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Composition and Functional Analysis of the *Saccharomyces cerevisiae* Trehalose Synthase Complex*

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In the yeast *Saccharomyces cerevisiae*, trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP), which convert glucose 6-phosphate plus UDP-glucose to trehalose, are part of the trehalose synthase complex. In addition to the *TPS1* (previously also called *GGS1*, *CIF1*, *BYP1*, *FDP1*, *GLC6*, and *TSS1*) and *TPS2* (also described as *HOG2* and *PFK3*) gene products, this complex also contains a regulatory subunit encoded by *TSL1*. We have constructed a set of isogenic strains carrying all possible combinations of deletions of these three genes and of *TPS3*, a homologue of *TSL1* identified by systematic sequencing. Deletion of *TPS1* totally abolished TPS activity and measurable trehalose, whereas deletion of any of the other genes in most cases reduced both. Similarly, deletion of *TPS2* completely abolished TPP activity, and deletion of any of the other genes resulted in a reduction of this activity. Therefore, it appears that all subunits are required for optimal enzymatic activity. Since we observed measurable trehalose in strains lacking all but the *TPS1* gene, some phosphatase activity in addition to Tps2 can hydrolyze trehalose 6-phosphate. Deletion of *TPS3*, in particular in a *tsl1*Δ background, reduced both TPS and TPP activities and trehalose content. Deletion of *TPS2*, *TSL1*, or *TPS3* and, in particular, of *TSL1* plus *TPS3* destabilized the trehalose synthase complex. We conclude that Tps3 is a fourth subunit of the complex with functions partially redundant to those of Tsl1. Among the four genes studied, *TPS1* is necessary and sufficient for growth on glucose and fructose. Even when overproduced, none of the other subunits could take over this function of Tps1 despite the homology shared by all four proteins. A portion of Tps1 appears to occur in a form not bound by the complex. Whereas TPS activity in the complex is inhibited by P_i, P_i stimulates the monomeric form of Tps1. We discuss the possible role of differentially regulated Tps1 in a complex-bound or monomeric form in light of the requirement of Tps1 for trehalose production and for growth on glucose and fructose.

In the yeast *Saccharomyces cerevisiae,* trehalose is synthesized by a large enzyme complex comprising the two catalytic activities of trehalose biosynthesis: trehalose-6-phosphate synthase (TPS)[1] and trehalose-6-phosphate phosphatase (TPP) (1–3). Several genes encoding subunits of the trehalose synthase complex have been cloned. The *TPS1*/*TSS1* gene has been cloned as encoding the smallest (56 kDa) subunit responsible for TPS activity (4, 5), the *TPS2* gene as encoding the 100-kDa subunit responsible for TPP activity (6), and the *TSL1* gene as encoding the large (123 kDa) regulatory subunit (5). The *TPS3* gene is a homologue of *TSL1*, identified by systematic sequencing (GenBank™/EMBL Data Bank accession number M88172). Two-hybrid analysis has indicated that it might also encode a functional component of the trehalose synthase complex (7), and its expression is coregulated with that of *TPS1*/*TSS1* and *TPS2* (8). The *TPS1*/*TSS1* gene has also been cloned independently by complementation of the *cif1* mutant (9) and the *fdp1* and *byp1* mutants (10, 11). In the latter case, we called it *GGS1* for "component of a general glucose sensing system" since these mutants were unable to grow on glucose and were deficient in a wide range of glucose-induced regulatory phenomena (10, 12–14).

The cloning of the *TPS1* gene has revealed a novel connection between trehalose metabolism and the control of glycolysis (for review, see Ref. 15). Three hypotheses have been proposed to explain this connection. 1) The Tps1 protein has, in addition to its function in trehalose 6-phosphate (Tre-6-P) synthesis, a separate regulatory function that is responsible for the restriction of glucose influx by interaction with glucose transport and sugar kinase activity (14). 2) Trehalose metabolism prevents overflow of glycolysis during the initiation of glucose fermentation by deviation of sugar phosphates into trehalose synthesis with concomitant recovery of free P_i required by the glyceraldehyde-3-phosphate dehydrogenase reaction (16). 3) Tre-6-P, the intermediate of trehalose biosynthesis, restricts sugar influx into glycolysis by inhibition of hexokinase activity (17).

All genes encoding subunits of the trehalose synthase complex display significant sequence homology to the *TPS1* gene (5, 6). In this study, we show that despite this homology, none of the other subunits of the trehalose synthase complex, singly or in combination, can take over the function of Tps1 in synthesizing Tre-6-P or in controlling glucose influx into glycolysis. Using a set of strains with all possible combinations of deletions in the four genes encoding the trehalose synthase complex, we report on the contribution of the different gene products to the two catalytic activities of the trehalose synthase complex *in vitro*, to regulatory properties of TPS activity *in*

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[1] The abbreviations used are: TPS, trehalose-6-phosphate synthase; TPP, trehalose-6-phosphate phosphatase; Tre-6-P, trehalose 6-phosphate; FPLC, fast protein liquid chromatography.

vitro, and to trehalose accumulation *in vivo*. In addition, we show that part of the Tps1 protein is apparently present as free protein, that this monomeric Tps1 protein has catalytic activity, and that it displays different regulatory properties compared with the Tps1 protein in the trehalose synthase complex.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—The *S. cerevisiae* strains used in this study are shown in Table I. Overexpression of *TPS1*, *TPS2*, *TPS3*, and *TSL1* was carried out with a YEp*lac*195 (*URA3*) plasmid containing the respective genes behind their own promoter. The cells were grown at 30 °C, as indicated, either to exponential phase or to stationary phase and on rich or synthetic medium with glucose (2%, w/v), galactose (2%, w/v) or glycerol (3%, v/v) as carbon source. Rich medium contained 1% (w/v) yeast extract and 2% (w/v) Bacto-peptone. Synthetic medium was prepared according to Sherman *et al.* (18). The growth measurements on solid agar medium (see Fig. 7) were performed on rich medium with 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, and the respective sugar as indicated.

Construction of Deletion Strains—The deletion approach for *TPS1* and *TPS2* has been described previously (16, 19). The coding region of the *TSL1* and *TPS3* genes was deleted completely using the same polymerase chain reaction approach (20) and replaced by the marker genes as indicated in Table I.

Determination of Trehalose and Tre-6-P Content and TPS and TPP Activities—Trehalose was determined as described by Neves *et al.* (21). Tre-6-P was quantitated by high pressure liquid chromatography as described previously (22). For the expression of trehalose and Tre-6-P content as estimated intracellular concentration, the following conversion factor was used: 7 mg of protein equals 100 μl of intracellular volume. TPS activity in crude cell extracts or after purification by Superose 6 FPLC (see below) was determined according to Hottiger *et al.* (23), and TPP activity according to De Virgilio *et al.* (6). Activity is expressed as microkatals/g of protein (1 microkatal/g of protein = 1 μmol/s/g of protein). Protein was determined by the biuret method (24).

Superose 6 FPLC of Cell Extracts and Western Blot Analysis—Extracts of stationary phase cells were loaded on a Superose 6 HR 16/50 FPLC column (Amersham Pharmacia Biotech) and eluted with buffer (pH 8.0) containing 150 mM NH_4HCO_3, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μg/ml pepstatin A. The flow rate used was 12 ml/h, and the volume of the fractions collected was 0.8 ml. The proteins in the samples from the gel filtration column were precipitated with trichloroacetic acid and washed once with cold acetone. The precipitate was dissolved in 0.1 M NaOH before addition of SDS-polyacrylamide gel electrophoresis loading buffer. Conditions for SDS-polyacrylamide gel electrophoresis were as described by Laemmli (25). After electrophoresis, the proteins were blotted by electroporation from the SDS gel onto nitrocellulose membranes. After transfer, the membranes were treated with blocking solution and incubated with the primary antibody (polyclonal rabbit anti-trehalose synthase complex antibody, kindly provided by Dr. J. Londesborough, VTT, Espoo, Finland) overnight at room temperature in a 1:2000 dilution. After washing, the bound primary antibodies were detected using monoclonal anti-rabbit alkaline phosphatase-conjugated immunoglobulin.

RESULTS

TPS Activity in Multiple Deletion Strains—We have constructed a set of 16 isogenic strains in the W303-1A background displaying all possible combinations of deletions in the four known genes encoding the trehalose synthase complex (Table I). For all genes, the entire open reading frame was deleted. The *TPS1* gene was essential to detect significant TPS activity whatever the combination of other genes present (Fig. 1). This was true in exponential as well as in stationary phase. However, in the presence of a functional *TPS1* gene, deletion of the other genes also affected TPS activity, both in exponential and in stationary phase. The single deletion of *TPS3* or *TSL1* had the least effect, but the double deletion caused a strong decrease in TPS activity. This is the first evidence that *TPS3* encodes an active functional component of the trehalose synthase complex. In the triple deletion strain *tps2*Δ *tsl1*Δ *tps3*Δ, significant TPS activity was detected, indicating that the *TPS1* gene product displays TPS activity in the absence of all other (known) subunits.

TABLE I
S. cerevisiae strains used in this study
All strains are isogenic to W303–1A and carry the same markers.

Strain	Genotype	Ref. source
W303–1A	*MAT***a** *ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 can1–100 GAL mal SUC2*	33
YSH6.127.-1C	*MAT***a**	This work
YSH6.127.-17C	*MAT***a** *tps1*Δ*::TRP1*	This work
YSH6.127.-6D	*MAT***a** *tps1*Δ*::TRP1 tsl1*Δ*::HIS3*	This work
YSH6.127.-3C	*MAT***a** *tps1*Δ*::TRP1 tps2*Δ*::LEU2*	This work
YSH6.127.-8D	*MAT***a** *tps1*Δ*::TRP1 tsp3*Δ*::URA3*	This work
YSH6.127.-12C	*MAT***a** *tps1*Δ*::TRP1 tps2*Δ*::LEU2 tsl1*Δ*::HIS3*	This work
YSH6.127.-11D	*MAT***a** *tps1*Δ*::TRP1 tps2*Δ*::LEU2 tps3*Δ*::URA3*	This work
YSH6.127.-9D	*MAT***a** *tps1*Δ*::TRP1 tps3*Δ*::URA3 tsl1*Δ*::HIS3*	This work
YSH6.127.-5D	*MAT***a** *tps1*Δ*::TRP1 tps2*Δ*::LEU2 tps3*Δ*::URA3 tsl1*Δ*::HIS3*	This work
YSH6.127.-4B	*MAT***a** tsl1Δ*::HIS3*	This work
YSH6.127.-14B	*MAT***a** tps2Δ*::LEU2 tsl1*Δ*::HIS3*	This work
YSH6.127.-6B	*MAT***a** tps3Δ*::URA3 tsl1*Δ*::HIS3*	This work
YSH6.127.-1A	*MAT***a** tps2Δ*::LEU2 tps3*Δ*::URA3 tsl1*Δ*::HIS3*	This work
YSH6.127.-9C	*MAT***a** tps2Δ*::LEU2*	This work
YSH6.127.-20C	*MAT***a** tps2Δ*::LEU2 tps3*Δ*::URA3*	This work
YSH6.127.-2D	*MAT***a** tps3Δ*::URA3*	This work

TPP Activity in Multiple Deletion Strains—TPP activity was entirely absent in all strains lacking *TPS2*, both in exponential and in stationary phase (Fig. 2). Deletion of *TSL1* and/or *TPS3* had only a limited effect on TPP activity, especially in exponential phase, but deletion of both genes reduced TPP activity under either growth condition. Deletion of *TPS1* severely reduced TPP activity, whatever other gene combination present.

Trehalose and Tre-6-P Content in Multiple Deletion Strains—Trehalose and Tre-6-P levels were determined only in stationary phase cells because, in wild-type strains, trehalose content is very low in exponential phase. In strains lacking *TPS1,* no trehalose or Tre-6-P was detected (Fig. 3). Deletion of *TSL1* or *TPS3* slightly reduced trehalose content, whereas the double deletion strain had <50% of the trehalose level in the wild-type strain. In addition, the *tsl1*Δ *tps3*Δ and *tps3*Δ strains showed a somewhat elevated Tre-6-P level compared with the wild-type strain. Very high increases in Tre-6-P were observed in all strains containing *tps2*Δ. Additional deletion of *TSL1* or *TPS3* had only a little effect on this elevated Tre-6-P level. All *tps2*Δ strains, however, still contained high levels of trehalose.

Superose 6 FPLC of Cell Extracts—We fractionated yeast cell extracts according to molecular mass of the proteins using Superose 6 FPLC, and we used Western blot analysis to detect the presence of the protein components of the trehalose synthase complex after separation of all proteins in each fraction by SDS gel electrophoresis (Figs. 4–6). Antibodies raised against the whole trehalose synthase complex (kindly provided by Dr. J. Londesborough) were used. We consistently detected the smallest component (Tps1) both as part of the trehalose synthase complex and as apparently free monomeric protein, at least not bound to the complex. The other subunits were mainly or only detected in the trehalose synthase complex. They seem to be present at lower levels than Tps1, although it cannot be excluded that the reactivity of the antibodies for the other components of the trehalose synthase complex is lower.

To check whether the presence of the free Tps1 protein might be the result of unspecific breakdown of the trehalose synthase complex, we incubated cell extracts for an extended period of time (30 min) at 30 °C. However, this treatment did not cause any change in the protein pattern of the FPLC-fractionated cell extracts (Fig. 4), indicating that the trehalose synthase com-

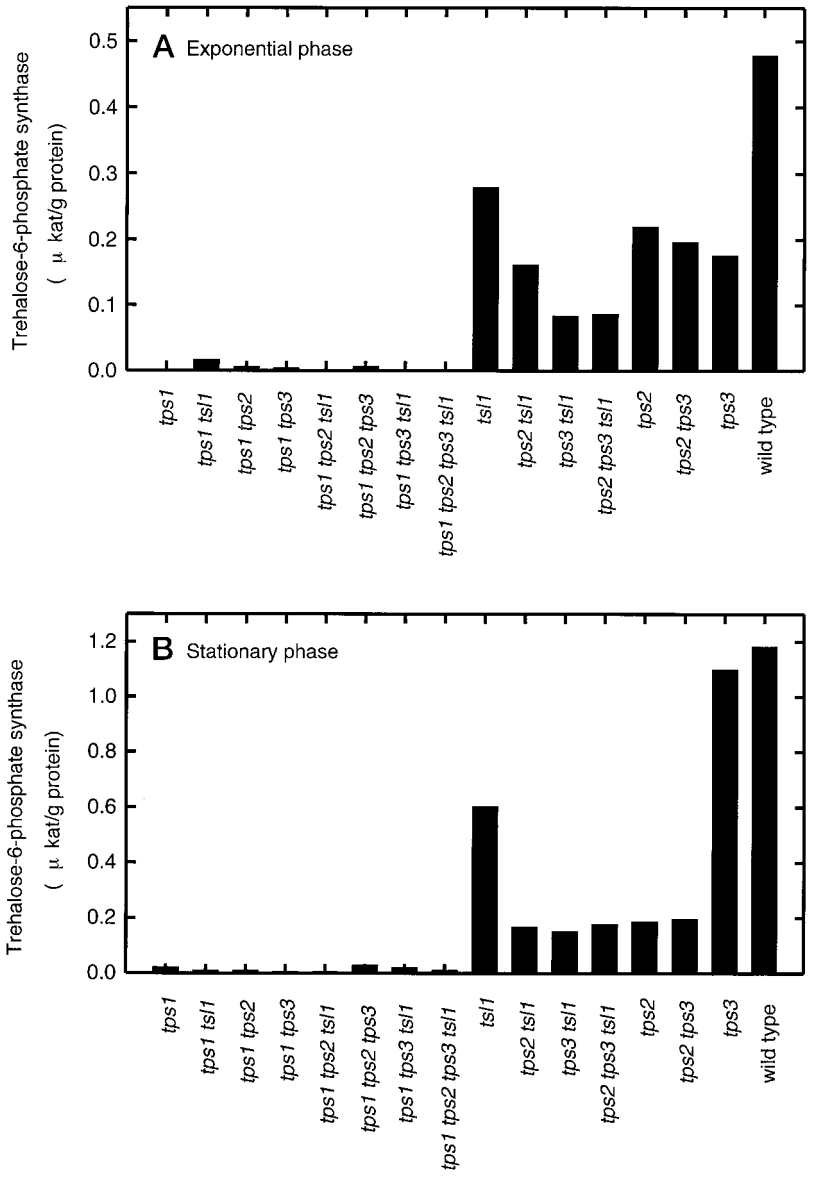

FIG. 1. **TPS activity in galactose-grown exponential (*A*) and stationary phase (*B*) cells of yeast strains containing all possible combinations of deletions in the four genes (*TPS1*, *TPS2*, *TPS3*, and *TSL1*) encoding the trehalose synthase complex.** *μkat*, microkatals.

plex is quite stable and that free Tps1 protein is probably also present *in vivo*.

Deletion of either *TSL1* or *TPS3* appeared to destabilize the trehalose synthase complex to some extent since more free Tps1 protein and more breakdown products were detected (Fig. 5, *A–C*). However, in both cases, Tps2 and a strong band of Tps1 could still be detected at the normal elution position of the trehalose synthase complex. Deletion of *TSL1* produced a more dramatic effect than deletion of *TPS3*, with a higher proportion of Tps1 and also Tps2 being detected free from the complex. In cell extracts of a *tsl1*Δ *tps3*Δ strain, on the other hand, no evidence for the presence of the trehalose synthase complex was observed (Fig. 5*D*). All Tps2 was detected free from the complex. The clear difference in complex formation in the presence and absence of Tps3 in the *tsl1*Δ strain confirms that it is part of the trehalose synthase complex. In all samples, a slight background of Tps1 was also detected, possibly caused by unspecific retention by the Superose beads. Deletion of *TPS2* caused the most dramatic effect, with only Tps1 free from the complex being present (Fig. 5*E*).

We also investigated whether overexpression of *TPS2, TSL1,* and *TPS3* separately or *TPS2* and *TSL1* simultaneously could reduce the level of free Tps1 protein. In none of these cases, however, was a reduction observed (data not shown). This also indicates that the expression of neither of these subunits is limiting for the formation of trehalose synthase complexes. Overexpression of *TPS2* and *TSL1*, both separately and combined, was clearly observed on the Western blots (data not shown). Overexpression of *TPS3*, on the other hand, did not result in the appearance of an additional band, probably because of the failure of the antibodies to react with the Tps3 protein. In a control experiment, the *TPS3*-containing plasmid was introduced into a *tsl1*Δ *tps3*Δ strain. This restored the presence of the trehalose synthase complex in the high molecular mass fractions, confirming that *TPS3* was truly expressed from this plasmid (data not shown).

In FPLC-fractionated extracts of wild-type cells, TPS activity could be measured only at the position of the trehalose synthase complex and not at the position of the free Tps1 protein (data not shown). However, in extracts from a *tps2*Δ *tsl1*Δ *tps3*Δ, strain TPS activity was clearly detected (see below), which fits with the presence of trehalose and Tre-6-P *in vivo* in cells of such a strain.

Growth of Multiple Deletion Strains—The growth properties of the 16 different deletion strains on galactose and fructose are shown in Fig. 6*A*. All strains lacking the *TPS1* gene were

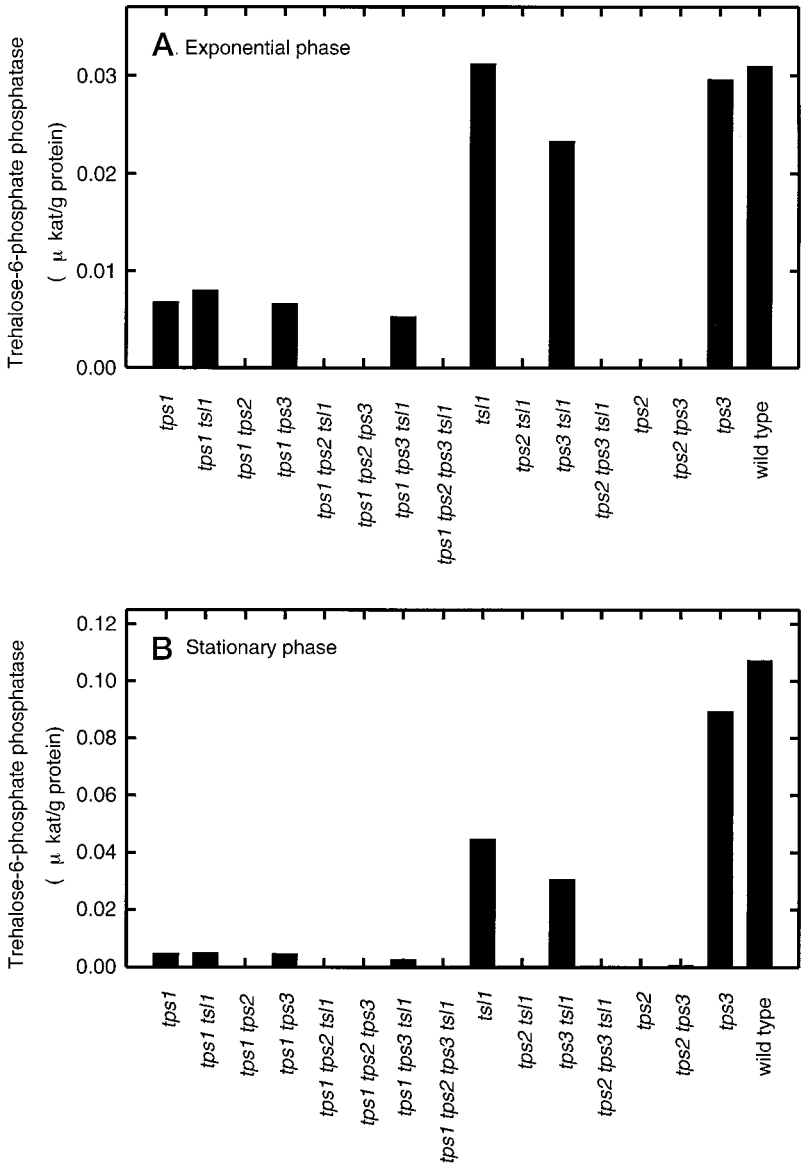

FIG. 2. **TPP activity in galactose-grown exponential (*A*) and stationary phase (*B*) cells of yeast strains containing all possible combinations of deletions in the four genes (*TPS1*, *TPS2*, *TPS3*, and *TSL1*) encoding the trehalose synthase complex.** *μkat*, microkatals.

unable to grow on fructose whatever other combination of genes present. In addition, the presence of the *TPS1* gene was sufficient for growth on fructose whatever other combination of genes present (Fig. 6*A*). Similar results were obtained for growth on glucose, except that the growth defect was somewhat less stringent than for growth on fructose (data not shown). Overexpression of any one of the four genes in a wild-type strain did not cause a noticeable growth defect on glucose or fructose (data not shown). In addition, overexpression of *TPS2*, *TPS3*, or *TSL1* in a *tps1*Δ strain was unable to restore or even improve growth on glucose or fructose (Fig. 6*B*).

Regulation of TPS Activity in Multiple Deletion Strains—We also investigated the effect of deletion of the subunits encoded by *TPS2*, *TSL1,* and *TPS3* on the regulation of *TPS1*-encoded TPS activity. The enzyme from wild-type cells is inhibited by inorganic P_i with a K_i of 1–2 mM (Fig. 7) (2, 26). Deletion of *TSL1,* but not *TPS3,* slightly reduced the inhibition by P_i (Fig. 7). This was observed both with cells grown on glucose until stationary phase and with exponential phase cells grown on glycerol (Fig. 7). Deletion of both *TSL1* and *TPS3*, deletion of *TPS2,* or deletion of all three genes eliminated P_i inhibition and switched it into P_i stimulation (Fig. 8). This was observed both for cells grown on glucose until stationary phase (Fig. 8) and with exponential phase cells grown on glycerol (data not shown). In the latter case, the activities in the strains *tsl1*Δ *tps3*Δ, *tps2*Δ, and *tsl1*Δ *tps3*Δ *tps2*Δ in the absence of P_i were 0.049, 0.130, and 0.024 microkatals/g, respectively, and they increased to 344.7, 261.3, and 438.8% of these values in the presence of 10 mM P_i. TPS activity as a function of the P_i concentration in the assay is shown in Figs. 7 and 8 as relative activity compared with the activity in the absence of P_i, which was taken as 100%. The absolute values for the activity in the absence of P_i are indicated in the legends of Figs. 7 and 8. We also measured phosphate inhibition of trehalose-6-phosphate synthase in crude cell extracts and after FPLC purification of the complex from cells grown on rich yeast extract/bacto peptone/glucose medium, and we found that, in this case, the activity was still inhibited by phosphate, but much less (maximum 50%) compared with enzyme from cells grown on synthetic glucose medium (data not shown).

TPS activity is strongly stimulated by fructose 6-phosphate (26). This effect can be observed clearly only in extracts from cells of a phosphoglucoisomerase mutant. In extracts of wild-type strains, phosphoglucoisomerase activity converts part of the TPS substrate glucose 6-phosphate into fructose 6-phosphate, which then acts as a stimulator of TPS activity. Our

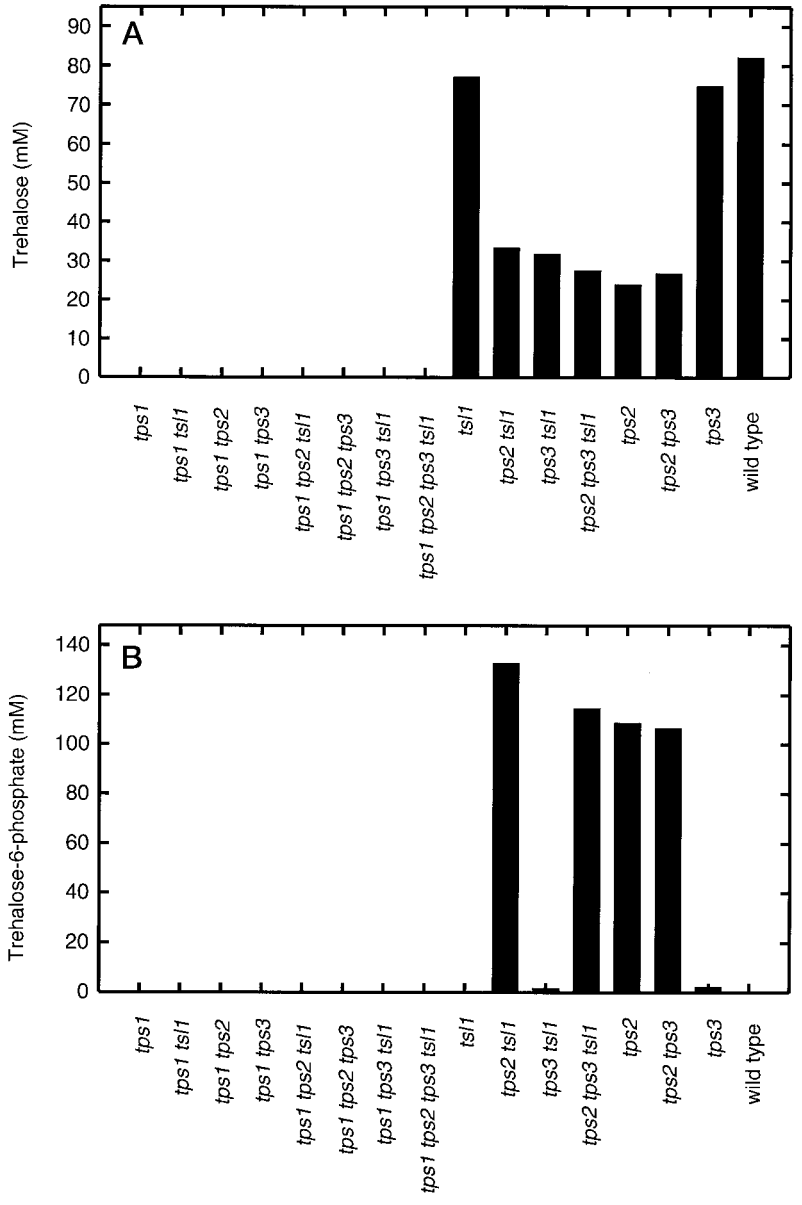

FIG. 3. **Trehalose (*A*) and Tre-6-P (*B*) content in galactose-grown stationary phase cells of yeast strains containing all possible combinations of deletions in the four genes (*TPS1*, *TPS2*, *TPS3*, and *TSL1*) encoding the trehalose synthase complex.**

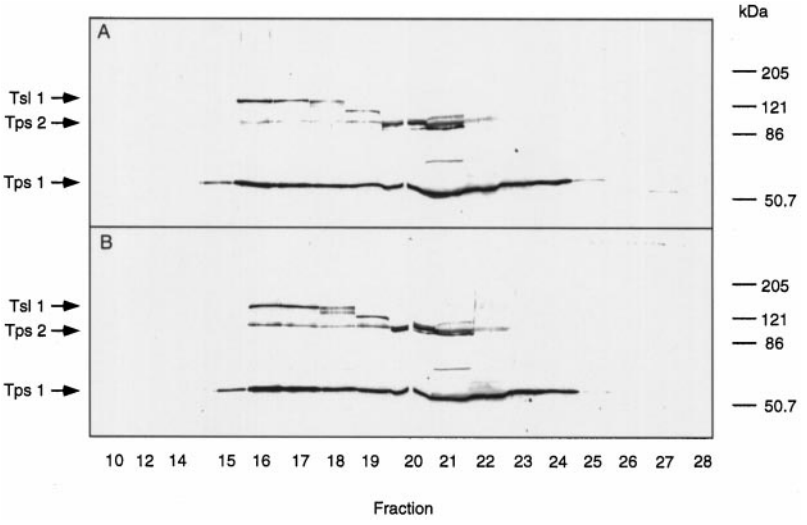

FIG. 4. **Western blot analysis of subunits of the trehalose synthase complex in cell fractions first separated by Superose 6 FPLC and subsequently submitted to SDS-gel electrophoresis.** Antibodies raised against the purified trehalose synthase complex were used. Tps1, Tsl1, and Tps2 were detected in fractions 16 and 17 (±600 kDa). Dimers of Tps2 and/or Tps1 and Tps2 might be present in fractions 20 and 21 (±200 kDa). Free Tps1 was present in fractions 23 and 24 (±50 kDa), with possible Tps1 dimers in fraction 22. The positions of Tps1, Tps2, and Tsl1 are indicated. (Tps3 is most probably not detected by the antibodies (see Fig. 5 and "Results").) *A,* extract of the wild-type strain; *B,* part of the same extract incubated for 30 min at 30 °C before separation by FPLC. The molecular mass standards used for calibration are indicated on the right.

measurements of inhibition by P_i were carried out in cell extracts of the wild-type strain without added fructose. However, because of the presence of phosphoglucoisomerase, an equilibrium mixture of glucose 6-phosphate and fructose 6-phosphate was rapidly formed in the test medium. Probably because of this reason, further addition of fructose 6-phosphate only

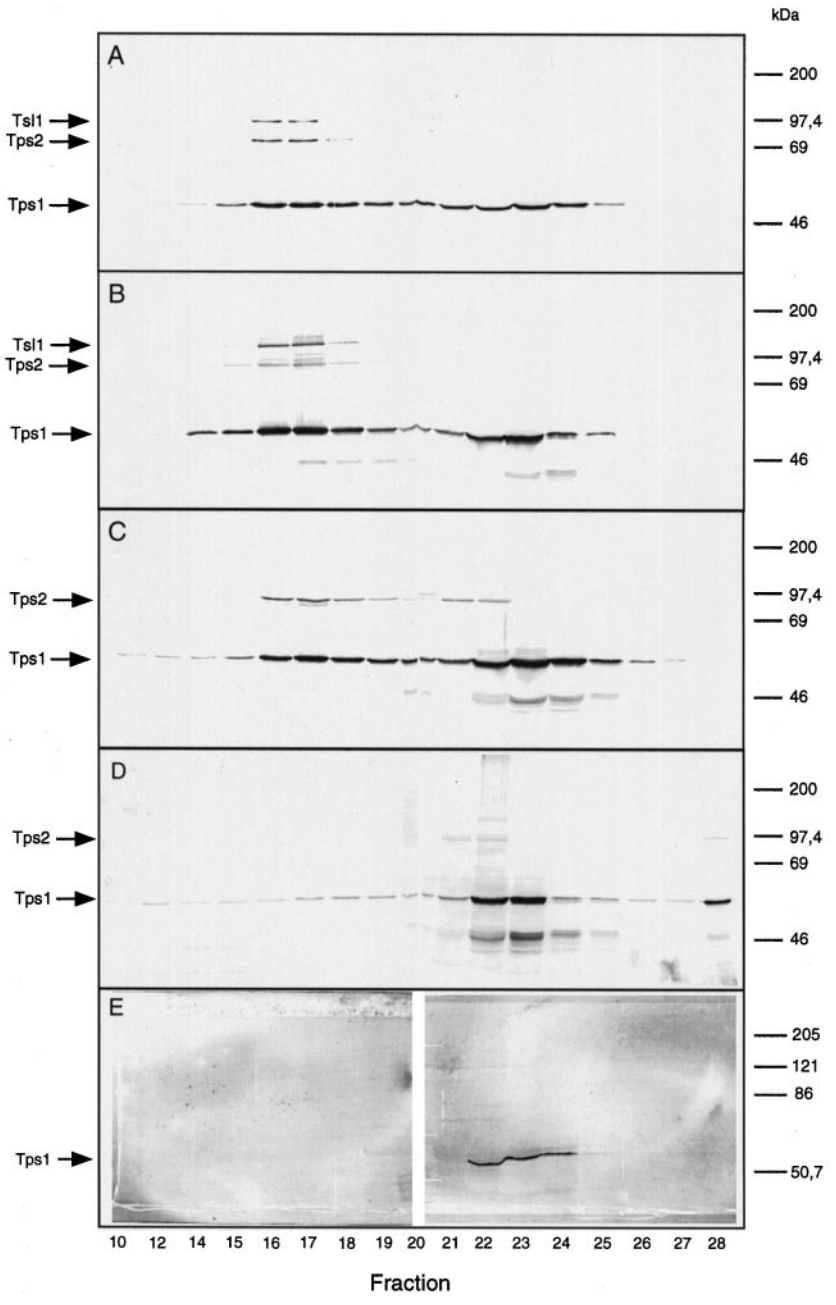

FIG. 5. **Western blot analysis of subunits of the trehalose synthase complex in cell fractions first separated by Superose 6 FPLC and subsequently submitted to SDS-gel electrophoresis.** Antibodies raised against the purified trehalose synthase complex were used. The trehalose synthase complex (±600 kDa) was present in fractions 16 and 17, whereas the free Tps1 protein (±50 kDa) was detected mainly in fractions 23 and 24. *A,* wild-type strain; *B, tps3*Δ strain; *C, tsl1*Δ strain; *D, tsl1*Δ *tps3*Δ strain; *E, tps2*Δ strain. The molecular mass standards used for calibration are indicated on the right.

slightly stimulated TPS activity (±20%). This additional activation did not appear to be affected by deletion of the other subunits of the trehalose synthase complex (data not shown).

DISCUSSION

Subunits of the Trehalose Synthase Complex—In the yeast *S. cerevisiae,* trehalose is synthesized by a large multisubunit complex comprising the two catalytic activities TPS and TPP (1–3). This is different from *Escherichia coli,* where the two enzymatic activities reside in separate enzymes encoded by the genes *otsA* (responsible for TPS activity) and *otsB* (responsible for TPP activity) (27–29). Up to now, four genes have been identified that appear to encode components of the trehalose synthase complex in *S. cerevisiae*: *TPS1*, *TPS2*, *TPS3,* and *TSL1*. *TPS1* is homologous to *otsA* from *E. coli* and remarkably also over its entire sequence to each of the other yeast subunits (~35% identity in each case). Interestingly, *otsB* shows homology only to the C-terminal part of the *TPS2* gene product, which is therefore probably responsible for the TPP activity of Tps2 (29). The comparison between the yeast and *E. coli* enzymes shows that the homology among the four genes in yeast might play a role in the formation of the trehalose synthase complex. Why yeast employs a complex to synthesize trehalose is not clear, but may have to do with improved efficiency and/or control of trehalose synthesis by a complex of the two enzymatic activities. In addition, it might also have to do with the role played by the *TPS1* gene product and/or the intermediate Tre-6-P in the control of glucose influx into yeast glycolysis (15). Possibly, trehalose synthesis by the complex allows a tighter control of the cellular Tre-6-P level, which otherwise might cause too much inhibition of hexokinase activity. Normally, one would expect most of the Tre-6-P to be channeled inside the complex and only small amounts to leak out into the cytosol. In this respect, it is remarkable that after addition of glucose to yeast cells, there is a large transient overshoot of the cellular Tre-6-P level up to 1–2 mM (22), which is severalfold higher than the steady-state level of 100–200 μM (17, 22). If

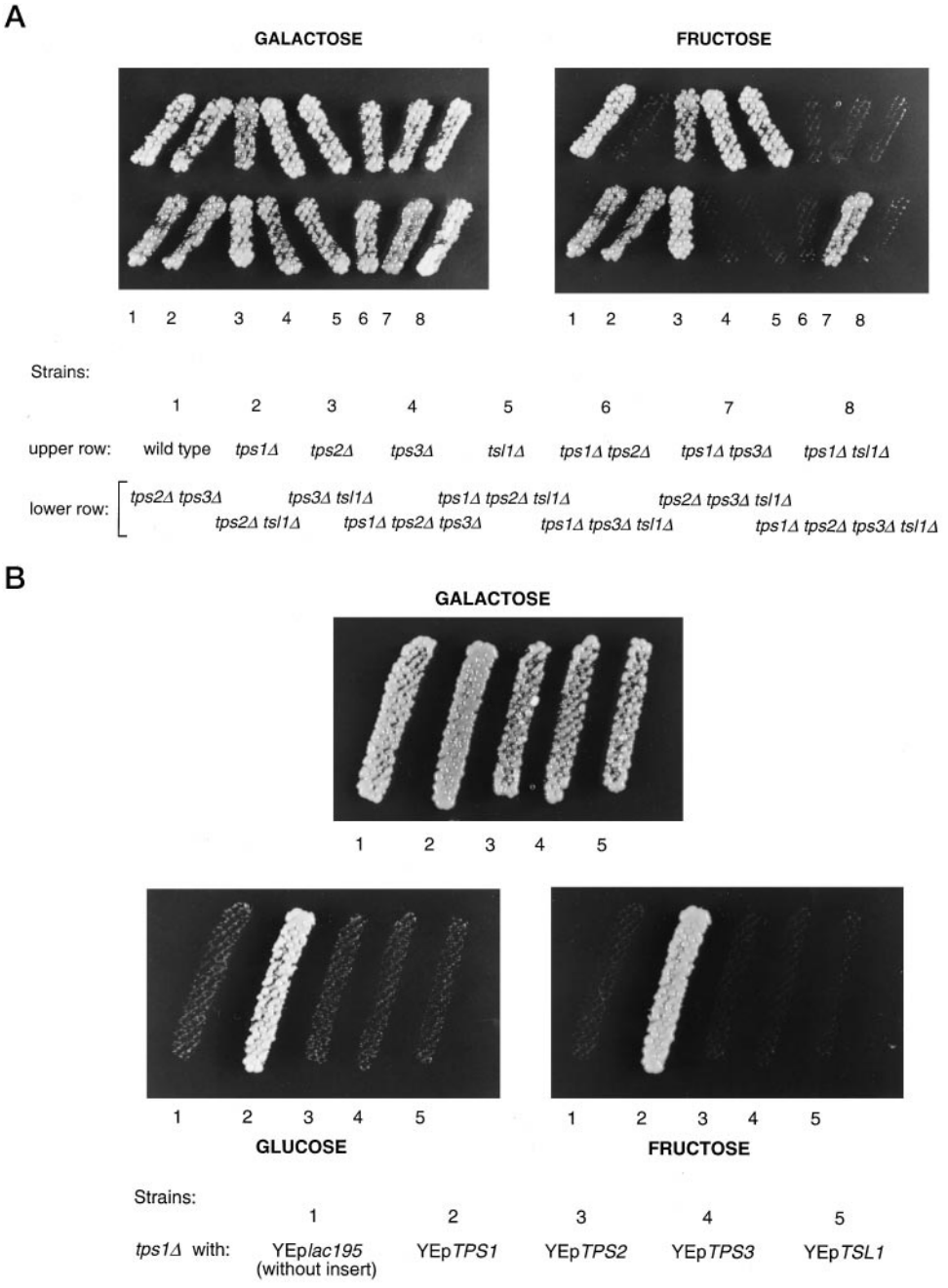

FIG. 6. *A,* growth of yeast strains containing all possible combinations of deletions in the four genes (*TPS1*, *TPS2*, *TPS3*, and *TSL1*) encoding the trehalose synthase complex on medium containing either galactose or fructose as carbon source; *B,* growth of a *tps1*Δ strain (YSH6.127.-17C) either with the YEp*lac*195 plasmid without insert or with overexpression using the same plasmid of *TPS1, TPS2*, *TPS3,* or *TSL1* on medium containing galactose, glucose, or fructose as carbon source.

trehalose synthesis were carried out only by the complex, it would indicate uncoupling of TPS and TPP activities, resulting in strong deviation of Tre-6-P into the cytosol rather than further hydrolysis to trehalose. However, our discovery of free monomeric Tps1 protein offers a new explanation for the rapid increase in Tre-6-P after glucose addition (see below).

It has been shown that the products of three of the four genes, *TPS1*, *TPS2,* and *TSL1*, are subunits of the trehalose synthase complex (4–6). For the *TPS3* gene, which has been identified by systematic sequencing, we have provided evidence using the yeast two-hybrid system that it encodes a structural subunit of the trehalose synthase complex. We showed that both Tps3 and Tsl1 interact *in vivo* with Tps1 and Tps2, whereas the latter two proteins also interact with each other (7). Now we provide evidence that *TPS3* encodes an active functional subunit of the trehalose synthase complex. The double deletion of *TSL1* and *TPS3* caused a stronger reduction in TPS activity *in vitro* and a stronger reduction in trehalose accumulation *in vivo* compared with the single deletion mutants (Figs. 1 and 3). The single deletion of *TSL1* reduced P_i inhibition of TPS activity, whereas the single deletion of *TPS3* had no effect in this respect (Fig. 7). On the other hand, the double deletion of *TSL1* and *TPS3* switched P_i inhibition into P_i stimulation (Fig. 8). The double deletion mutant displayed a strong destabilization of the trehalose synthase complex as opposed to the single deletion mutants, providing further evidence for a role of Tps3 as structural subunit. These results support the conclusion that *TSL1* and *TPS3* encode redundant genes, with *TSL1* playing a somewhat more important function, at least for the parameters that we have measured. Pre-

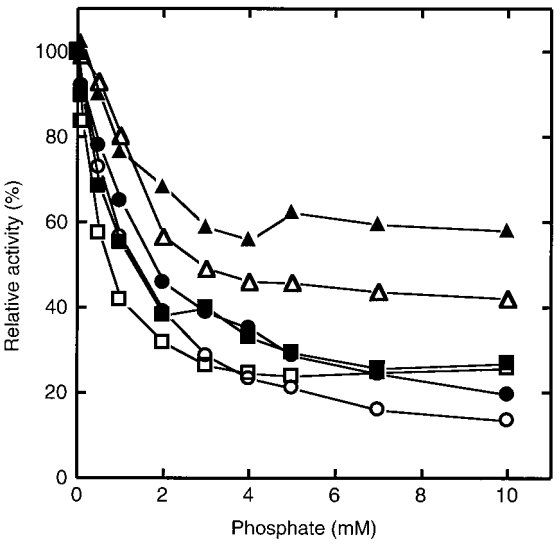

FIG. 7. **Inhibition of TPS activity by P_i.** ● and ○, wild-type strain; ▲ and △, *tsl1*Δ strain; ■ and □, *tps3*Δ strain. ●, ▲, and ■, cells grown on synthetic medium with glucose until stationary phase; ○, △, and □, exponential phase cells grown on synthetic medium with glycerol. The absolute values for the activity (expressed as microkatals/g of protein) in the absence of P_i for glucose-grown stationary phase cells were 0.518 (wild-type strain), 0.498 (*tsl1*Δ), and 0.391 (*tps3*Δ), and those for exponential phase cells grown on glycerol were 0.713 (wild-type strain), 0.372 (*tsl1*Δ), and 0.779 (*tps3*Δ).

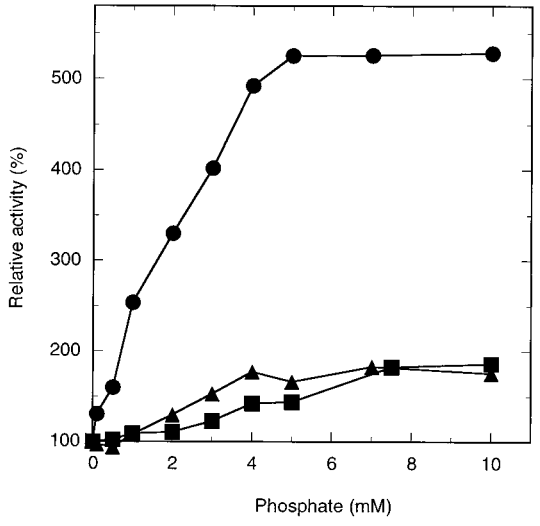

FIG. 8. **Stimulation of TPS activity by P_i in strains with a deletion of both *TSL1* and *TPS3* (●), a deletion of *TPS2* (▲), or a deletion of *TPS2*, *TSL1*, and *TPS3* (■).** The cells were grown on synthetic medium with glucose until stationary phase. The absolute values for the activity (expressed as microkatals/g of protein) in the absence of P_i were 0.036 (*tsl1*Δ *tps3*Δ), 0.123 (*tps2*Δ), and 0.097 (*tps2*Δ *tsl1*Δ *tps3*Δ).

viously, Ferreira *et al.* (30) were unable to detect a significant effect of *TPS3* deletion, as opposed to *TSL1* deletion, on the increase in TPS activity that occurs after glucose exhaustion. This fits with our observation that in stationary phase cells, the effect of *TPS3* deletion is minimal (Fig. 1). Since the expression of *TSL1* differs from that of the other three genes during growth on glucose, the function of Tsl1 and Tps3 might be different under these conditions (8). The relative differences in trehalose and Tre-6-P content and the activities of TPS and phosphatase in the same 16 deletion strains were also observed after heat shock, supporting our conclusions and indicating that if Tsl1 has a specific role, it is not associated with the response to heat shock (7).

Although all genes of the trehalose synthase complex display striking homology, the other gene products are not capable of taking over the function of Tps1 in Tre-6-P synthesis and in conferring ability to grow on glucose or fructose. This is true for whatever combination of genes present. In addition, overexpression of the other genes is unable to restore the growth on glucose or fructose of a strain lacking *TPS1*. The inability of the other subunits to take over the function of Tps1 suggests that the only function of the parts with homology to Tps1 in the other subunits might be the formation of the trehalose synthase complex. We did not investigate whether the parts of the other genes homologous to Tps1 could take over the function of Tps1 when separated from the non-homologous part. If they were able to do so, however, the physiological meaning would be rather unclear.

The actual subunit composition of the trehalose synthase complex is not clear. Since its total estimated molecular mass (600–800 kDa) exceeds the sum of the molecular mass of the known gene products (3), at least one of the genes is expected to encode more than one subunit. The antibodies used for the Western blot analysis of the FPLC-purified cell extracts were raised against the whole trehalose synthase complex. Apparently, these antibodies are capable of reacting with all components, except for the Tps3 subunit. This is indicated by the failure to detect Tps3 in a *tsl1*Δ strain and in a *TPS3* overexpression strain. Because the reactivity of the antibodies with the different subunits is not known, it is not possible to draw firm conclusions concerning the actual composition of the trehalose synthase complex. However, since the Tps1 band was always much stronger than the others, it appears to be the first candidate for being present with more than one molecule in the trehalose synthase complex.

Presence of Tps1 Not Bound to the Trehalose Synthase Complex—The Superose gel fractionation experiments indicated that a significant part of the Tps1 protein might be present in the cells as free monomeric protein or at least not bound to the trehalose synthase complex. This was an unexpected finding. The appearance of the two pools was not abolished by single overexpression of the other subunits, Tps2, Tsl1, or Tps3, or by combined overexpression of Tps2 and Tsl1. This indicates that the amount of one of the other subunits is not simply limiting for complex formation and that the separation of Tps1 into two pools might be controlled in a more precise way. The dual location of Tps1 might reflect a dual function. Possibly, the Tps1 protein in the trehalose synthase complex is involved only in trehalose synthesis, whereas the free Tps1 protein might have a function in controlling the influx of glucose into glycolysis, *e.g.* by interaction with hexokinase and/or sugar carriers (14, 15). The presence of the free Tps1 protein provides an alternative explanation to uncoupling of TPS and TPP activities in the trehalose synthase complex for the large increase in cellular Tre-6-P content after addition of glucose. The free Tps1 protein might be responsible for this increase.

The presence of free Tps1 protein does not appear to be an artifact of the extraction procedure since incubation of the extract for an extended period of time at 30 °C did not change the proportion of free and complex-associated Tps1 protein. Hence, the trehalose synthase complex appears to be quite stable. This agrees with previous reports on the purification of the complex. Vandercammen *et al.* (2) were unable to separate TPS and TPP activities using several purification procedures. In addition, Londesborough and Vuorio (3) were able to purify a complex in which the TPP subunit and the regulatory subunit were proteolytically reduced in size, although the complex still retained all subunits and both enzymatic activities. (Simple addition of a protease inhibitor, as was also done in our exper-

iments, prevented the limited reduction in size.) This shows that the subunits remain together even after limited proteolysis. This stability of the trehalose synthase complex supports the conclusion that the presence of free Tps1 protein in the cell extracts is not due to the extraction procedure and that therefore free Tps1 protein is also present *in vivo*.

Regulation of Tps1 Bound to and Free from the Trehalose Synthase Complex—The finding that deletion of *TPS2*, of both *TSL1* and *TPS3,* or of all three genes switches P_i inhibition of TPS activity into P_i stimulation without compromising the capacity to grow on glucose and fructose indicates that P_i inhibition of TPS is not essential for growth on rapidly fermented sugars. It also indicates that any free Tps1 protein (synthesizing Tre-6-P) would be stimulated by P_i, whereas the Tps1 protein present in the trehalose synthase complex (synthesizing trehalose in combination with Tps2) would be inhibited by P_i. Free P_i could play a role in the control of glucose influx into glycolysis (15). P_i is a substrate for glyceraldehyde-3-phosphate dehydrogenase in glycolysis, and it is precisely at this point that glycolysis becomes blocked in the *tps1* mutants after addition of glucose (11, 31). A high free P_i level is indicative of slow sugar influx into glycolysis (slow sequestration of P_i into sugar phosphates), whereas a low P_i level indicates rapid sugar influx (rapid sequestration of P_i into sugar phosphates). The highest free P_i level is observed before the initiation of sugar fermentation. Addition of fermentable sugar to wild-type cells is always followed by a rapid decline in the free P_i level to ~40% of the initial concentration (11). A rapid increase in the Tre-6-P level has also been observed during the initiation of fermentation (22), whereas at the same time, the trehalose level rapidly decreases (32). Hence, under these conditions, the synthesis of trehalose and Tre-6-P is clearly regulated in an opposite way *in vivo*. The opposite control by P_i of Tps1 in the trehalose synthase complex and of free Tps1 might offer an explanation for this opposite behavior of Tre-6-P and trehalose *in vivo* after addition of glucose. Activation of the free Tps1 protein by P_i would result in accumulation of Tre-6-P, whereas at the same time, the inhibition of Tps1 in the complex by P_i would result in the down-regulation of trehalose synthesis.

Phosphatase Activity Not Encoded by Tps2—We have observed that all *tps2*Δ strains, including the *tps2*Δ *tsl1*Δ *tps3*Δ strain, still accumulate high levels of trehalose. This indicates that other phosphatases apparently independent of the trehalose synthase complex are able to hydrolyze Tre-6-P with relatively high efficiency. The finding that a significant part of the *TPS1* gene product appears to be present as free protein gives this result more importance. Possibly, *TPS2*-encoded TPP activity is responsible only for the hydrolysis of the Tre-6-P produced "internally" in the trehalose synthase complex. The free Tps1 protein displays less catalytic activity than the Tps1 subunit in the complex, and the Tre-6-P that it produces might be hydrolyzed *in vivo* by other phosphatases. Strong stimulation of TPS activity by the other subunits in the trehalose synthase complex is supported by the phenotype of the single and multiple *TPS2*, *TSL1,* and *TPS3* deletion strains. They always display less TPS activity *in vitro* and accumulate less trehalose *in vivo*.

In conclusion, our results have more precisely established the structural and functional importance of the four subunits of the trehalose synthase complex, Tps1, Tps2, Tsl1, and Tps3, for trehalose synthesis. In addition, they have revealed that the enzymatic machinery of trehalose metabolism contains additional components and regulatory controls than previously known. These novel controls might at least in part be responsible for some of the previous unexpected observations concerning trehalose metabolism, such as the opposite behavior of the trehalose and Tre-6-P levels after addition of glucose and the uncoupling of glucose influx control and the capacity to grow and ferment on glucose.

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