Three pathways for trehalose biosynthesis in mycobacteria

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Trehalose is present as a free disaccharide in the cytoplasm of mycobacteria and as a component of cell-wall glycolipids implicated in tissue damage associated with mycobacterial infection. To obtain an overview of trehalose metabolism, we analysed data from the *Mycobacterium tuberculosis* genome project and identified ORFs with homology to genes encoding enzymes from three trehalose biosynthesis pathways previously characterized in other bacteria. Functional assays using mycobacterial extracts and recombinant enzymes derived from these ORFs demonstrated that mycobacteria can produce trehalose from glucose 6-phosphate and UDP-glucose (the OtsA–OtsB pathway) from glycogen-like $\alpha(1 \rightarrow 4)$ -linked glucose polymers (the TreY–TreZ pathway) and from maltose (the TreS pathway). Each of the pathways was found to be active in both rapid-growing *Mycobacterium smegmatis* and slow-growing *Mycobacterium bovis* BCG. The presence of a disrupted *treZ* gene in *Mycobacterium leprae* suggests that this pathway is not functional in this organism. The presence of multiple biosynthetic pathways indicates that trehalose plays an important role in mycobacterial physiology.

Keywords: genome, mycobacteria, osmotic stress, trehalose, tuberculosis

INTRODUCTION

Trehalose is a non-reducing disaccharide (1-O- α -D-glucopyranosyl- α -D-glucopyranoside) found in insects, plants and microbial cells. It has a protective effect on proteins and biological membranes during cryopreservation or desiccation *in vitro*, and has been implicated in survival of micro-organisms exposed to environmental stresses *in vivo*. *Escherichia coli* and *Saccharomyces cerevisiae* contain little or no trehalose during exponential growth under standard culture conditions, for example, but the free sugar rapidly accumulates in response to osmotic stress or heat shock, and during the stationary phase of culture (Strom & Kaasen, 1993; De Virgilio *et al.*, 1994). In *Streptomyces venezuela*, trehalose is absent from the mycelium in vegetative cultures, but is abundant in spores (Ranade & Vining, 1993).

In contrast, trehalose is constitutively present in mycobacteria cultured in routine laboratory media; it is the only free sugar readily detectable in the cytoplasm and accounts for 1.5–3 % of the total dry weight of the cells (Elbein & Mitchell, 1973). The function of the free trehalose is unknown. Metabolic studies in the rapid-growing *Mycobacterium smegmatis* showed that the trehalose pool is subject to constant turnover, suggesting it is not simply accumulated as an energy reserve (Winder *et al.*, 1972). Trehalose is also incorporated into a range of mycobacterial glycolipids, including cord factor (trehalose 6,6'-dimycolate), sulpholipids (acylated trehalose-2'-sulphate derivatives) and trehalose-containing lipooligosaccharides (Besra & Chatterjee, 1994). Toxic activities associated with these cell-wall components have led to the suggestion that they may play a role in tissue damage resulting from infection with pathogenic mycobacteria such as *Mycobacterium tuberculosis*.

The emergence of multidrug-resistant strains of *M. tuberculosis* (Pablos-Mendez *et al.*, 1998) highlights the need to develop novel drugs for treatment of tuberculosis; particularly compounds that could act against the bacterial population that persists during prolonged

Abbreviations: MOT, maltooligosyltrehalose.

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Fig. 1. Trehalose biosynthesis pathways. Three pathways for trehalose biosynthesis have been identified in bacteria. (a) The OtsA-OtsB pathway is found in a wide range of microbes including *E. coli* and *Sac. cerevisiae*. (b) The TreY-TreZ pathway is used by *Arthrobacter*, *Rhizobium* sp. and *Sulfolobus acidocaldarius*. (c) The TreS pathway has been characterized in *Pimelobacter* sp. and in *T. aquaticus*.

therapy with currently available drugs. The prominence of trehalose in the cytoplasm, and its presence in toxic glycolipids suggests that disruption of trehalose metabolism might represent a useful drug target. Pan *et al.* (1996) demonstrated that several antibiotics that inhibit growth of *M. smegmatis* had an effect on a trehalose biosynthetic enzyme, and Belisle *et al.* (1997) demonstrated inhibition of mycobacterial growth and disruption of a trehalose mycolyltransferase enzyme by 6-azido-6-deoxy- α,α' -trehalose. We have also noticed that trehalosamine (2-amino-2-deoxy- α -D-glucopyranosyl α -D-glucopyranoside) inhibits the growth of *M. smegmatis*, which may be due to interference with trehalose metabolism. In the present study, we wished to identify enzymes involved in biosynthesis of trehalose in mycobacteria, with the eventual goal of assessing their potential as drug targets.

The best characterized pathway for trehalose biosynthesis involves condensation of glucose 6-phosphate with UDP-glucose to form trehalose 6-phosphate, with subsequent dephosphorylation generating the free disaccharide (Fig. 1a). In *E. coli*, these reactions are catalysed by trehalose-6-phosphate synthase (the product of the *otsA* gene) and trehalose-6-phosphate phosphatase (*otsB*) (Kaasen *et al.*, 1992). Transcription of the *otsA* and *otsB* genes is induced during stationary phase and osmotic stress in *E. coli* (Kaasen *et al.*, 1994). In *Sac. cerevisiae*, the two reactions are catalysed by TPS1 and

TPS2, which form a multimeric trehalose synthase complex together with additional subunits, which regulate activation in response to stress (Reinders *et al.*, 1997). An alternative pathway for generation of trehalose from glycogen has been identified in *Arthrobacter* (Maruta *et al.*, 1996a), *Rhizobium* (Maruta *et al.*, 1996b) and in the archaeon *Sulfolobus acidocaldarius* (Maruta *et al.*, 1996c). In this pathway, the terminal $\alpha(1 \rightarrow 4)$ -linked residue of the glucose polymer is converted to an $\alpha(1 \rightarrow 1)$ linkage by maltooligosyltrehalose synthase (MOT synthase, encoded by *treY*). The terminal disaccharide is subsequently cleaved by a hydrolase enzyme (MOT trehalohydrolase, encoded by *treZ*), releasing a free trehalose molecule (Fig. 1b). Finally, a third pathway has been identified in *Pimelobacter* (Tsusaki *et al.*, 1996) and *Thermus aquaticus* (Tsusaki *et al.*, 1997), in which the $\alpha(1 \rightarrow 4)$ link in maltose is converted to an $\alpha(1 \rightarrow 1)$ link to form trehalose (Fig. 1c). This reaction is catalysed by trehalose synthase, encoded by the *treS* gene. Both TreY and TreS are involved in rearranging the attachment at O-4 to O-1, but at least in *Pimelobacter* (Nishimoto *et al.*, 1996) and *Arthrobacter* (Nakada *et al.*, 1995) are constrained by the number of additional sugar residues attached to the penultimate glucose.

In mycobacteria, biosynthesis of trehalose from glucose 6-phosphate and UDP-glucose has been demonstrated. Trehalose-6-phosphate synthase (equivalent to OtsA of

E. coli) has been purified from *M. smegmatis*, with a molecular mass of 59–60 kDa estimated by gel filtration and PAGE (Pan *et al.*, 1996). Trehalose-6-phosphate phosphatase (OtsB) has also been partially purified, but its molecular mass was not determined (Matula *et al.*, 1971).

The availability of complete bacterial genome sequence information makes it possible to obtain a general overview of metabolic pathways within a particular organism in order to construct a framework for subsequent biological characterization (Moxon, 1995). In the present study, we have screened the genome sequence of *M. tuberculosis* (Cole *et al.*, 1998) for the presence of ORFs with homology to genes involved in trehalose biosynthesis in other bacteria. We report on evidence that all three of the previously described biosynthetic pathways are functional in mycobacteria.

METHODS

Genomics. *M. tuberculosis* genome sequence data were analysed using the BLAST server at the Sanger Centre (http://www.sanger.ac.uk/Projects/M_tuberculosis/). Data from the partially completed *Mycobacterium leprae* genome sequence was similarly searched (http://www.sanger.ac.uk/Projects/M_leprae/). The identified mycobacterial ORFs were then used to search the non-redundant GenEMBL database with the BLAST2 algorithm through a gateway provided by EMBL (<http://www.bork.embl-heidelberg.de:8080/Blast2/>), using TBLASTN, the BLOSUM62 matrix and an EXPECT value of 10, without filtering. Further analysis was performed using the GCG suite of programs sited at the HGMP Resource Centre, Hinxton, UK.

Growth of mycobacteria and preparation of extracts. *M. smegmatis* mc²6 was grown with shaking at 37 °C in LB medium (Gibco BRL). *Mycobacterium bovis* BCG (Pasteur) was grown in Middlebrook 7H9 medium with ADC supplement (Difco) and 0.5% (v/v) glycerol. Bacteria from exponentially growing cultures (200 ml) were harvested by centrifugation, washed with PBS and resuspended in 1/100 vol. PBS. Cells were disrupted by ultrasonication using a Soniprep 150 (MSE) for 10 s at an amplitude of 8 µm, and cell debris was removed by centrifugation for 5 min in a microfuge. The resulting supernatant was subjected to gel filtration using a PD10 column (Pharmacia) to remove small molecules such as trehalose and other substrates that may interfere with enzyme assays. Protein levels were determined using the Coomassie Plus Protein Assay (Pierce). For routine enzyme assays, cell extracts were used at 0.5 mg protein ml⁻¹. All enzyme assays were performed at least twice using extracts prepared from different cultures, and each assay was performed in duplicate.

Assay of trehalose biosynthesis

Measurement of trehalose. Trehalose was measured by assaying the release of free glucose following incubation of samples with a specific recombinant trehalase from *E. coli* prepared as described below. Samples for analysis (10 µl aliquots) were added to 10 µl 1 M NaH₂PO₄, pH 6.0, 80 µl water and 1 µl recombinant trehalase in microtitre-plate wells, and incubated at 37 °C for 1 h. Glucose was then measured by adding 100 µl 50 mM NaH₂PO₄, pH 6.0, containing 0.75 mg o-dianisidine ml⁻¹, 2 U glucose oxidase and 0.1 U peroxidase (all from Sigma). Colour development was measured after 30 min at 450 nm using a microtitre-plate reader, and a linear re-

lationship was obtained between trehalose concentration (0.2–1 mM) and absorbance.

Preparation of recombinant trehalase (TreA). The trehalase enzyme used in the assay was a partially purified preparation of recombinant *E. coli* TreA. The *treA* gene, cloned in the expression vector pBAD24, was kindly donated by Michael Ehrmann (University of Konstanz, Germany) together with an *E. coli* host strain (CS12) defective in several dextrin-cleaving enzymes (Prinz *et al.*, 1996). A periplasmic extract containing the recombinant trehalase was obtained using cold osmotic shock, as described by Nossal & Heppel (1966). Although other proteins were present in the recombinant preparation, no glucose was produced from maltose, maltoheptaose, sucrose, fructose, galactose or glycogen.

Trehalose biosynthesis assay. The assay mixture consisted of mycobacterial extract (40 µl) with appropriate substrates in a final volume of 100 µl in 100 mM NaH₂PO₄ at pH 6.0. For the OtsA–OtsB pathway, the substrates were UDP-glucose (10 mM) and glucose 6-phosphate (5 mM), with 10 mM MgCl₂. For the TreY–TreZ pathway, maltoheptaose (Sigma) was added at a concentration of 1% (w/v). For the TreS pathway, maltose (Sigma) was added at 1% (w/v). Assays were incubated at 37 °C for up to 1 h and the reaction stopped by boiling for 10 min. Denatured protein was removed by centrifugation for 2 min in a microfuge and aliquots of the supernatant were used to determine the level of trehalose using the assay described above.

Other enzyme assays

Trehalose-6-phosphate synthase. Trehalose-6-phosphate synthase (OtsA) activity was measured by determining the release of UDP from UDP-glucose in the presence of glucose 6-phosphate. Mycobacterial cell extract or purification fraction was added to a final volume of 100 µl in 20 mM Tris/HCl, pH 7.5, containing 10 mM glucose 6-phosphate, 5 mM UDP-glucose, 0.04 mg heparin ml⁻¹ (which enhances the enzyme activity; Pan *et al.*, 1996) and 10 mM MgCl₂, and incubated at 37 °C for 20 min. The sample was then boiled for 5 min, denatured proteins removed by centrifugation, and the amount of UDP in the supernatant measured in terms of oxidation of NADH in a linked assay with pyruvate kinase and lactic acid dehydrogenase. The assay was carried out in microtitre-plate wells containing 2.5 mM phosphoenolpyruvate, 0.5 mM NADH, 10 mM MgCl₂, 50 mM Tris/HCl, pH 7.5, 3 U pyruvate kinase ml⁻¹ and 3 U lactic acid dehydrogenase ml⁻¹ (both from Sigma). The decrease in absorbance at 340 nm was measured continuously in a microtitre-plate reader over a period of 20 min.

Trehalose-6-phosphate phosphatase. Phosphatase activity was measured in a final volume of 50 µl containing 10 mM Tris/HCl, pH 7.0, 2 mM MgCl₂, 2 mM trehalose 6-phosphate (Sigma) and an appropriate amount of enzyme preparation, in a microtitre plate at 37 °C for 0–30 min. To this, 200 µl filtered 1% ammonium molybdate, 0.15% malachite green and 12.5% (v/v) concentrated HCl was added and the absorbance at 630 nm measured to determine the amount of free phosphate produced (Matula *et al.*, 1971).

Maltose synthesis. Conversion of trehalose to maltose by *M. tuberculosis* trehalose synthase (TreS) was measured in an assay format analogous to that used to measure trehalose biosynthesis. Trehalose (1%, w/v) replaced maltose as the substrate, and glucose release was measured following incubation with maltase (Sigma) in place of trehalase.

Purification and N-terminal sequencing of *M. smegmatis* OtsA. Trehalose-6-phosphate synthase (OtsA) was purified

Table 1. Sequence of primers used in this study

Name	Sequence (5'–3')
OtsA-F	AAAAGGATCCATGACTTCGCGGGGTAAC
OtsA-R	GATCTTAAGCTTATGTCGCGTCGCGCGC
OtsB-F	AAGGATCCATGCCGGTCACCATAGAG
OtsB-Fcore	AAGGATCCATGTCGGAACCTCCCGAC
OtsB-R	AAAAGCTTGCGTTGAGTGCTACTAAG
TreS-F	TTGCTGATCAGTGCAGGGCAGTCACGTCGAA
TreS-R	TTGCAAGCTTGTCATAGGCGCCGCTCTCCC
TreY-F	AAGGATCCATGGCATTTCGCGTTATTTCC
TreY-R	AGCTAAGCTTTCAGGCATTGTCGCGCAC
TreZ-F	AGCTAAGCTTTCAGGCCGGCTCCGCAGC
TreZ-R	CGGGATCCATGCCTGAATTCCGAGTATGGG

from *M. smegmatis* using a procedure adapted from that described by Pan *et al.* (1978). *M. smegmatis* cell paste (25 g wet weight) was suspended in 30 ml 50 mM Tris/HCl buffer, pH 7.5, and lysed by sonication. Intact cells and insoluble debris were removed by centrifugation. Protein was precipitated in 50% saturated ammonium sulphate, collected by centrifugation, dissolved in 8 ml 50 mM Tris/HCl, pH 7.5, and dialysed overnight against the same buffer. The sample was fractionated by ion-exchange chromatography on a Mono Q-Sepharose column using a gradient of 0–500 mM KCl by HPLC. Fractions with enzyme activity were pooled and concentrated by 70% saturated ammonium sulphate precipitation and dialysis. The product was further fractionated on a heparin–agarose affinity column using a 50–1000 mM KCl gradient. All fractions were analysed for enzyme activity and by SDS-PAGE and Coomassie staining. Only those positive for trehalose-6-phosphate synthase activity were found to contain a band of 56 kDa. The positive fractions were pooled, separated by Tricine gel electrophoresis, blotted onto a PVDF membrane, the 56 kDa protein was eluted and N-terminal sequence determined on an automated ABI 476A protein sequencer (Applied Biosystems).

Production of recombinant proteins

Histidine-tagged recombinants. ORFs and partial ORFs with homology to *otsA*, *otsB*, *treY*, *treZ* and *treS* genes were amplified from *M. tuberculosis* and *M. leprae* chromosomal DNA by PCR using Deep Vent DNA polymerase (New England Biolabs) and the primers shown in Table 1.

The PCR products were cloned either directly into the expression vector pQE30 (Qiagen), or into the vector pPCR-Script-SK (Stratagene), prior to subcloning into pQE30. Recombinant clones were grown and expression induced by addition of IPTG according to the manufacturer's recommendations. In the case of the TreS construct, a fraction of the recombinant protein was found to be soluble in the bacterial lysate. This was purified from a 1 ml cleared sonicate obtained from an induced 100 ml culture, using an Ni-NTA spin column (Qiagen) according to the manufacturer's instructions. For the remaining clones, insoluble protein products were denatured with 8 M urea and purified by affinity chromatography under denaturing conditions on a Ni-NTA column, according to the manufacturer's recommended conditions (Qiagen). Purified recombinant proteins were renatured by stepwise dialysis against 50 mM Tris/HCl (pH 8.0) solutions containing 5 M, 2.5 M, 1 M and no urea, followed by another dialysis against 50 mM Tris/HCl, pH 8.0.

Recombinants in *M. smegmatis*. For expression in recombinant mycobacterial systems, PCR products containing the *M. tuberculosis treY* and *treZ* genes were cloned in the vector pACE (De Smet *et al.*, 1999). This is based on the mycobacterial–*E. coli* shuttle vector pOLY-G with the addition of a 3 kb fragment from the region upstream of the acetamidase gene of *M. smegmatis* (Parish & Stoker, 1997). Recombinant *M. smegmatis* clones were grown in minimal medium with 0.2% (w/v) succinate as the carbon source and 50 µg hygromycin ml⁻¹ (Boehringer). For protein expression, acetamide (0.2%, w/v) was added to exponential cultures which were grown for a further 24 h prior to harvesting and preparation of extracts by sonication.

RESULTS

Analysis of the *M. tuberculosis* genome sequence

Sequences generated from the *M. tuberculosis* genome project were screened for the presence of ORFs sharing homology with genes encoding components of trehalose biosynthesis pathways in other microbes. The results are summarized in Table 2.

A search based on the known *E. coli* trehalose biosynthesis genes identified ORF Rv3490 as a homologue of trehalose-6-phosphate synthases from *E. coli* (OtsA) and yeast (TPS1). A closely related ORF was found in the partially completed genome sequence of *M. leprae*. Two *M. tuberculosis* ORFs showed similarity to the phosphatase gene encoded by *otsB* in *E. coli*. Rv2006 encodes a protein with 1327 aa (named *otsB* by Cole *et al.*, 1998), and Rv3372 a smaller protein of 391 aa (*otsB2*). Both ORFs share a 260 aa 'core' region which is closely related to *E. coli* OtsB (266 aa) and to a similar core region of yeast TPS2 (896 aa). A related ORF in *M. leprae* encodes a 429 aa protein, again sharing the same core region. The different structures of the OtsB homologues are illustrated diagrammatically in Fig. 2. The additional N-terminal and C-terminal domains of the *M. tuberculosis otsB* gene share homology with two other *M. tuberculosis* ORFs, Rv3400 (35% identity over 238 aa) and Rv3401 (27% identity over 777 aa), respectively. A more limited homology is observed with N-terminal domains of *M. tuberculosis otsB2* (residues 190–282, 48% identity) and *M. leprae otsB* (residues

Table 2. Mycobacterial homologues of trehalose biosynthesis enzymes

<i>M. tuberculosis</i> ORF	Length (aa)	Name	Matching sequences	Accession no.	Length (aa)	Identity (%)	<i>M. leprae</i> homologue accession no.
Rv3490	500	OtsA	<i>E. coli</i> OtsA	P31677	495	33	Q50167
			<i>Sac. cerevisiae</i> TPS1	Q00764	473	36	
Rv2006	1327*	OtsB	<i>E. coli</i> OtsB	P31678	266	29	Q49734
			<i>Sac. cerevisiae</i> TPS2	P31688	896	28	
Rv3372	391	OtsB2	<i>E. coli</i> OtsB	P31678	266	32	Q49734
			<i>Sac. cerevisiae</i> TPS2	P31688	896	25	
			<i>M. tuberculosis</i> OtsB	Rv2006	1327	57	
Rv1563c	765	TreY	<i>Rhizobium</i> sp.	Q53237	772	45	Cosmid L458
			<i>Arthrobacter</i> sp.	Q44315	775	46	
Rv1562c	580	TreZ	<i>Rhizobium</i> sp.	Q53238	596	53	Cosmid L458
			<i>Arthrobacter</i> sp.	Q44316	598	53	
Rv0126	601	TreS	<i>Pimelobacter</i> sp.	P72235	573	71	Cosmid B1610
			<i>Streptomyces</i> sp.	O54207	572	69	

*Identities listed are for the core region of OtsB from residue 283 to 532.

Fig. 2. OtsB homologues in mycobacteria. ORFs encoded by *M. tuberculosis* *otsB* and *otsB2*, and *M. leprae* *otsB* share a conserved 260 aa core region closely related to *E. coli* *otsB* and *S. cerevisiae* TPS2. The additional N-terminal and C-terminal domains of the *M. tuberculosis* *otsB* gene share homology with two other *M. tuberculosis* ORFs, Rv3400 (35 % identity over 238 aa) and Rv3401 (27 % identity over 777 aa), respectively. A more limited homology is observed with N-terminal domains of *M. tuberculosis* *otsB2* (residues 190–282, 48 % identity) and *M. leprae* *otsB* (residues 81–160, 28 % identity).

81–160, 28 % identity). ORFs Rv3400 and Rv3401 have been annotated by Cole *et al.* (1998) as a possible phosphoglucomutase and an unknown protein, respectively.

Two ORFs (Rv1563c and Rv1562c) were identified in the *M. tuberculosis* genome as having 45 % and 53 % overall identity at amino acid level with MOT synthase (TreY) and MOT trehalohydrolase (TreZ) from *Rhizobium* and *Arthrobacter* (Table 2). The genes have

been named *glgY* and *glgZ* by Cole *et al.* (1998). We have used the designations *treY* and *treZ* in this study, to reflect the close similarity to the genes in *Rhizobium*, *Arthrobacter* and *Sulfolobus* [also, *glgY* has previously been used as an alternative designation for glycogen phosphorylase, *glgP*, in *E. coli* (Rimmele & Boos, 1994)]. The *M. tuberculosis* genes overlap by 7 bp, and are closely linked to a third gene, homologous to *glgX* of *E. coli* and *treX* of *Sulfolobus acidocaldarius*, encoding an α -amylase involved in glycogen debranching (Romeo *et*

Table 3. Biosynthesis of trehalose in mycobacterial extracts

The functional activity of the three trehalose biosynthesis pathways was assayed by incubation of mycobacterial protein extracts ($0.5 \text{ mg protein ml}^{-1}$) with appropriate substrates. Reactions were stopped at time points from 0–60 min, and glucose concentrations were measured before and after incubation with recombinant *E. coli* trehalase. The rate of trehalose biosynthesis was calculated by regression analysis over the linear phase of the reaction.

Substrate	Pathway	Trehalose generated*	
		<i>M. smegmatis</i>	<i>M. bovis</i> BCG
Glucose 6-phosphate + UDP-glucose	OtsAB	9.5	5.3
Maltoheptaose	TreYZ	41.6	18.5
Maltose	TreS	14.1	24.4

* nmol trehalose $\text{min}^{-1} (\text{mg protein})^{-1}$.

al., 1988). *M. leprae* cosmid L458 contains homologues of both *treY* and *treZ*, although both are interrupted by frameshifts and stop codons.

Finally, ORF Rv0126 was found to share approximately 70% overall identity with trehalose synthase (TreS) from *Pimelobacter* (Tsusaki *et al.*, 1996) and *Streptomyces* (Bruton *et al.*, 1995). A similar ORF is present in *M. leprae* (Table 2).

Analysis of enzyme function

Analysis of the *M. tuberculosis* genome identified genes for three distinct trehalose-biosynthesis pathways. We then set out to determine whether these three pathways could be detected by functional assays based on enzyme activities using mycobacterial extracts and recombinant proteins expressed from the corresponding ORFs.

Trehalose from glucose monosaccharide. To measure activities of the OtsA–OtsB pathway, glucose 6-phosphate and UDP-glucose were incubated with cell-free extracts from *M. smegmatis* and *M. bovis* BCG. Formation of trehalose was detected by adding recombinant *E. coli* trehalase (TreA) and measuring the glucose produced over a 1 h incubation period. Trehalose synthesis was observed using both extracts (Table 3); there was no detectable synthesis in extracts incubated without substrate. Constant readings in samples that were not treated with trehalase showed that there was no detectable release of free glucose from the glucose 6-phosphate or UDP-glucose substrates during the incubation period. The optimum pH for the trehalose biosynthesis reaction was between pH 6 and 7 (Fig. 3).

To determine whether the ORFs identified by genome analysis were responsible for the biosynthetic activity observed in crude extracts, trehalose-6-phosphate synthase (OtsA) was purified from *M. smegmatis* using a minor modification of a procedure described by Pan *et al.* (1978). The N-terminal amino acid sequence of the purified protein, PESGHETISGTSDFFVVANRLPVzE-

Fig. 3. Effect of pH on trehalose biosynthesis. Trehalose biosynthesis was measured in an *M. smegmatis* extract ($0.5 \text{ mg protein ml}^{-1}$) incubated with glucose 6-phosphate and UDP-glucose (●), maltoheptaose (□), or maltose (○). The pH of the reaction mixture was varied by changing the ratio of Na_2HPO_4 to NaH_2PO_4 in the assay buffer. Trehalose formation was measured as described in the legend to Table 3, and the percentage of the maximum activity is plotted for each pathway.

zLPDGQT, was identical to the *M. tuberculosis* OtsA ORF in 18 out of 30 residues, including an exact match over a stretch of 12 amino acids. This is consistent with the amino acid sequence of three internal peptides of the *M. smegmatis* enzyme determined by Pan *et al.* (1996), each of which showed over 75% identity to *M. tuberculosis* *otsA*. None of these peptide sequences produced comparable matches to other regions of the *M. tuberculosis* genomic sequence. Taken together, these results provide strong support for the conclusion that the *M. tuberculosis* *otsA* gene encodes the functional homologue of the trehalose-6-phosphate synthase activity detected in *M. smegmatis*. Direct attempts to measure activity of the *M. tuberculosis* gene product were frustrated by the fact that expression as a histidine-tagged recombinant protein in *E. coli* resulted in the generation of insoluble protein lacking detectable enzyme activity.

Mycobacterial sequences with OtsB homology – including the complete N-terminal region and the central OtsB core domain – were also cloned in *E. coli* using the expression vector pQE30 to generate proteins with an N-terminal affinity tag suitable for rapid purification. Again, all of the constructs formed insoluble inclusion bodies when expression was induced by IPTG. These were solubilized by denaturation in 8 M urea, purified by affinity chromatography, and then dialysed against decreasing concentrations of urea with the aim of allowing renaturation of functional enzymes. Activity was detected in one construct, which contained a 275 aa fragment from the *M. leprae* gene corresponding to the OtsB core domain. The purified renatured preparation catalysed the release of phosphate from trehalose 6-phosphate, with specificity demonstrated by lack of activity when glucose 6-phosphate, galactose 6-phosphate or fructose 6-phosphate was used as substrate (data not shown). Renaturation was inefficient, and several micrograms of protein were needed to detect a small amount of activity, with poor reproducibility between different batches of recombinant protein. These results suggest that the sequence homology with the OtsB core region is functionally significant, though they do not allow us to determine whether one or both of the *M. tuberculosis* homologues contributes to trehalose-6-phosphate phosphatase activity in cell extracts.

Trehalose from $\alpha(1 \rightarrow 4)$ -linked glucose polymer. To test for the presence of a functional TreY–TreZ pathway, *M. smegmatis* and *M. bovis* BCG extracts were incubated with maltoheptaose as substrate. Maltoheptaose is a heptameric oligosaccharide composed of glucose residues connected by $\alpha(1 \rightarrow 4)$ linkages identical to those found in glycogen. Again, biosynthesis of trehalose was observed (Table 3). The reaction was optimal at pH 6.5 (Fig. 3). Formation of trehalose from maltoheptaose could occur by formation of a terminal $\alpha(1 \rightarrow 1)$ linked intermediate with subsequent cleavage to release the disaccharide (the TreY–TreZ pathway), or by initial hydrolysis to generate maltose, followed by conversion to trehalose by the trehalose synthase activity described below. The assay system based on unfractionated cell extract does not distinguish between these two potential pathways.

With the aim of direct measurement of specific enzyme activities, the *M. tuberculosis* *treY* and *treZ* homologues were cloned in *E. coli* using the pQE30 expression vector. Again, the products were insoluble, and no functional activity could be recovered following affinity purification. Soluble products were obtained, however, when the genes were cloned into a mycobacterial expression vector, pACE, in *M. smegmatis* (De Smet *et al.*, 1999). In this system, the genes are placed under the control of an acetamidase promoter, with expression induced by addition of acetamide to cultures. Recombinant protein was not visible on Coomassie blue-stained gels, but production of trehalose from maltoheptaose was measured in soluble extracts from recombinant cultures, using a concentration selected to dilute out the background activity associated with the

Fig. 4. Trehalose biosynthesis from maltoheptaose by recombinant mycobacterial TreY and TreZ enzymes. Biosynthesis of trehalose from maltoheptaose was assayed using extracts (0.5 mg protein ml⁻¹) prepared from recombinant *M. smegmatis* clones expressing the *treY* and *treZ* genes of *M. tuberculosis*. Trehalose concentrations were calculated on the basis of glucose released following incubation with recombinant *E. coli* trehalase; results are corrected for free glucose observed in the absence of trehalase treatment. There was no detectable trehalose synthesis in the extract from vector control *M. smegmatis* (△) or in that from the TreY recombinant (●). Trehalose synthesis was observed using the extract from the TreZ recombinant (●), with enhanced activity when this was mixed with the TreY extract (□).

Fig. 5. Trehalose biosynthesis from maltose by recombinant mycobacterial TreS enzyme. Activity of the purified TreS recombinant is shown. The assay was carried out using maltose (○) or trehalose (●) as the substrate, and glucose was measured following treatment with trehalase or maltase respectively. Values are corrected for glucose detected prior to hydrolase treatment.

endogenous *M. smegmatis* enzymes in the vector-control extract (Fig. 4). There was no significant increase in trehalose synthesis using the extract from the recombinant expressing the *M. tuberculosis* MOT synthase (TreY) gene. Production of trehalose was observed, however, using the MOT trehalohydrolase (TreZ) recombinant, suggesting that the second step may be rate limiting in the pathway. This activity was enhanced when extract from the TreY recombinant was mixed with the TreZ extract. Enhanced activity was not observed if the TreY extract was replaced by the same concentration of the corresponding extract from the vector control. Fig. 4 shows results in trehalase-treated

samples, corrected for background levels measured without trehalase. Direct release of free glucose was detected when extracts from the TreZ recombinant were incubated with maltoheptaose, but this activity was reduced following addition of the TreY extract. This suggests that the TreZ enzyme has a preference for hydrolysis of maltooligosyltrehalose, but also has additional α -glucosidase activity.

Trehalose from maltose. Incubation of maltose with extracts from *M. smegmatis* and *M. bovis* BCG also resulted in production of trehalose (Table 3). The assay had a pH optimum of 6 (Fig. 3). Expression of the *M. tuberculosis* *treS* homologue as a His-tagged recombinant in *E. coli* using pQE30 generated mainly inactive, insoluble protein, but sufficient protein remained soluble for detection of enzyme activity. Enzyme activity able to convert maltose to trehalose was present in recombinant but not vector-control extracts (data not shown). The soluble recombinant protein retained trehalose synthase activity following purification by nickel affinity chromatography. Fig. 5 illustrates activity of the purified recombinant TreS using either trehalose or maltose as substrate. Although production of trehalose from maltose was some 2.5-fold greater than that of maltose from trehalose, the recombinant enzyme was capable of catalysing the reaction in both directions.

DISCUSSION

Biochemical experiments support the prediction from genome analysis that there are three routes for the synthesis of trehalose in mycobacteria. *M. tuberculosis* is unusual amongst the microbial genomes completed to date in having multiple pathways for trehalose biosynthesis; other organisms rely on a single pathway, most commonly OtsA–OtsB. Even the presence of two parallel pathways for the synthesis of a single product appears to be a rare occurrence, and to our knowledge has only been described in detail for diaminopimelate synthesis (Wehrmann *et al.*, 1998) and glutamate (Helling, 1998).

Interestingly, analysis of the partially completed genome sequence of *M. leprae* suggests that this closely related pathogen has dispensed with the TreY–TreZ pathway. Cosmid L458 contains a genomic region similar to that encoding TreY–TreZ in *M. tuberculosis*, but with regions homologous to the *M. tuberculosis* ORFs disrupted by multiple frame shifts and stop codons. Comparison of the genetic organization in the two genomes suggests that *M. leprae* has evolved a lifestyle in which it no longer requires a third trehalose-biosynthesis pathway, thus removing the selective pressure for retention of the functionally active *treY* and *treZ* genes.

In addition to having multiple distinct pathways for trehalose biosynthesis, *M. tuberculosis* also has two possible routes through the OtsA–OtsB pathway, with genome analysis identifying two enzymes containing the core functional domain of trehalose-6-phosphate phosphatase (OtsB). Both of the *otsB* genes are larger than

their *E. coli* counterpart. Both have an additional N-terminal domain. In the case of the larger *M. tuberculosis* OtsB, the N-terminal domain resembles ORF Rv3400, encoding a potential phosphoglucomutase, while the additional C-terminal domain has sequence similarity to a protein of unknown function encoded by Rv3401. Thus the structure of the *M. tuberculosis* *otsB* gene appears to combine sequences related to two other ORFs that are adjacent in the genome (Rv3400 and Rv3401) fused together by insertion of a copy of the OtsB core region. Biochemical studies carried out so far have addressed the functional activity only of the OtsB core; it will be of interest to determine whether *M. tuberculosis* OtsB is in fact a multifunctional enzyme encoded as a single polypeptide. The significance of having two OtsB enzymes is unknown. It can be reasoned that the N-terminal domain with potential phosphoglucomutase activity could facilitate trehalose biosynthesis by provision of the glucose 6-phosphate substrate, but the function of the C-terminal domain in the larger OtsB protein is unclear. Findings from genome-sequencing programmes indicate that the combination of multiple enzymes from a metabolic pathway within a single multifunctional polypeptide may be a relatively common phenomenon (Marcotta *et al.*, 1999).

Accumulation of trehalose in *E. coli* and *Sac. cerevisiae* under stress conditions is thought to contribute to maintenance of cell integrity and protein stability (Strom & Kaasen, 1993; De Virgilio *et al.*, 1994). The constitutive presence of trehalose in apparently unstressed mycobacteria may likewise have a protective function. Trehalose may act as an osmoprotectant, for example (Poolman & Glaasker, 1998). Consistent with such a role, the concentration of free trehalose relative to total protein increased 5–10-fold during growth of *M. smegmatis* in hyperosmotic medium (K. A. L. De Smet, unpublished observations). Bacteria are able to utilize a range of solutes to allow them to maintain their cytoplasmic volume and turgor pressure during exposure to osmotic stress (Csonka & Hanson, 1991), and analysis of the *M. tuberculosis* genome reveals the presence of additional systems that could modulate solute transport in response to osmotic stress. These include homologues of the glycine-betaine transporter genes of *Corynebacterium glutamicum* (*betP*, Rv0917) and *Bacillus subtilis* (*proW*, *proZ*, *proV* and *proX*; Rv3756c, Rv3757c, Rv3758c and Rv3579c), and the *trkA* potassium transporter of *Streptomyces coelicolor* (Rv2691, Rv2692). However, the ability to regulate trehalose concentrations may have a particular advantage in allowing response to changing osmotic pressure while avoiding the need for transport of solutes through the relatively impermeable mycobacterial cell wall.

If trehalose is indeed required for maintenance of mycobacterial cell integrity in response to external stress, disruption of trehalose metabolism could provide a target for drugs which will act against both actively dividing cells and quiescent or non-dividing populations. It will be of interest to exploit recent refinements in

techniques for mutagenesis of mycobacteria (Bardarov *et al.*, 1997; Pelicic *et al.*, 1998) to construct strains with disruptions in the genes described in the present study. While it might be anticipated that mutations will be compensated in part by complementary pathways, mutants might display unusual phenotypes under particular growth conditions which could provide insights into the basic function of trehalose in mycobacterial physiology.

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