

Structural analysis of the subunits of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae* and their function during heat shock

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Summary

Synthesis of trehalose in the yeast *Saccharomyces cerevisiae* is catalysed by the trehalose-6-phosphate (Tre6P) synthase/phosphatase complex, which is composed of at least three different subunits encoded by the genes *TPS1*, *TPS2*, and *TSL1*. Previous studies indicated that Tps1 and Tps2 carry the catalytic activities of trehalose synthesis, namely Tre6P synthase (Tps1) and Tre6P phosphatase (Tps2), while Tsl1 was suggested to have regulatory functions. In this study two different approaches have been used to clarify the molecular composition of the trehalose synthase complex as well as the functional role of its potential subunits. Two-hybrid analyses of the *in vivo* interactions of Tps1, Tps2, Tsl1, and Tps3, a protein with high homology to Tsl1, revealed that both Tsl1 and Tps3 can interact with Tps1 and Tps2; the latter two proteins also interact with each other. In addition, trehalose metabolism upon heat shock was analysed in a set of 16 isogenic yeast strains carrying deletions of *TPS1*, *TPS2*, *TSL1*, and *TPS3* in all possible combinations. These results not only confirm the previously suggested roles for Tps1 and Tps2, but also provide, for the first time, evidence that Tsl1 and Tps3 may share a common function with respect to regulation and/or structural stabilization of the Tre6P synthase/phosphatase complex in exponentially growing, heat-shocked cells.

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Introduction

The non-reducing disaccharide trehalose (α -D-glucopyranosyl α -D-glucopyranoside) is widespread in nature and has been found in many prokaryotes, fungi, and some lower plants and animals (Elbein, 1974). Interestingly, trehalose synthesis is induced in most of these organisms in response to a small set of specific environmental conditions. In particular, trehalose is accumulated in phases of nutrient starvation, desiccation, and after exposure to a mild heat shock (for reviews see Van Laere, 1989; Wiemken, 1990; Crowe *et al.*, 1992). It has been suggested, therefore, that trehalose plays a role as a stabiliser of cellular structures under stress conditions (Keller *et al.*, 1982; Crowe *et al.*, 1984). In accordance with this suggestion, *in vitro* studies have revealed the exceptional capability of trehalose in protecting biological membranes and enzymes from freezing- or drying-induced dehydration (for a review see Crowe *et al.*, 1992) and heat stress (Hottiger *et al.*, 1994).

As early as 1958 (Cabib and Leloir, 1958) trehalose biosynthesis was found to be catalysed by a two-step process involving trehalose-6-phosphate (Tre6P) synthase and Tre6P phosphatase. Since then, several studies have dealt with the purification and kinetic properties of Tre6P synthase and Tre6P phosphatase in yeast (Panek *et al.*, 1987; Vandercammen *et al.*, 1989; Bell *et al.*, 1992; De Virgilio *et al.*, 1993; Londesborough and Vuorio, 1993). The overall picture that emerged from these studies is that both enzymes are part of a multimeric protein complex with an approximate molecular mass of 630–800 kDa. This complex, the Tre6P synthase/phosphatase complex, is composed of at least three different subunits with apparent molecular masses of 56, 102, and 123 kDa. The corresponding genes, *TPS1* (encoding the 56 kDa subunit; Bell *et al.*, 1992), *TPS2* (encoding the 102 kDa subunit; De Virgilio *et al.*, 1993), and *TSL1* (encoding the 123 kDa subunit; Vuorio *et al.*, 1993), have been identified and sequenced. Based on its homology to Tsl1 (55% identity upon optimal alignment over the entire amino acid sequence; Manning *et al.*, 1992), Tps3 may constitute a fourth subunit of the complex. Remarkably, the deduced amino acid sequence of *TPS1* shows striking similarity over its

entire sequence to parts of the deduced amino acid sequences of all three (*TPS2*, *TSL1*, and *TPS3*).

Several observations indicate that Tps1 may carry the catalytic activity of the Tre6P synthase: (i) *tps1Δ* strains lack any detectable Tre6P synthase activity (Bell *et al.*, 1992); (ii) *TPS1* expression in *Escherichia coli* (Vuorio *et al.*, 1993) and in yeast (W. Bell *et al.*, submitted) results in greatly increased Tre6P synthase activity; (iii) *TPS1* complements the trehalose-synthesis defect of an *E. coli otsA* mutant (McDougall *et al.*, 1993); and (iv) transgenic tobacco plants expressing *TPS1* are able to synthesize trehalose (Holmström *et al.*, 1996). It was therefore surprising that *TPS1* was found to be identical to the previously sequenced *CIF1* (catabolite inactivation of fructose-1,6-bisphosphatase), a gene reported to be essential for growth on glucose in *Saccharomyces cerevisiae* (González *et al.*, 1992). Several mutations described earlier as *fdp1*, *cif1*, *byp1*, and *glc6* have been shown to be alleles of the same gene and to exert a wide range of pleiotropic phenotypes (reviewed in Thevelein and Hohmann, 1995). An important phenotype of all these mutants (except *glc6*), however, is that they are unable to grow on glucose, apparently because of an uncontrolled influx of glucose into the glycolytic pathway (Van Aelst *et al.*, 1991; Hohmann *et al.*, 1993; Blázquez *et al.*, 1993; Blázquez and Gancedo, 1994). At least three different models for this unexpected role of Tps1 (also called Ggs1 in this context) in the control of glycolysis have been presented, as follows. (i) Trehalose synthesis could serve as a metabolic buffer system by draining off excessively synthesized sugar phosphates into trehalose and thereby releasing phosphate which is required further along the glycolytic pathway for the glyceraldehyde-3-phosphate dehydrogenase reaction. (ii) Tre6P has been shown to inhibit *S. cerevisiae* hexokinases *in vitro* and its absence in *tps1Δ* strains could result in unrestrained activity of hexokinases, leading to an excessive flux of glucose through the early steps of glycolysis. (iii) Finally, Tps1 could directly regulate sugar transport and/or sugar phosphorylation, in addition to its role as a subunit of the Tre6P synthase/phosphatase complex (see Thevelein and Hohmann, 1995, for a review of these models).

Disruption of *TPS2* causes loss of Tre6P phosphatase activity and accumulation of Tre6P under conditions in which wild-type cells normally accumulate trehalose (heat shock and stationary phase), suggesting that Tps2 carries the catalytic activity of Tre6P phosphatase (De Virgilio *et al.*, 1993). While *TPS1* and *TPS2* encode Tre6P synthase and Tre6P phosphatase, respectively, the role of the *TSL1* (for Tre6P synthase long chain) gene product with respect to trehalose synthesis is not yet understood. It was reported that partial proteolytic degradation of Tsl1, the 123 kDa subunit of the complex, reduces the phosphate-mediated inhibition as well as the fructose-6-phosphate-mediated

activation of Tre6P synthase (Vuorio *et al.*, 1993). Thus, Tsl1 may be involved in modification of the kinetic properties of Tre6P synthase activity. Recent studies on *TPS3* and *TSL1* expression levels suggest that Tsl1 and Tps3 may to some extent act as interchangeable regulators of the Tre6P synthase/phosphatase complex, but that they also may exert different regulation under specific growth conditions (Winderickx *et al.*, 1996). The precise function of both proteins, however, is not clear. The present study was undertaken in order to clarify the molecular composition of the Tre6P synthase complex as well as the functional role of its potential subunits. Our results using the two-hybrid system (Fields and Sternglanz, 1994) show that both Tsl1 and Tps3 can interact with Tps1 and Tps2 *in vivo*, while the latter two proteins also interact with each other. In addition, we show, by analysis of the heat-induced responses of trehalose metabolism in deletion mutants, that all four potential subunits indeed have a functional role in the synthesis of trehalose.

Results

Two-hybrid analysis of interactions between Tps1, Tps2, Tps3, and Tsl1

A variety of biochemical data suggest that Tps1, Tps2, and Tsl1 interact intimately with each other to form the Tre6P synthase/phosphatase complex (Bell *et al.*, 1992; De Virgilio *et al.*, 1993; Vuorio *et al.*, 1993; Londesborough and Vuorio, 1993). Based on its homology to Tsl1, a fourth protein, Tps3, may also be part of this complex (De Virgilio *et al.*, 1993). To investigate the possible interactions of these four proteins, directed two-hybrid analysis (Fields and Sternglanz, 1994) was performed. Strong interactions (based on β-galactosidase activities) were determined for all possible combinations of pairs between Tps1-AD, Tps2-AD, and Tps3-AD and Tps1-DBD, Tps2-DBD, and Tps3-DBD (Table 1). Thus, Tps1, Tps2, and Tps3 appear to interact not only directly with each other, but also with themselves, suggesting that each of these subunits may be present as a homodimer or homomultimer in the Tre6P synthase/phosphatase complex. It must be pointed out that these data provide the first experimental evidence that Tps3 may in fact be a part of this complex.

As a full-length Tsl1-AD did not interact with any of the other Tps-DBD fusions (data not shown), we tested a truncated Tsl1-AD (Tsl1-I-AD; amino acids 60–332), which was originally isolated in a two-hybrid screen for proteins interacting with Tps2, for interaction with the other proteins. Accordingly, Tsl1-I-AD showed weak interaction with Tps1-DBD, strong interaction with Tps2-DBD, and very weak or no interaction with Tps3-DBD and Tsl1-DBD. The full-length Tsl1-DBD interacted weakly with both Tps1-AD and Tps2-AD, but not with Tps3-AD or

Table 1. Two-hybrid interactions among the subunits of the trehalose-6-phosphate synthase complex in *Saccharomyces cerevisiae*.

AD fusions	DBD Fusions					pEG202
	<i>TPS1</i>	<i>TPS2</i>	<i>TPS3</i>	<i>TSL1</i>	<i>MSB2</i>	
<i>TPS1</i>	548	150	696	49	1	5
<i>TPS2</i>	208	528	411	16	2	17
<i>TPS3</i>	708	239	425	3	18	8
<i>TSL1-I</i>	53	346	20	3	12	6
pJG4-5	2	1	3	2	18	10

Possible interactions among Tps1, Tps2, Tps3, and Tsl1 were detected using the two-hybrid system described in the *Experimental procedures*. Numbers represent mean β -galactosidase activities (in Miller units) from three independent transformants for each pair of plasmids. *TSL1-I* codes for an internal part of Tsl1 (amino acids 60–332). pJG4-5 indicates the AD vector with no insert; pEG202 is the DBD vector with no insert. Values that were at least 20-fold higher than the corresponding control (pJG4-5 without insert) are shown in bold.

Tsl1-I-AD. Thus, Tps3 and Tsl1 appear to interact with both Tps1 and Tps2, but not with each other.

Effects of *TPS1*, *TPS2*, *TPS3*, and *TSL1* deletions

To determine the consequences of the loss of Tps1, Tps2, Tps3, and Tsl1 for trehalose metabolism during heat shock, we replaced the complete *TPS1*, *TPS2*, *TPS3*, and *TSL1* coding sequences in the wild-type strain YSH 6.106.-3A with *TRP1*, *LEU2*, *URA3*, and *HIS3*, respectively. An isogenic set of 15 strains carrying these four deletions in all possible combinations was constructed as described in the *Experimental procedures*. While strains with an intact

Tps1 were able to grow on glucose, all *tps1Δ* strains were defective for growth on glucose, irrespective of additional mutations in *TPS2*, *TPS3*, or *TSL1*, or any combination of these deletions (W. Bell *et al.*, submitted). As none of the strains was defective for growth on galactose, the following experiments were all carried out on media containing galactose as carbon source. The 15 strains and their isogenic wild-type strain YSH 6.106.-3A were grown to exponential phase at 27°C, subjected to a heat shock for 1 h at 42°C, and assayed for their trehalose and Tre6P contents as well as for their activities of Tre6P synthase and Tre6P phosphatase (Table 2). As expected, wild-type cells had very high Tre6P synthase (0.543 $\mu\text{kat g}^{-1}$ protein) and Tre6P phosphatase (0.051 $\mu\text{kat g}^{-1}$ protein) activities *in vitro* and contained high levels of trehalose (0.327 g g^{-1} protein) but very low levels of Tre6P (<0.010 g g^{-1} protein) *in vivo* (Table 2, row 1).

Previous studies suggested that the catalytic subunits of Tre6P synthase and Tre6P phosphatase are encoded by *TPS1* and *TPS2*, respectively (Bell *et al.*, 1992; De Virgilio *et al.*, 1993). In accordance with these suggestions, all strains harbouring *tps1Δ* were found to have virtually no Tre6P synthase activity and to be unable to accumulate detectable amounts of trehalose or Tre6P during heat shock (Table 2, rows 2, 6, 7, 8, 12, 13, 14, and 16). In addition, all strains carrying *tps2Δ* had drastically reduced Tre6P phosphatase activities and were found to accumulate high levels of Tre6P during heat shock (between 0.257 and 0.199 g g^{-1} protein) provided that they contained a functional *TPS1* (Table 2, rows 3, 9, 10, and 15). A residual level of Tre6P phosphatase activity (below 9 ngat g^{-1} protein) was found in all *tps2Δ* strains including

Table 2. Trehalose and trehalose-6-phosphate levels and activities of trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in heat-shocked wild-type yeast and mutants with various combinations of *tps1Δ*, *tps2Δ*, *tps3Δ*, and *tsl1Δ*.

Row no.	Strain	Relevant genotype	Trehalose (g g^{-1} protein)	Tre6P (g g^{-1} protein)	Tre6P synthase ($\mu\text{kat g}^{-1}$ protein)	Tre6P phosphatase ($\mu\text{kat g}^{-1}$ protein)
1	YSH 6.106.-3A	Wild type	0.327 $\pm$ 0.016	< 0.010	0.543 $\pm$ 0.133	0.051 $\pm$ 0.002
2	YSH 6.106.-1A	<i>tps1Δ</i>	< 0.005	< 0.010	< 0.001	0.015 $\pm$ 0.006
3	YSH 6.106.-8C	<i>tps2Δ</i>	0.022 $\pm$ 0.003	0.199 $\pm$ 0.041	0.069 $\pm$ 0.036	0.004 $\pm$ 0.002
4	YSH 6.106.-2B	<i>tps3Δ</i>	0.344 $\pm$ 0.014	< 0.010	0.336 $\pm$ 0.083	0.051 $\pm$ 0.016
5	YSH 6.106.-19B	<i>tsl1Δ</i>	0.352 $\pm$ 0.056	< 0.010	0.427 $\pm$ 0.029	0.055 $\pm$ 0.014
6	YSH 6.106.-16D	<i>tps1Δ tps2Δ</i>	< 0.005	< 0.010	< 0.001	0.008 $\pm$ 0.007
7	YSH 6.106.-5B	<i>tps1Δ tps3Δ</i>	< 0.005	< 0.010	< 0.001	0.013 $\pm$ 0.005
8	YSH 6.106.-6A	<i>tps1Δ tsl1Δ</i>	< 0.005	< 0.010	< 0.001	0.014 $\pm$ 0.007
9	YSH 6.106.-19A	<i>tps2Δ tps3Δ</i>	0.020 $\pm$ 0.006	0.220 $\pm$ 0.020	0.031 $\pm$ 0.028	0.005 $\pm$ 0.001
10	YSH 6.106.-14B	<i>tps2Δ tsl1Δ</i>	0.015 $\pm$ 0.004	0.257 $\pm$ 0.010	0.027 $\pm$ 0.026	0.004 $\pm$ 0.001
11	YSH 6.106.-16C	<i>tps3Δ tsl1Δ</i>	0.128 $\pm$ 0.018	0.015 $\pm$ 0.010	0.011 $\pm$ 0.008	0.051 $\pm$ 0.006
12	YSH 6.106.-2D	<i>tps1Δ tps2Δ tps3Δ</i>	< 0.005	< 0.010	< 0.001	0.007 $\pm$ 0.004
13	YSH 6.106.-10A	<i>tps1Δ tps2Δ tsl1Δ</i>	< 0.005	< 0.010	< 0.001	0.008 $\pm$ 0.004
14	YSH 6.106.-8A	<i>tps1Δ tps3Δ tsl1Δ</i>	< 0.005	< 0.010	0.004 $\pm$ 0.003	0.008 $\pm$ 0.002
15	YSH 6.106.-1D	<i>tps2Δ tps3Δ tsl1Δ</i>	0.030 $\pm$ 0.013	0.252 $\pm$ 0.036	0.011 $\pm$ 0.005	0.008 $\pm$ 0.002
16	YSH 6.106.-4C	<i>tps1Δ tps2Δ tps3Δ tsl1Δ</i>	< 0.005	< 0.010	0.002 $\pm$ 0.002	0.005 $\pm$ 0.002

Log-phase cells of the individual strains were grown at 27°C, transferred to a water bath at 42°C, and incubated for 1 h. Results shown represent the mean $\pm$ SE of three to four independent experiments. Detection limits for the levels of trehalose, Tre6P, Tre6P synthase activity, and Tre6P phosphatase activity were 0.005 g g^{-1} protein, 0.010 g g^{-1} protein, 0.001 $\mu\text{kat g}^{-1}$ protein, and 0.002 $\mu\text{kat g}^{-1}$ protein, respectively.

the quadruple *tps1Δ tps2Δ tps3Δ tsl1Δ* strain (Table 2, rows 3, 6, 9, 10, 12, 13, 15 and 16), indicating that Tps1, Tps3, and Tsl1 are not responsible for this activity. The residual Tre6P phosphatase activity may be due to unspecific phosphatases.

Surprisingly, all strains carrying *tps1Δ* and a functional *TPS2* were not only defective for Tre6P synthase activity but also had greatly reduced levels of Tre6P phosphatase activity (Table 2, rows 2, 7, 8 and 14). Conversely, all strains carrying *tps2Δ* and a functional *TPS1* were not only defective for Tre6P phosphatase activity but also had significantly reduced levels of Tre6P synthase activity (Table 2, rows 3, 9, 10 and 15). Despite the low levels of *in vitro* detectable Tre6P synthase activity, all *TPS1 tps2Δ* strains were found to accumulate high amounts of Tre6P during heat shock (see above), indicating that their *in vivo* Tre6P synthase activities were sufficient to sustain this accumulation and are relatively unaffected by the loss of Tps2. Therefore, the low Tre6P synthase activities in *TPS1 tps2Δ* strains measured *in vitro* may be a reflection of an impaired structural integrity of the entire Tre6P synthase/phosphatase complex in extracts lacking Tps2. Similarly, the low activity of Tre6P phosphatase activity in *tps1Δ TPS2* strains may also be a result of the destabilization of the complex in extracts where Tps1 is absent. The triple deletion strain *tps2Δ tps3Δ tsl1Δ* (Table 2, row 15) was found to have low, but detectable, Tre6P synthase activity and to accumulate large amounts of Tre6P. Thus, the presence of Tps2, Tps3, and Tsl1 seems not to be a prerequisite for normal functioning of the Tre6P synthase *in vivo* under heat-shock conditions.

Earlier studies suggested that Tsl1 may be a regulatory protein which mediates fructose-6-phosphate activation and P_i inhibition of Tre6P synthase (Londesborough and Vuorio, 1993). Given the sequence similarity between Tsl1 and Tps3, both proteins may actually perform redundant regulatory functions or perform their functions under different physiological conditions. We found that deletion of *TPS3* (Table 2, row 4) resulted in only moderate reduction of Tre6P synthase ($0.336 \mu\text{kat g}^{-1}$ protein) and did not affect the Tre6P phosphatase ($0.051 \mu\text{kat g}^{-1}$ protein) activity when compared with the wild type. In accordance with these results, trehalose (0.344 g g^{-1} protein) and Tre6P ($<0.010 \text{ g g}^{-1}$ protein) levels also did not differ significantly from those in the wild-type strain (Table 2, row 1). Deletion of *TSL1* (Table 2, row 5) also resulted in a moderate reduction of Tre6P synthase activity ($0.427 \mu\text{kat g}^{-1}$ protein) but no reduction of Tre6P phosphatase activity ($0.055 \mu\text{kat g}^{-1}$ protein), and also had no significant effect on the amounts of trehalose (0.352 g g^{-1} protein) or Tre6P ($<0.010 \text{ g g}^{-1}$ protein) accumulated during heat shock, compared to the wild-type strain (Table 2, row 1). Thus, both single mutants, *tps3Δ* and *tsl1Δ*, were not significantly reduced in their Tre6P synthase activities or in

their ability to accumulate trehalose. In contrast, deletion of both *TPS3* and *TSL1* (Table 2, row 11) was found to cause not only a dramatic reduction in Tre6P synthase activity ($0.011 \mu\text{kat g}^{-1}$ protein) but also a significant reduction in the amount of accumulated trehalose (0.128 g g^{-1} protein) during heat shock, relative to the wild-type strain (Table 2, row 1). Tre6P phosphatase activity ($0.051 \mu\text{kat g}^{-1}$ protein) and Tre6P levels (0.015 g g^{-1} protein) were not significantly altered in the *tps3Δ tsl1Δ* strain. These results indicate that Tps3 and Tsl1 indeed share a functional role with respect to the regulation of Tre6P synthase activity.

Discussion

A number of publications have dealt with the structural and functional analysis of the Tre6P synthase complex in *S. cerevisiae*. The biochemical and genetic data available suggest that the three proteins Tps1, Tps2, and Tsl1 form a multimeric protein complex (Bell *et al.*, 1992; De Virgilio *et al.*, 1993; Vuorio *et al.*, 1993; Londesborough and Vuorio, 1993). In the present study we have performed a series of experiments to further elucidate the structural composition of the Tre6P synthase complex as well as the specific role of its potential subunits. Using the two-hybrid system, we show that these three proteins can physically interact with each other *in vivo*. A fourth protein with high homology to Tsl1, namely Tps3, was also found to interact with both Tps1 and Tps2 in the directed two-hybrid assay. Interestingly, while Tps1 and Tps2 interacted with all other subunits, including themselves, we could not detect any direct interaction between Tsl1 and Tps3, which may simply be explained by the potentially altered tertiary structure, lower protein stability, or low expression level of one of these two-hybrid constructs (see also Fields and Sternglanz, 1994). However, the absence of detectable Tsl1–Tps3 interaction may also indicate that binding of these two proteins to the Tre6P synthase complex may be either spatially or temporally separated, thus allowing the cells to regulate trehalose synthesis in response to a broader range of different physiological conditions. In accordance with such a model, *TPS3* and *TSL1* have been reported to be differentially expressed in *S. cerevisiae*. While *TPS3* is expressed at a constant rate in exponentially growing and stationary-phase cells, *TSL1* expression is greatly enhanced upon entrance into stationary phase (Winderickx *et al.*, 1996). Thus, the two proteins may indeed mediate, to some extent, differential regulation of the Tre6P synthase complex according to their relative abundance under different physiological conditions (e.g. exponential phase and stationary phase). Nevertheless, our finding that the *tps3Δ tsl1Δ* double mutant, in contrast to the corresponding single mutants, was seriously impaired in Tre6P

synthase activity indicates that both proteins must also share some common functions with respect to trehalose synthesis in exponentially growing, heat-shocked cells. In this context, it must be pointed out that our analysis of the *tps3Δ tsl1Δ* double mutant together with the results of the two-hybrid assays for the first time indicate that Tps3 is a subunit of the Tre6P synthase complex. Whether Tps3 is also involved in mediation of fructose-6-phosphate activation and P_i inhibition of the Tre6P synthase, as has been suggested for Tsl1 (Londesborough and Vuorio, 1993), and whether this regulation may vary according to the physiological conditions, remain to be elucidated.

Despite detailed biochemical analyses of the Tre6P synthase complex, the stoichiometry of the various subunits is still unclear. Gel filtration experiments showed that the molecular mass of the complex is around 630–800 kDa (Londesborough and Vuorio, 1991; Bell *et al.*, 1992). As the sum of the molecular masses of the four putative subunits only adds up to ≈ 400 kDa, either some or all of the subunits must exist in more than one copy in the complex. In support of this hypothesis, we found that Tps1, Tps2, and Tps3 were able to interact with themselves in the two-hybrid system, indicating that they may be present as homodimers or homomultimers in the Tre6P synthase complex. Gel filtration experiments revealed that defined fractions representing proteins with an approximate molecular mass of 100 kDa contained only free Tps1 (56 kDa; W. Bell *et al.*, submitted), indicating that Tps1 may form a homodimer. Further detailed biochemical studies will be necessary to elucidate the exact stoichiometry of the subunits of the Tre6P synthase complex and to determine whether the conclusions drawn from the results of our two-hybrid analyses reflect the situation *in vivo*. The interactions measured by the two-hybrid method now can be used to identify the domains on the subunits responsible for the interaction and, in particular, to find out whether the Tps1-homologous sections of Tps2, Tsl1, and Tps3 serve a function in the assembly of the complex.

Analysis of the isogenic set of strains carrying deletions of *TPS1*, *TPS2*, *TPS3*, and *TSL1* in all possible combinations confirmed the previously suggested roles for Tps1 and Tps2 (Bell *et al.*, 1992; De Virgilio *et al.*, 1993). As all strains deleted for *TPS1* lost their Tre6P synthase activity and were unable to accumulate trehalose or Tre6P during heat shock, we can conclude that *TPS1* codes for the Tre6P synthase. In addition, the finding that all *tps2Δ* strains had strongly reduced Tre6P phosphatase activities and accumulated high amounts of Tre6P, as long as they contained a functional *TPS1* gene, confirms that *TPS2* codes for the Tre6P phosphatase. Despite the absence of the specific Tre6P phosphatase activity, all *tps2Δ* strains including the quadruple *tps1Δ tps2Δ tps3Δ tsl1Δ* mutant were found to have

residual Tre6P phosphatase activity. As none of the known subunits can be responsible for this residual activity in the quadruple deletion strain, we suggest that this activity is due to unspecific phosphatases. This would also explain the small amount of trehalose found in the *tps2Δ TPS1* strain.

A further interesting aspect of our studies is the finding that all strains deleted for *TPS1* have greatly reduced Tre6P phosphatase activities. Similar results were reported by Vuorio *et al.* (1993) and led to the speculation that Tps1 may also carry Tre6P phosphatase activity. This seems unlikely, however, as *tps2Δ* strains with an intact *TPS1* only accumulated residual amounts of trehalose (see above). It is therefore more likely that deletion of *TPS1* leads to a destabilization of the trehalose synthase complex, and hence to a decrease in Tre6P phosphatase activity, which is especially apparent under the conditions of the *in vitro* assay. Likewise, strains deleted for *TPS2* also lost most of their Tre6P synthase activity *in vitro*, even though they were able to synthesize Tre6P during heat shock, indicating that the Tre6P synthase was functional *in vivo*. Thus, it is possible that the absence of Tps2 also leads to destabilization of the Tre6P synthase complex, especially during the *in vitro* assay. In accordance with this suggestion, we previously found that Tre6P synthase activity in *tps2Δ* strains was not significantly reduced if determined in permeabilized cells (De Virgilio *et al.*, 1993), a method much less disruptive than the cell-extraction method used in this study. As particularly small proteins (<80 kDa, e.g. Tps1) may be lost during sample preparation in permeabilized cells (Miozzari *et al.*, 1978), use of cell extracts was clearly the method of choice in the present study, where the occurrence of free Tps1 in the extracts of some mutant strains could be anticipated. From these observations it is obvious that the Tre6P synthase activities measured *in vitro* often do not reflect the actual *in vivo* situation. A further example of the discrepancy between *in vitro* and *in vivo* activity of the Tre6P synthase is provided by the triple *tps2Δ tps3Δ tsl1Δ* mutant which has almost no detectable Tre6P synthase activity and yet is able to accumulate high amounts of Tre6P during a mild heat shock. This triple-deletion mutant is especially interesting as Tps1 seems to be able to synthesize a large amount of Tre6P even though it is probably present as a free subunit in this strain. Based on the finding that the *tps3Δ tsl1Δ* double mutant, in contrast to the *tps2Δ tps3Δ tsl1Δ* triple mutant, is seriously defective for Tre6P and trehalose synthesis one may speculate that the free Tps1 subunit may be negatively regulated by binding to Tps2. In this context, future studies should undoubtedly focus on the potential regulation of Tre6P synthase activity by the other subunits of the Tre6P synthase/phosphatase complex. In particular, it will be interesting to determine the exact biochemical

Table 3. List of strains used in this work.

Strain	Genotype	Source
YSH 6.106.-3A	α <i>leu2 ura3 trp1his3 ade2</i>	This work ^a
YSH 6.106.-1A	α <i>tps1Δ::TRP1</i>	This work ^a
YSH 6.106.-8C	α <i>tps2Δ::LEU2</i>	This work ^a
YSH 6.106.-2B	α <i>tps3Δ::URA3</i>	This work ^a
YSH 6.106.-19B	α <i>tsl1Δ::HIS3</i>	This work ^a
YSH 6.106.-16D	α <i>tps1Δ::TRP1 tps2Δ::LEU2</i>	This work ^a
YSH 6.106.-5B	α <i>tps1Δ::TRP1 tps3Δ::URA3</i>	This work ^a
YSH 6.106.-6A	α <i>tps1Δ::TRP1 tsl1Δ::HIS3</i>	This work ^a
YSH 6.106.-19A	α <i>tps2Δ::LEU2 tps3Δ::URA3</i>	This work ^a
YSH 6.106.-14B	α <i>tps2Δ::LEU2 tsl1Δ::HIS3</i>	This work ^a
YSH 6.106.-16C	α <i>tps3Δ::URA3 tsl1Δ::HIS3</i>	This work ^a
YSH 6.106.-2D	α <i>tps1Δ::TRP1 tps2Δ::LEU2 tps3Δ::URA3</i>	This work ^a
YSH 6.106.-10A	α <i>tps1Δ::TRP1 tps2Δ::LEU2 tsl1Δ::HIS3</i>	This work ^a
YSH 6.106.-8A	α <i>tps1Δ::TRP1 tps3Δ::URA3 tsl1Δ::HIS3</i>	This work ^a
YSH 6.106.-1D	α <i>tps2Δ::LEU2 tps3Δ::URA3 tsl1Δ::HIS3</i>	This work ^a
YSH 6.106.-4C	α <i>tps1Δ::TRP1 tps2Δ::LEU2 tps3Δ::URA3</i> <i>tsl1Δ::HIS3</i>	This work ^a
EGY48	α <i>his3 trp1ura3 LEU2::pLexAop6-LEU2</i>	Zervos <i>et al.</i> (1993)

a. Isogenic to YSH 6.106.-3A except for the individual deletions. YSH 6.106.-3A is isogenic to, and a direct sibling of, W303-1A (a *leu-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2*; Thomas and Rothstein, 1989).

nature as well as the physiological relevance of the suggested Tps3/Tsl1-mediated regulation of trehalose synthesis. Our two-hybrid studies as well as our functional analysis of the subunits of the trehalose synthase complex provide an excellent basis for such future studies.

Experimental procedures

Strains, media, and microbiological and recombinant DNA methods

The *S. cerevisiae* strains used in this study are listed in Table 3. *E. coli* strain JMB9 ($(r^{-}m^{+}) \Delta trpF$) (Sterner *et al.*, 1995) was used to rescue pJG4-5-based plasmids from strain EGY48. The transformed cells were plated directly onto Vogel–Bonner minimal plates (Davis *et al.*, 1980) supplemented with 0.2% (w/v) glucose, 0.5% (w/v) casamino acid hydrolysate, 0.01 mM FeCl₃ and 100 mg ampicillin l⁻¹. Other plasmid manipulations were performed in *E. coli* strain DH5 α (Gibco BRL) using standard procedures (Sambrook *et al.*, 1989). Standard procedures of yeast genetics and molecular biology (Guthrie and Fink, 1991; Sambrook *et al.*, 1989) were used. Yeast transformations were performed using a modification of the Li⁺-ion method (Gietz *et al.*, 1992).

Yeast and *E. coli* media, including defined media (SD with appropriate amino acid supplements) were prepared according to standard recipes (Sambrook *et al.*, 1989; Rose *et al.*, 1990). Yeast cell cultures were incubated on a rotary shaker (140 r.p.m.) at 27°C, taking care that the cell densities were below 5 × 10⁶ cells ml⁻¹ at the beginning of the heat-shock experiments (1 h at 42°C).

The complete open reading frames of *TPS1*, *TPS2*, *TSL1*, and *TPS3* were deleted by the method of Eberhardt and Hohmann (1995). The strategies for deletion of *TPS1* (Van Aelst *et al.*, 1993) and *TPS2* (Hohmann *et al.*, 1996) have been described elsewhere. For deletion of *TSL1*, a plasmid carrying the *TSL1* open reading frame as well as 5' and 3' flanking

sequences cloned into the *Sma*I site of pBluescript (Stratagene) (Vuorio *et al.*, 1993) was amplified by the polymerase chain reaction (PCR), using primers that yield a PCR product lacking the complete open reading frame. *Bgl*II sites that had been introduced in the primers were used to ligate this PCR product to a *Bam*HI–*HIS3* fragment, which was derived from the YDp set (Berben *et al.*, 1991). From the resulting plasmid a *Bam*HI–*Eco*RI fragment, containing *TSL1* flanking sequences separated by *HIS3* and *HIS3* flanking sequences, was isolated and used for yeast transformation. For the deletion of *TPS3*, a 4.2 kb *Xba*I–*Sma*I fragment containing the *TPS3* open reading frame including flanking sequences was cloned into the *Xba*I–*Sma*I sites of pUC19. The plasmid was amplified by PCR, using primers that yield a PCR product lacking the complete *TPS3* open reading frame. *Bam*HI sites that had been introduced into the primers were used to ligate this PCR product to a *Bam*HI–*URA3* fragment, which was derived from the YDp set (Berben *et al.*, 1991). From the resulting plasmid an *Xba*I–*Eco*RI fragment, containing *TPS3* flanking sequences separated by *URA3* and *URA3*-flanking sequences, was isolated and used for yeast transformation. All deletions were confirmed by Southern blot analysis. Strains carrying multiple deletions were generated by crossing and tetrad analysis.

Two-hybrid analyses

The interactions of the subunits of the Tre6P synthase complex were tested by two-hybrid analysis (Fields and Sternglanz, 1994), using the LexA system described in detail elsewhere (Gyuris *et al.*, 1993). To fuse the various full-length Tre6P synthase complex subunits to the LexA DNA-binding domain (DBD) coding sequences in plasmid pEG202 (Zervos *et al.*, 1993) and to the activation domain (AD) coding sequences in a modified version of plasmid pJG4-5 (Gyuris *et al.*, 1993) that contains the polylinker region from pEG202 (C. De Virgilio and D. DeMarini, unpublished), *S. cerevisiae* *TPS1*, *TPS2*, *TPS3*, and *TSL1* (accession numbers X61275, X70694, M88172,

and X72788, respectively) full-length coding sequences were amplified by PCR using Vent DNA polymerase (New England Biolabs) and either cloned genes (*TSL1* and *TPS3*) or genomic DNA (*TPS1* and *TPS2*) as templates. Appropriate restriction sites were introduced with the primers. The PCR products were cloned at the *Bam*HI site (*TPS1*), the *Not*I site (*TPS2*), the *Xho*I site (*TPS3*), or the *Eco*RI–*Sal*I site (*TSL1*) of pEG202. The constructs contain either 2 (EF for pEG202–*TSL1*), 5 (EFGI for pEG202–*TPS1*), 12 (EFGIRRPWRPQ for pEG202–*TPS2*), or 13 (EFGIRRPWRPLE for pEG202–*TPS3*) additional amino acids between the LexA DBD and the first amino acid of the fused protein (M for all but Tps1; L, corresponding to the 10th residue, for Tps1). As a negative control, *MSB2* was fused to the DBD in pEG202 as described earlier (Simon *et al.*, 1995). For construction of a fusion of Tps1 to the AD in pJG4-5, the *TPS1* sequence was isolated from pEG202–*TPS1* by partial digestion with *Eco*RI and *Xho*I (*TPS1* has an internal *Eco*RI site) and cloned into the *Eco*RI site of pJG4-5.

To fuse Tps2 and Tps3 to the activation domain in pJG4-5, the defined sequences were amplified as above and cloned into the *Not*I site (for *TPS2*) of a pJG4-5 that had been modified to include a *Not*I site in its polylinker, or into the *Xho*I site of pJG4-5 (for *TPS3*). The Tsl1–AD fusion (*TSL1-I*) was originally isolated in a two-hybrid screen for proteins that interact with Tps2. The fused sequence in pJG4-5 codes for the amino acids 60–332 of the original Tsl1 sequence.

Strain EGY48 (Table 3) containing the *LexAop–lacZ* reporter plasmid pSH18-34 (Gyuris *et al.*, 1993) was cotransformed with pEG202 or a pEG202-derived plasmid expressing a LexA DBD fusion protein and with pJG4-5 or a pJG4-5-derived plasmid expressing an AD fusion protein. Beta-galactosidase activities were then assessed in three independent clones of each strain grown for 16 h at 27°C in minimal medium containing 2% (w/v) galactose, 1% (w/v) raffinose, and 20 µg leucine ml⁻¹.

Enzyme assays and determination of metabolite levels

Enzyme activities were measured in crude extracts made from cells harvested by centrifugation and washed once with distilled water. After resuspension in imidazole buffer (1 mM EDTA, 1 mM PMSF, 2 mM MgCl₂, 50 mM imidazole-HCl, pH 6.3), glass beads (0.5 mm diameter) were added and the cells were broken by four cycles of vortexing and cooling on ice, each for 1 min. The extracts were transferred into fresh tubes and centrifuged for 15 min at 20 000 × *g*. Supernatants were desalted on Sephadex G-25 columns (bed volume 2 ml) and then used for enzyme assays. All procedures were carried out at 4°C. Tre6P synthase activity was measured using the coupled assay described by Hottiger *et al.* (1987). The activity of Tre6P phosphatase was determined by incubation of extracts with Tre6P (Sigma) and quantification of the product trehalose by high-performance liquid chromatography (HPLC) analysis as described below (see also De Virgilio *et al.*, 1993).

For the determination of trehalose and Tre6P, 10 ml of exponentially growing cells was filtered (Whatman GF/C), washed three times with 5 ml of distilled H₂O, resuspended in 1 ml of H₂O and transferred to a boiling water bath for 10 min.

After centrifugation (three times for 10 min at 20 000 × *g*, trehalose and Tre6P were determined in the supernatant by HPLC analysis as described by De Virgilio *et al.* (1993) using an anion-exchange column (CarboPac PA-1, Dionex) and a Dionex DX-300 Gradient Chromatography System. Peaks were detected with a pulsed amperometric detector (Dionex).

Protein concentrations in crude extracts were measured using the Bio-Rad protein assay according to the manufacturer's instructions, using BSA as the standard. Protein concentrations in the cultures were determined by means of a modified Lowry assay (Peterson, 1977), using BSA as the standard.

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