed to act against the multiple M. tuberculosis phenotypes encountered in vivo, there is a need to develop experimental systems that will allow us to selectively silence targeted genes at different stages of the infection process. Experiments are in progress to address this issue using constructs in which otsB2 is placed under the control of a tetracycline regulated antisense system (unpublished data).

In contrast to the ΔotsA phenotype, the ΔtreS mutant shows growth comparable to wild type during the initial phase, followed by a late stage defect in bacterial load and, more markedly, time-to-death. The TreS requirement for persistent infection with M. tuberculosis could reflect either a need for production of additional trehalose or, conversely, for mobilisation of stored trehalose into maltose and then into usable glucose. Several studies suggest that lipids rather than carbohydrates provide the major source for nutrition of persistent M. tuberculosis. Key enzymes required for gluconeogenesis from lipid precursors – isocitrate lyase, phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase – are upregulated in vivo and are essential for virulence of M. tuberculosis in the mouse model (27, 30, 31). It is possible that trehalose provides an additional source of glucose during persistent infection. In some organisms (including mammals) trehalose can be converted to glucose by a trehalase enzyme, but no trehalase homologue has been identified in the genome of M. tuberculosis (8). M. tuberculosis does have a homologue of trehalose phosphorylase (Rv3401), which can convert trehalose to glucose and glucose-1-phosphate in other organisms (32), although this is apparently non-essential for mycobacterial growth in vitro or during acute infection in mice (16, 27). The ability of TreS to interconvert α-1,1 and α-1,4 linkages (7, 12) may therefore provide an important pathway for generation of glucose from trehalose. Pan et al (12) have described an alternative pathway for TreS-mediated production of glucose. They found that M. smegmatis TreS produced glucose as a by-product of a two-step reaction to produce trehalose from maltose, involving the cleavage of maltose to release two molecules of glucose, one of which is transferred to another enzyme-bound glucose to give trehalose.

In summary, we have shown that OtsAB is the dominant pathway for trehalose biosynthesis in M. tuberculosis and that its loss cannot be compensated by either of the two alternative pathways. The trehalose-6-phosphate phosphatase encoded by the otsB2 gene represents an attractive and tractable antituberculosis drug target.

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Figure Legends

Figure 1
Multiplication of mutant and wild-type mycobacteria in the lungs (panel A) and spleens (panel B) of mice following intravenous challenge with 10⁶ colony forming units (CFU). Organs were harvested from four mice at each time point and CFU load in lungs and spleen determined for the parent strain (H37Rv, diamonds) and for each mutant: ΔotsA, squares; ΔtreS, triangles; ΔtreY, circles. Error bars are set at ±30% to reflect the largest error. An identical inoculum dose was ensured by monitoring CFU counts by microcolonies prior to infection.

Figure 2
Mortality counts for mice infected with 10⁶ CFU of trehalose biosynthesis mutants. Wild type and mutant strains are indicated as follows: H37Rv, diamonds; ΔotsA, squares; ΔtreS, triangles; ΔtreY, circles. The percentage of animals surviving is plotted against time.

Figure 3
Effect of trehalose-6-phosphate concentration on trehalose-6-phosphate phosphatase (OtsB2) activity. Activity is represented as the mean phosphate released ± sd.

Figure 4
One-dimensional 500 MHz ¹H NMR spectra of the substrate’s and product’s α-anomeric proton resonances before (0 minutes) and at time points 5, 120 and 240 minutes after addition of TreS to (a) maltose (M) and (b) trehalose (T). M’ is the reducing sugar’s α-anomeric proton. Peak G appears to correspond to free glucose and is also present at later time-points. The ¹H chemical shifts of the α-anomeric proton resonances relative to 3-(Trimethylsilyl)-Propionic acid (0.0 ppm) are 5.42 ppm (M), 5.24 ppm (M’), 5.20 ppm (T) and 5.23 ppm (G).
### Table 1
Primer sequences used for knock-out and expression plasmid construction.

| Gene   | Region | Purpose | Primer | RE  | Sequence (5’-3’) |
|--------|--------|---------|--------|-----|-----------------|
| otsA   | up     | KO      | A1F    | BamHI| CGGGATCCGGAGAGATGCCACTATTGCC |
| otsA   | down   | KO      | A3F    | BglII| GGAATCTTACGGTCAAGCCGCTCCCG |
| otsA   | down   | KO      | A4R    | XbaI | GCTCTAGAAACCCGTTCGTAACAGGCACC |
| otsB   | up     | KO      | B1F    | BamHI| CGGGATCCGGCCGGCCGCGCCGCAACG |
| otsB   | up     | KO      | B2R    | SpeI | GGACTAGTCAGCTTGAAGATGGCCACTATTGCC |
| otsB   | down   | KO      | B3F    | XbaI | GCTCTAGAAACCCGTTCGTAACAGGCACC |
| otsB   | down   | KO      | B4R    | PstI | AAAGATCTTACGGTCAAGCCGCTCCCG |
| otsB2  | up     | KO      | B21F   | BamHI| CGGGATCCGGCCGGCCGCGCCGCAACG |
| otsB2  | up     | KO      | B22R   | SpeI | GGACTAGTCAGCTTGAAGATGGCCACTATTGCC |
| otsB2  | down   | KO      | B23F   | BglII| GGAATCTTACGGTCAAGCCGCTCCCG |
| otsB2  | down   | KO      | B24R   | XbaI | GCTCTAGAAACCCGTTCGTAACAGGCACC |
| treS   | up     | KO      | S1F    | BamHI| CGGGATCCGGCCGGCCGCGCCGCAACG |
| treS   | down   | KO      | S3F    | BglII| GGAATCTTACGGTCAAGCCGCTCCCG |
| treS   | down   | KO      | S4R    | XbaI | GCTCTAGAAACCCGTTCGTAACAGGCACC |
| treS   | Exp    | KO      | SFor1  | NdeI | CCCTCTAGACGGCAAGTGGCACGTGC |
| treS   | Exp    | KO      | SRev1  | XhoI | CCCTCTAGACGGCAAGTGGCACGTGC |
| treY   | up     | KO      | Y1F    | BamHI| CGGGATCCGGGATATAACGGGATGCACC |
| treY   | up     | KO      | Y2R    | SpeI | GGACTAGTTGAATTCCGAGTATGGGACC |
| treY   | down   | KO      | Y3F    | BglII| GGAATCTTACGGTCAAGCCGCTCCCG |
| treY   | down   | KO      | Y4R    | XbaI | GCTCTAGAAACCCGTTCGTAACAGGCACC |

* Rv gene designations: *otsA*, Rv3490; *otsB*, Rv2006; *otsB2*, Rv3372; *treY*, Rv1563c; *treS*, Rv0126.

1.5kb upstream and downstream were cloned either side of the hygromycin cassette for knock-out plasmid construction.

RE, restriction endonucleases.

Restriction endonuclease recognition sites are underlined.

### Table 2
Specificity of *M. tuberculosis* trehalose-6-phosphate phosphatase (OtsB2).
Specificity was determined by measuring the release of phosphate from various sugar-6-phosphate substrates. After 10 minutes the assay was saturated with respect to the amount of phosphate released from trehalose-6-phosphate.

| substrate          | Abs630nm 5 min | Abs630nm 10 min |
|--------------------|----------------|-----------------|
| Trehalose-6P       | 1.27           | 1.62            |
| Fructose-6P        | 0.06           | 0.09            |
| Galactose-6P       | 0.04           | 0.05            |
| Glucose-6P         | 0.03           | 0.03            |
| Mannose-6P         | 0.01           | 0.01            |
| Maltose-6P         | 0.00           | 0.00            |
| Sucrose-6P         | 0.00           | 0.00            |
Table 3
Assessment of co-factor requirements for trehalose-6-phosphate phosphatase. Cations were added to the reaction buffer at a final concentration of 2 mM and phosphate released from trehalose-6-phosphate by the enzyme was measured after 5 minutes at 37°C. Values are expressed as a percentage of the phosphate released using Mg\(^{2+}\) as the co-factor.

| Co-factor | Mg\(^{2+}\) | None | Ca\(^{2+}\) | Co\(^{2+}\) | Fe\(^{2+}\) | Mn\(^{2+}\) | Mg\(^{2+}\)/Ca\(^{2+}\) | Mg\(^{2+}\)/EDTA |
|-----------|-------------|------|------------|-----------|----------|----------|----------------|---------------|
| % Activity| 100.0       | 26.0 | 0.0        | 30.0      | 65.0     | 26.0     | 71.6           | 3.8           |
Figure 3

[Graph showing the relationship between Trehalose-6-phosphate (mM) and phosphate released, with a Km value of 0.6 mM.]

Figure 4

[Diagram showing the structures of Malose and Trehalose with labels for M and T. A chromatogram is also shown with peaks labeled M' and T at different time points (0, 5, 120, 240 minutes).]
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