Synergy Between Trehalose and Hsp104 for Thermotolerance in *Saccharomyces cerevisiae*

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ABSTRACT

We isolated a mutant strain unable to acquire heat shock resistance in stationary phase. Two mutations contributed to this phenotype. One mutation was at the *TPS2* locus, which encodes trehalose-6-phosphate phosphatase. The mutant fails to make trehalose and accumulates trehalose-6-phosphate. The other mutation was at the *HSP104* locus. Gene disruptions showed that *tps2* and *hsp104* null mutants each produced moderate heat shock sensitivity in stationary phase cells. The two mutations were synergistic and the double mutant had little or no stationary phase-induced heat shock resistance. The same effect was seen in the *tps1* (trehalose-6-phosphate synthase) *hsp104* double mutant, suggesting that the extreme heat shock sensitivity was due mainly to a lack of trehalose rather than to the presence of trehalose-6-phosphate. However, accumulation of trehalose-6-phosphate did cause some phenotypes in the *tps2* mutant, such as temperature sensitivity for growth. Finally, we isolated a high copy number suppressor of the temperature sensitivity of *tps2*, which we call *PMU1*, which reduced the levels of trehalose-6-phosphate in *tps2* mutants. The encoded protein has a region homologous to the active site of phosphomutases.

YEAST deprived of nutrients stop growing and dividing and arrest as 1N, unbudded cells in a state called stationary phase (PRINGLE and HARTWELL 1981). Cells in stationary phase accumulate the storage carbohydrates glycogen and trehalose that provide a source of energy during starvation (LILLIE and PRINGLE 1980). They also become resistant to shocks of high temperature and to other stresses. The resistance is quite dramatic; at times when survival of stationary cells is ~50%, survival of identically treated exponential cells is ~10,000 times lower (*e.g.*, SCHULZE 1895; SHERMAN 1956; SCHENBERG-FRASCINO and MOUSTACCHI 1972).

Heat shock proteins (hsps) are good candidates for molecules that protect cells from extreme temperatures since hsp synthesis and the acquisition of thermotolerance are closely correlated (LINDQUIST and CRAIG 1988). Some hsps are essential for viability (WERNER-WASHBURNE *et al.* 1987) and one hsp, Hsp104, is known to be important for heat-induced and stationary phase-induced thermotolerance (SANCHEZ and LINDQUIST 1990; SANCHEZ *et al.* 1992). However, the known hsps do not seem to fully account for all thermotolerance. For example, strains with a mutant heat shock factor (HSF), and consequently low-level transcription of hsp genes, can still acquire thermotolerance (SMITH and YAFFE 1991). Other studies show that thermotolerance is partly independent of hsp synthesis (BARNES *et al.* 1990; DE VIRGILIO *et al.* 1991). Furthermore there is no *hsp* mutation or combination of mutations that makes stationary phase cells behave like exponential cells with regard to heat shock resistance. Therefore it seems likely that molecules in addition to the known heat shock proteins are important for heat shock resistance.

Another candidate thermoprotectant is the disaccharide trehalose. High concentrations of trehalose are correlated with resistance to heat and desiccation in many organisms; *e.g.*, slime molds, insects, resurrection plants, yeast and other fungi (KILLICK and WRIGHT 1972; ELBIEN 1974; CROWE *et al.* 1984; HOTTIGER *et al.* 1987, 1989, 1992; DE VIRGILIO *et al.* 1990, 1991; NEVES *et al.* 1991; NEVES and FRANÇOIS 1992). Trehalose may be partly able to replace water in forming a hydration shell around proteins, and it is now well established that many if not most enzymes become resistant to heat denaturation and to desiccation in the presence of trehalose (*e.g.*, COLAÇO *et al.* 1992; HOTTIGER *et al.* 1994; ALDRIDGE 1995). In yeast, high concentrations of trehalose are well correlated with resistance to heat shock (HOTTIGER *et al.* 1987, 1989, 1992; DE VIRGILIO *et al.* 1991; NEVES and FRANÇOIS 1992). Yeast synthesizes trehalose in a two-step reaction as shown in Figure 1 (CABIB and LELOIR 1958). These reactions are carried out by a complex consisting of three subunits: (1) a 56-kDa subunit that is trehalose-6-phosphate synthase encoded by *TPS1* (or *CIF1*) (BELL *et al.* 1992), (2) a 100-kDa subunit that is trehalose-6-phosphate phosphatase encoded by *TPS2* (DE VIRGILIO *et al.* 1993), and (3) a 123-kDa subunit encoded by *TSL1* and thought to have

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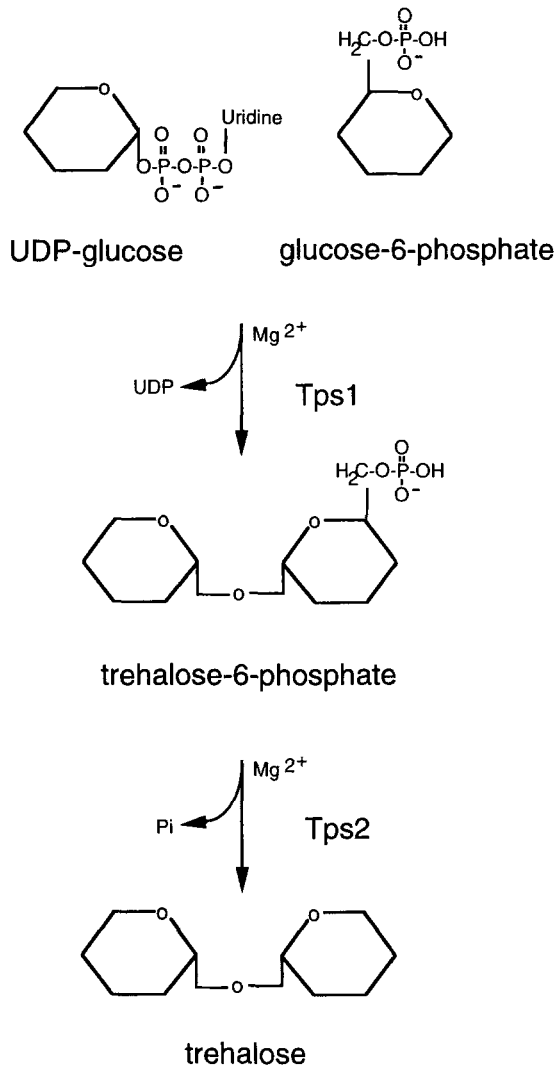


FIGURE 1.—The biosynthesis of trehalose. In the first step, Tps1 (trehalose-6-phosphate synthase) transfers the glucosyl residue from UDP-glucose to glucose-6-phosphate yielding trehalose-6-phosphate. In the second step, Tps2 (trehalose-6-phosphate phosphatase) cleaves the phosphate from trehalose-6-phosphate resulting in trehalose.

a regulatory function as well as some trehalose-6-phosphate phosphatase activity (VUORIO *et al.* 1993). In addition, there is a *TPS1/CIF1* homologue in the database that is extremely similar to *TSL1* (VUORIO *et al.* 1993). Curiously, *TPS1*, *TPS2*, *TSL1* and the *CIF* homologue share about a 33% identity over a stretch of ~500 amino acids, with *TSL1* and the *CIF* homologue sharing a 55% identity over their entire length (DE VIRGILIO *et al.* 1993; VUORIO *et al.* 1993). It is not clear why these four proteins have this common region. The *TPS1* and *TPS2* mRNAs are heat shock inducible (BELL *et al.* 1992; DE VIRGILIO *et al.* 1993; SUR *et al.* 1994). The *TPS2* promoter contains the C_4T heat shock/stress response element, while *TPS1* and *TSL1* contain this C_4T element and also the canonical heat shock element, HSE (VUORIO *et al.* 1993).

Genetic evidence suggests that trehalose is important for thermotolerance of yeast cells (DE VIRGILIO *et al.* 1994) and of *Escherichia coli* cells (HENGGE-ARONIS *et al.* 1991). Mutants lacking *TPS1* or *TPS2* are somewhat heat shock sensitive, and neutral trehalase (*nth*) mutants maintain acquired thermotolerance for longer than do wild-type cells (DE VIRGILIO *et al.* 1994). However, interpretation of these results is complicated by the fact that trehalose metabolism affects other processes, and *tps1* and *tps2* mutations are pleiotropic. For instance, *tps1* mutants cannot grow on glucose (HOHMANN *et al.* 1993). The reason for this is not clear, but one possibility is that *tps1* mutants completely lack trehalose-6-phosphate, and there is evidence that trehalose-6-phosphate is a feedback inhibitor of hexokinases (BLÁZQUEZ *et al.* 1993); therefore, *TPS1* may be important in regulating the early steps of glycolysis. *tps2* mutants, in addition to being heat shock sensitive, are unable to grow at 37°. Again, the reason for this has been unclear, but one possibility is that trehalose-6-phosphate that accumulates in *tps2* mutants is toxic at high temperatures (PIPER and LOCKHEART 1988; DE VIRGILIO *et al.* 1993, 1994; HOTTIGER *et al.* 1994; THEVELEIN and HOHMANN 1995).

We have searched for mutants unable to acquire heat shock resistance in stationary phase. Our results suggest that trehalose and Hsp104 somehow cooperate to produce heat shock resistance. Mutants lacking both Hsp104 and trehalose are extremely heat shock sensitive, and this is true even at moderate temperatures where neither *hsp104* mutants nor trehalose mutants show a large defect.

MATERIALS AND METHODS

Strains and culture conditions: *Saccharomyces cerevisiae* strains used are listed in Table 1. RTT100 was the original Hs^- Ts^- mutant isolated, and was *tps2* and *hsp104*. RTT102 was an Hs^- Ts^- segregant from a cross between RTT100 and TF14.1. RW-5b was an Hs^- Ts^- segregant from a cross between RTT102 and W303. Yeast were grown on YEP (1% yeast extract, 2% peptone) with 2% glucose (YEPD), 2% galactose (YEPGal), or 2% ethanol (YEPEtOH) as the carbon source. Cultures were grown for 4–6 days to achieve stationary phase, or overnight to a density of $1-5 \times 10^6$ cells/ml for log phase. *tps2* strains and control strains were grown at room temperature (RT) since the *tps2* mutant is Ts^-. *tps1* strains were grown on YEPGal or YEPEtOH medium since the *tps1* strain cannot grow on YEPD medium (HOHMANN *et al.* 1993). *E. coli* strains (JA226, DH10B, and XL1-Blue) were used as hosts for plasmid construction and amplication, and were grown in standard bacterial media (SAMBROOK *et al.* 1989).

Isolation of mutants: Two related but not isogenic "wimp" strains of opposite mating types, RTF1.5-2 and TF14.1, were mutagenized with EMS to ~50% survival (SHERMAN *et al.* 1986). (A wimp strain has a mutationally weakened cAMP-dependent protein kinase that is constitutively active at a low level.) Mutagenized cells were spread on plates, colonies were grown to stationary phase, and these were screened for heat shock resistance. This was done by replica plating to a pair of YEPD plates. One plate from each pair was heat shocked for 2 hr in a 50° air incubator then moved to 30° for 3 days, while the other plate was grown at 30°. About 18,000 colonies

TABLE 1

***S. cerevisiae* strains**

Strain	Genotype	Source
RTF1.5-2	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w2} tpk2::HIS3 tpk3::TRP1 bcy1::URA3 hsp104*	M. WIGLER
TF14.1	*MAT***a** *his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 cyr1:URA3*	M. WIGLER
RTT100[a]	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w2} tpk2::HIS3 tpk3::TRP1 bcy1::URA3 hsp104 tps2*	This study
RTT102[b]	*MAT***a** *his3 leu2 ura3 trp1 ade8 tpk1^{w1}* (or ^{w2}) *tpk2::HIS3 tpk3::TRP1 bcy1::URA3 hsp104 tps2*	This study
RW-5b	*MAT***a** *his3 leu2 ura3 trp1 tpk1^{w1}* (or *w2* or *TPK1*) *tpk2::HIS3 tps2 hsp104*	
W303a	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-52 can1-100 [psi$^+$] ssd1-d*	R. ROTHSTEIN
W303α	*MATα ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-52 can1-100 [psi$^+$] ssd1-d*	R. ROTHSTEIN
W303a/α	W303a × W303α	This study
W101	Isogenic with W303α, *tps1::TRP1*	This study
W102	Isogenic with W303**a**, *tps2::URA3*	This study
W103	Isogenic with W303, *tps1::TRP1 tps2::URA3*	This study
W105	W102 with high-copy number plasmid carrying *PMU1*	This study
YS483	Isogenic with W303**a**, *hsp104::LEU2*	S. LINDQUIST
YS484	Isogenic with W303α, *hsp104::LEU2*	S. LINDQUIST
BE1[c]	Isogenic with W303, *tps1::TRP1 hsp104::LEU2*	This study
BE2[d]	Isogenic with W303, *tps2::URA3 hsp104::LEU2*	This study
BE3[e]	Isogenic with W303, *tps1::TRP1 tps2::URA3 hsp104::LEU2*	This study

[a] Derived from RTF1.5-2 by EMS mutagenesis.
[b] Segregant from RTT100 × TF14.1
[c] Tetrad segregant from W101 × YS483.
[d] Tetrad segregant from W102 × YS484.
[e] Tetrad segregant from W103 × YS483.

were examined, and 52 colonies were picked from the master plates as potential mutants. These colonies were retested for heat shock sensitivity in a more quantitative heat shock assay. After retesting, there were only four clones with a strong Hs$^-$ phenotype. Two of these were genetically intractable. The two remaining clones were analyzed. Genetic crosses showed that these two clones had mutations in the same complementation group. One clone, RTT100, was chosen for all further analysis.

Temperature sensitivity for growth (Ts$^-$): Ts$^-$ was determined by replica plating cell patches onto a pair of plates. One plate from the pair was incubated at 37° (or 38°, Figure 5) for 2 days, and the other plate was incubated at room temperature. Growth was scored after 2 days.

Heat shock assays: For quantitative heat shock assays yeast cells were grown to stationary phase, sonicated, and counted. Samples were diluted in YEP medium at room temprature to a density of ~1 × 10^5 cells/ml. One milliliter from each sample was heated to 48° (or 50° or 51°) in a waterbath. Small aliquots from these samples were taken at specific times and further diluted in cold YEP medium for plating later. The 0 time sample was done in duplicate, and it was taken immediately before heat treatment of the cultures. Samples were spread onto YEPD (or YEPGal) plates, grown at room temperature for a few days, and then colonies were counted. Survival was calculated by normalizing to the 0 time controls. Qualitative heat shock assays were performed by following the same procedure described above, but instead of plating out individual cells, cells were spotted onto plates in droplets (10 μl) containing ~1000 cells. Induced thermotolerance heat shock assays were performed by growing cultures to midlog phase in YEPD (or YEPGal) medium at room temperature. The cultures were diluted to ~1 × 10^5 cells/ml and incubated in a 37° (or 39°) waterbath for 60 min. Next they were transferred to a 48° waterbath and aliquots were taken at various times, diluted and stored on ice. Cells were spread on plates and survival was calculated as described above. All heat shock assays were performed at least twice and one of these assays was chosen for illustration.

Genetic techniques and nucleic acid analysis: Standard procedures were used for yeast genetics (SHERMAN *et al.* 1986) and molecular biology (SAMBROOK *et al.* 1989).

Gene disruptions: For construction of the *tps1::TRP1* allele, the 2.75-kb *Sph*I fragment from pMB14 (kindly donated by M. GANCEDO) containing *TPS1* was cloned into plasmid YEp351 at the *Sph*I site. Next the 1.2-kb *Bsp*EI-*Stu*I fragment representing 80% of the *TPS1* ORF was deleted, the *Bsp*EI end of the cut plasmid was modified, and a 0.84-kb *Stu*I-*Sma*I fragment of *TRP1* was used to replace the deleted sequence. The resulting plasmid, pBE19, was digested with *Sph*I to yield a 2.3-kb fragment containing the *tps1::TRP1* allele and this was used for transformation. The *tps2::URA3* allele was obtained from plasmid pME-2DA (kindly donated by M. GUSTIN). In this plasmid the 2.1-kb *Msc*I-*Sal*I fragment representing 75% of the *TPS2* ORF was deleted and replaced with a 1.8-kb *Eco*RI-*Sal*I fragment of *URA3*. Plasmid pME-2DA was digested with *Xba*I and *Sac*I yielding a 2.4-kb fragment containing the *tps2::URA3* allele that was used for transformation. Transposon mutagenesis was used to disrupt the *PMU1* gene (KLECKNER *et al.* 1991). A mini-Tn10 mutagenized 5C2 plasmid was isolated that could no longer complement the Ts$^-$ defect of strain RTT102, and that gave the correct restriction digestion pattern for the insertion of the 3.2-kb mini-Tn10 transposon (see RESULTS). Sequence analysis later showed that the transposon had inserted into the *PMU1* gene. The transposon-tagged 7.2-kb *Pvu*II fragment containing *URA3* from the 5C2 plasmid was transformed into strain W303a/α. The diploid was sporulated, and tetrad analysis showed that all tetrad segregants were Ts$^+$. One Ura$^+$ Ts$^+$ tetrad segregant was mated to strain RW-5b (Ura$^-$ Ts$^-$), and tetratype tetrads were obtained showing *PMU1* is not the Ts$^-$ locus.

Chromosomal mapping: A lambda clone filter library containing an ordered array of overlapping genomic inserts was received from L. RILES and M. OLSON (OLSON *et al.* 1986). A 4-kb *Pvu*II fragment carrying *PMU1* was labeled and hybridized to the filter library. It hybridized to clone #4091, which indicated the fragment resides on the long arm of chromo-

some *XI*. Mapping of fragments from *TPS2* and *HSP104* are included in RESULTS.

Sequencing: For *TPS2*, the 2.8-kb *Bgl*II-*Sal*I fragment of plasmid 5H1 was subcloned into pUC18 and nested deletions were made and sequenced (SANGER *et al.* 1977; SAMBROOK *et al.* 1989). For *PMU1*, a 4-kb *Pvu*II fragment from the 5C2 plasmid was subcloned into pUC118, and nested deletions were made and sequenced. For *pmu1::Tn10*, the transposon-tagged 5C2 plasmid was sequenced using oligonucleotides to the *PMU1* gene. This showed that the transposon had inserted into the *PMU1* ORF between nucleotides +223 and +224. For *HSP104*, subclones from the complementing plasmid, 3C6, were partially sequenced by Lark Sequencing Technologies Inc. (Houston, Texas), showing that *HSP104* was present. Restriction mapping showed that plasmid 3C6 contained the entire *HSP104* gene.

HPLC analysis of carbohydrate levels: Yeast cells were grown at RT to stationary phase in YEPGal medium. For each sample 1 ml of stationary phase cells was collected in an Eppendorf tube and washed four times in deionized water. The wet weight of cells per sample was determined by weighing the Eppendorf tube containing a pellet of cells and later subtracting the weight of the empty tube. Each pellet was resuspended in 1 ml of deionized water and boiled for 5 min in a water bath. After centrifugation (5 min at 15,000 × *g*) the supernatant was collected and stored at −20°. An aliquot of this supernatant was subjected to HPLC analysis (DE VIRGILIO *et al.* 1993). The quantity of trehalose and trehalose-6-phosphate per sample was determined by converting the peak areas of trehalose and trehalose-6-phosphate from each chromatogram to nanomoles by use of a standard curve for each carbohydrate.

RESULTS

Isolation of a mutant strain defective in stationary phase heat shock resistance: We reasoned that cells might have multiple mechanisms for heat shock resistance, and that to find mutations in unknown mechanisms, it might be helpful to work in a background in which a known mechanism was defective. Since the RAS/cAMP signalling pathway is known to affect the acquisition of stationary phase characteristics (for review see BROACH and DESCHENES 1990), we decided to do a mutant screen in a wimp background (*bcy1 tpk1^{w} tpk2 tpk3*) that has a weak, unregulated cAMP-dependent kinase (CAMERON *et al.* 1988). These wimp strains, like wild-type strains, acquire glycogen and acquire heat shock resistance when nutrients are exhausted, demonstrating that there is a cAMP-independent signalling pathway for at least some stationary phase events. We hoped to find mutations in this cAMP-independent pathway, which might be rendered nonredundant by the lack of the RAS/cAMP signalling pathway.

A heat-shock sensitive (Hs$^-$) strain, RTT100, was found as described in MATERIALS AND METHODS. This strain and its derivative RTT102 had little or no stationary phase-induced heat shock resistance (Figure 2A). Pretreatment of log phase cells at 37° also failed to induce thermotolerance (Figure 2B). A further phenotype was that the mutant strain failed to grow at 37° (*i.e.*, it was Ts$^-$). We were concerned that the mutant might fail to grow at 37° because even this moderate temperature might produce a lethal heat shock. However, this did not appear to be the case, because when mutant cells were shifted to 37° for several hours their growth would arrest, but if these cells were returned to room temperature their growth would resume efficiently (data not shown). On the other hand, the arrest due to 10 min of heat shock at 45° or higher was irre-

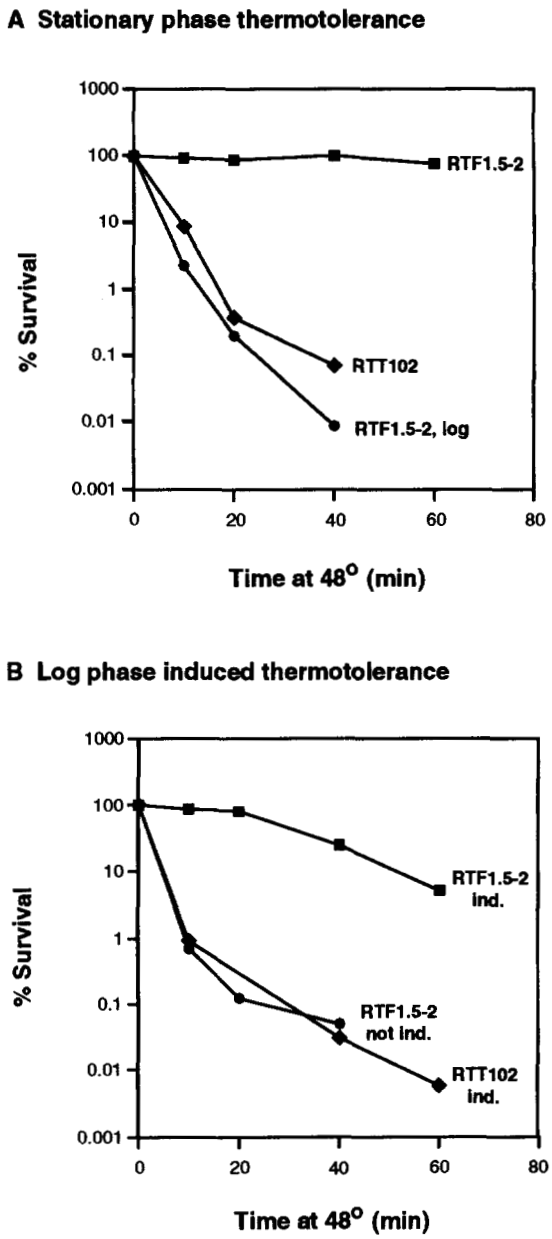


FIGURE 2.—Survival after heat shock treatment of wimp strains. RTF1.5-2 is the parental strain, and RTT102 is the Hs$^-$ Ts$^-$ strain that contains the *tps2* mutation. (A) Cells were grown in YEPD medium to stationary phase and were heat shocked for various lengths of time. Log indicates that the cells were in the logarithmic (rapid) phase of growth. (B) Cells were grown at room temperature in YEPD medium to midlog phase. ind. (induced) means cells were pretreated at 37° for 1 hr before a 48° heat shock (MATERIALS AND METHODS). not ind. (not induced) means cells were not pretreated.

versible (Figure 2B). Stronger evidence that the Ts$^-$ phenotype and the Hs$^-$ phenotype are distinct (though due to the same mutation) is given below.

Both the Hs$^-$ and the Ts$^-$ phenotypes were recessive. Within the genetic background of the parental wimp strain, RTF1.5-2, the Hs$^-$ phenotype segregated 2:2 in crosses, and the Ts$^-$ phenotype cosegregated with the Hs$^-$ phenotype. However, when the mutant was crossed to other genetic backgrounds, including the wimp strain TF14.1, segregation became irregular. The Ts$^-$ phenotype segregated 2:2, but only half of the Ts$^-$ segregants were strongly Hs$^-$, while the other half of the Ts$^-$ segregants were weakly Hs$^-$. This suggested that there were two relevant genes: the first gene causing the Ts$^-$ phenotype and a weak Hs$^-$ phenotype, and the second gene, which was presumably already present in the parental RTF1.5-2 strain before mutagenesis, modifying the first gene to intensify the Hs$^-$ phenotype. Surprisingly, neither of these phenotypes cosegregated with any of the mutations in the cAMP pathway.

Genetic analysis within the wimp background was extremely difficult, partly because the wimp strains did not have isogenic WIMP$^+$ counterparts, partly because sporulation and spore germination were both poor, and partly because of the many segregating cAMP pathway loci. Since it appeared that mutations in the cAMP pathway were not involved in the Hs$^-$ phenotype, we decided to identify the mutant genes, and then reconstruct the situation in the W303 background, where genetics would be easier.

Cloning and analysis of relevant genes: We cloned the relevant genes by transforming an Hs$^-$ Ts$^-$ mutant strain with a single copy *LEU2 CEN ARS* library (F. SPENCER and P. HIETER, personal communication) and a high copy *LEU2* YEp213 library (TODA *et al.* 1987). In separate screens, we selected for complementation of the Ts$^-$ phenotype, and the Hs$^-$ phenotype. These screens yielded multiple clones of a total of three different genes. The single copy library, when screened for complementation of the Ts$^-$ phenotype, gave clones carrying the *TPS2* gene (trehalose-6-phosphate phosphatase). When screened for complementation of the Hs$^-$ phenotype, the same library gave clones carrying the *TPS2* gene and clones carrying the *HSP104* gene. Finally, the high copy library, when 2000 clones were screened for complementation of the Ts$^-$ phenotype, gave a new gene which we call *PMU1*. Analysis of these clones is presented below.

Identification and analysis of *TPS2*: A *LEU2 CEN ARS* single copy library was transformed into strain RTT102 (Hs$^-$Ts$^-$) and selected for complementation of the Ts$^-$ phenotype. Plasmid clone 5H1 complemented both the Ts$^-$ phenotype and the Hs$^-$ phenotype. This plasmid carried a 9-kb insert. A *Bgl*II-*Sal*I fragment of ~3 kb was subcloned into the integrating vector pRS305 (*LEU2*). The resulting plasmid was cut at a unique *Bam*HI site within the insert, and this was used to direct integration in strain W303α. This marked strain was then crossed to RW-5b (Hs$^-$Ts$^-$). In six tetrads analyzed, all tetrads were parental ditypes and segregated 2:2 for Leu$^+$ Ts$^+$ and Leu$^-$ Ts$^-$. This shows that the insert in clone 5H1 is linked to the Ts$^-$ mutation.

A 5.7-kb *Hin*dIII fragment of 5H1 was labeled with ^{32}P and used to probe a lambda clone filter library (OLSON *et al.* 1986). The fragment hybridized strongly to lambda clone #1635 and weakly to lambda clone #6876, indicating that the fragment resides on the right arm of chromosome *IV*, near the *RAD55* locus.

Subclones from the 9-kb insert of 5H1 were tested for complementation. The smallest complementing fragment was a *Bgl*II-*Sal*I fragment of ~3 kb. Upon sequencing, it was found that this contained the *TPS2* gene (2.7 kb) (DE VIRGILIO *et al.* 1993; MATERIALS AND METHODS). Mutations in *TPS2* are known to be Ts$^-$ (PIPER and LOCKHEART 1988; DE VIRGILIO *et al.* 1993).

Phenotypic analysis of tps2: Gene disruption was used to make a *tps2* null strain (W102) in the W303 background, and also in an isogenic W303 wimp strain (MATERIALS AND METHODS; TOKIWA *et al.* 1994). *tps2* null mutants, whether WIMP$^+$ or wimp$^-$, were somewhat more sensitive in stationary phase to a heat shock at 48° than the isogenic wild-type strain (Figure 3A; data not shown). When the heat shock was done at 51°, the *tps2* mutants showed significant heat shock sensitivity (Figure 3B), but still much less than seen in RTT102.

Previous workers have also noted that *tps2* mutants are somewhat heat shock sensitive, but the interpretation of this has been controversial (DE VIRGILIO *et al.* 1994). One possibility is that trehalose acts as a thermoprotectant. Another possibility is that the *tps2* mutants accumulate trehalose-6-phosphate. This may be toxic to cells and may induce heat shock sensitivity. To distinguish these hypotheses, we used the *tps1* mutant, which is defective in trehalose-6-phosphate synthase. These mutants cannot make trehalose-6-phosphate, and of course cannot make trehalose. We constructed isogenic *tps1* and *tps1 tps2* null strains in the W303 background to compare to the *tps2* strain. These strains were grown to stationary phase in YEPGal medium instead of YEPD medium because *tps1* mutants cannot grow on glucose (HOHMANN *et al.* 1993). The *tps1* mutant (W101) and *tps1 tps2* double mutant (W103) were both somewhat heat shock sensitive when the heat shocks were done at 51° (data not shown), but were not as sensitive as the *tps2* mutant (W102) (Figure 4, and data not shown) suggesting that some of the heat shock sensitivity of the *tps2* mutant is due to lack of trehalose, and some is due to the presence of trehalose-6-phosphate.

Trehalose content of mutant strains: HPLC analysis of carbohydrates in stationary phase of these strains is shown in Table 2. As expected neither the *tps1* strain nor the *tps1 tps2* strain accumulated trehalose or trehalose-6-phosphate. The *tps2* strain accumulated trehalose-6-phosphate and low levels of trehalose. The conversion

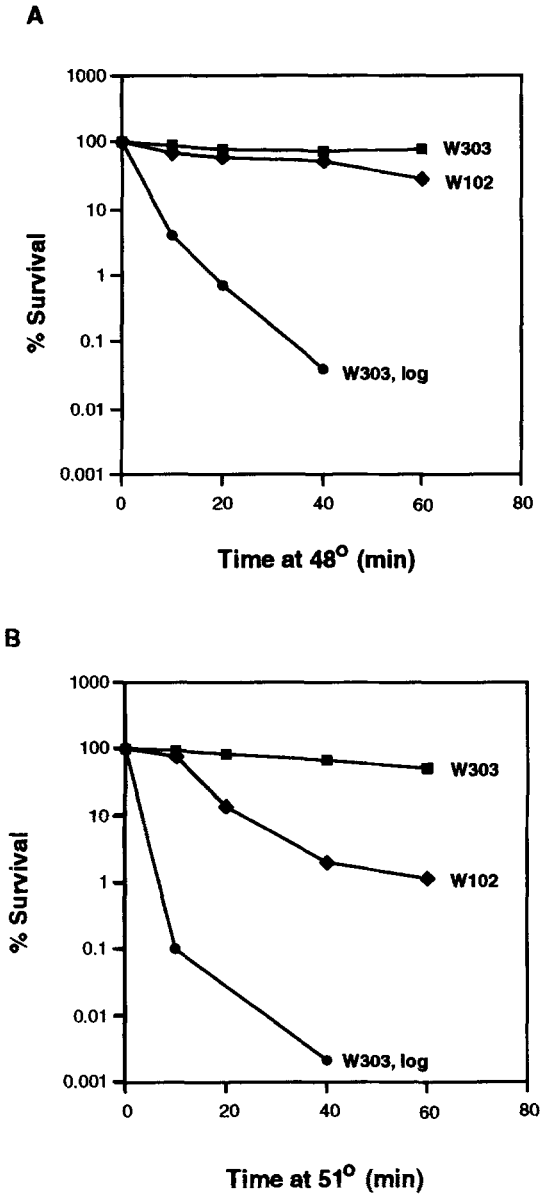


FIGURE 3.—Heat shock assays on stationary phase cultures of the *tps2* strain in the W303 genetic background. W303 is the wild-type strain, and the W102 strain contains the *tps2 URA3* mutation. Cells were grown in YEPD medium to stationary phase and heat shocked at 48° (A) or 51° (B). Log indicates control cells in the logarithmic (rapid) phase of growth.

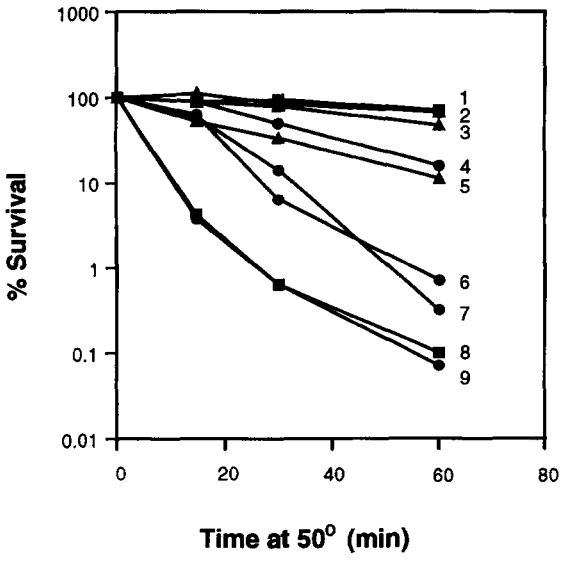
of some trehalose-6-phosphate to trehalose may be due to the *TSL1* gene, which may have some overlapping function with *TPS2* (VUORIO *et al.* 1993). These results are consistent with the idea that lack of trehalose causes some heat shock sensitivity, and that accumulation of trehalose-6-phosphate causes additional sensitivity.

The tps2 mutant is Ts⁻ due to the accumulation of trehalose-6-phosphate: Although *tps2* mutants were Ts⁻ on all tested carbon sources, the *tps1* mutant (W101) and the *tps1 tps2* double mutant (W103) were not Ts⁻ (Figure 5). This suggests that the Ts⁻ phenotype of *tps2* mutants is due to the accumulation of trehalose-6-phosphate and not to the lack of trehalose.

Identification and analysis of *HSP104*: Strain RTT102 was transformed with a *LEU2 CEN ARS* library, and we selected for complementation of the Hs⁻ phenotype (rather than the Ts⁻ phenotype, as above). We isolated 2.7×10^4 Leu⁺ transformants and did heat shock assays on these transformants by pooling them into 70 groups. Each group was diluted and samples were then heat shocked at 48° for various times. Cells were further diluted, then spotted onto plates in droplets containing 1000 cells, and later examined for survival. Five clones were consistently heat shock resistant and these fell into two classes. One class consisted of two Hs⁺ Ts⁺ transformants and contained complementing plasmids harboring the *TPS2* gene. The second class consisted of three Hs⁺ Ts⁻ transformants and contained complementing plasmids with common restriction fragments. Partial sequencing of the complementing plasmid 3C6 from one of the Hs⁺ Ts⁻ transformants showed that this plasmid contained the *HSP104* gene (SANCHEZ and LINDQUIST 1990). It has previously been shown that *hsp104* null mutants are somewhat defective in stationary phase heat shock resistance (SANCHEZ *et al.* 1992). We found that *HSP104* on a *URA3 CEN* plasmid (pYS104, from S. LINDQUIST) also complemented the Hs⁻ phenotype of Hs⁻ Ts⁻ mutant cells such as RTT102 (data not shown). *HSP104* (pYS104) did not increase the heat shock resistance of wild-type W303 stationary phase cells even at very high temperatures, showing that the complementation was specific for our mutant strain (data not shown). These results suggested that the RTT100 and RTT102 strains probably contained an *hsp104* mutation as well as a *tps2* mutation, and it is the double mutation that leads to the extreme heat shock sensitivity (see below).

The *HSP104* gene was located on the physical map of the yeast genome by labeling and hybridizing the 2.4-kb *Pvu*II fragment from the 3C6 plasmid to a lambda clone library (OLSON *et al.* 1986). This fragment hybridized to the overlapping lambda clones #3465 and #3487 that indicated that *HSP104* resides on the left arm of chromosome *XII* near the *TRX1* and *PDC1* genes.

Interaction of hsp104 with tps1 and tps2: We constructed isogenic *hsp104, tps1 hsp104, tps2 hsp104,* and *tps1 tps2 hsp104* null mutants in the W303 background to see if we could duplicate the effects seen in RTT102. The *hsp104* mutant was not heat shock sensitive in stationary phase at 48°, but when the heat shock temperature was raised to 50°, some sensitivity could be seen, consistent with previous results (SANCHEZ *et al.* 1992; Figure 4; data not shown). However, the *tps2 hsp104* double mutant (BE2) was extremely heat shock sensitive at both 48° and 50° (Figure 4; data not shown). It was far more sensitive than either the *tps2* mutant (W102) or the *hsp104* single mutant (YS483). It was comparable to the original mutant, RTT100. The *tps1 hsp104* strain (BE1) and *tps1 tps2 hsp104* strain (BE3) were also extremely heat shock sensitive, though not



1 W303
2 W101 (*tps1::TRP1*)
3 W103 (*tps1::TRP1 tps2::URA3*)
4 YS483 (*hsp104::LEU2*)
5 W102 (*tps2::URA3*)
6 BE1 (*tps1::TRP1 hsp104::LEU2*)
7 BE3 (*tps1::TRP1 tps2::URA3 hsp104::LEU2*)
8 W303, log
9 BE2 (*tps2::URA3 hsp104::LEU2*)

FIGURE 4.—Heat shock assay of stationary phase strains containing the *tps1*, *tps2* and *hsp104* mutations in the W303 genetic background. These strains were grown in YEPGal medium to stationary phase and then heat shocked at 50°. W303 log phase cells were included as a control. The geometric mean of the data from two heat shock assays was used to construct the plots.

quite as sensitive as the *tps2 hsp104* strain (BE2). When the *tps2 hsp104* double mutant (BE2) was pretreated at 37° and then heat shocked, it failed to display any induced thermotolerance (data not shown). Thus there appeared to be a synergistic effect of lack of trehalose and lack of *HSP104* function on heat shock sensitivity since the double mutants had a more severe heat shock phenotype than the expected product of the two single mutants. In addition, accumulation of trehalose-6-phosphate probably contributes to heat shock sensitivity in *tps2* strains, since these are invariably more sensitive than *tps1* strains.

We then did several experiments to show more directly that the RTT102 strain contained a mutant *hsp104* gene. When RTT102 was crossed to a *tps2 hsp104* strain in the W303 background, the resulting diploid was about as heat shock sensitive as RTT102 itself (data not shown). Furthermore, when RTF1.5-2, the original wimp strain, was crossed to an *hsp104* mutant in the W303 background, this diploid displayed the moderate heat shock sensitivity expected of an *hsp104* mutant (SANCHEZ *et al.* 1992) (data not shown) whereas after a cross with the *HSP104* version of W303, the diploid was more resistant (data not shown). (We also tried to show the presence of an *hsp104* mutation in RTT102 by integrating a marked *HSP104*, crossing to a *tps2* strain, and sporulating, but unfortunately sporulation failed, which is typical for *tps2* homozygotes; SUR *et al.* 1994.) From these results, and from the fact that *HSP104* on a plasmid suppresses the extreme heat shock sensitivity of RTT102, we believe that the parental strain RTF1.5-2 and its mutant derivatives RTT100 and RTT102 carry a mutant allele of *hsp104*. The wimp strain TF14.1, however, must be *HSP104* to explain the irregular segregation of the Hs^- phenotype in crosses with RTT100 (see above).

Isolation and characterization of the high copy suppressor *PMU1*: Strain RTT102 was transformed with a high copy number library in vector YEp213, and ~2000 Leu^+ transformants were obtained. Four plasmid clones with common restriction fragments were isolated by complementation of the Ts^- phenotype. Restriction mapping and chromosomal mapping experiments with one of the clones, 5C2, showed that it did not contain the *TPS2* gene. Gene tagging experiments showed that the locus defined by the new DNA did not cosegregate with the Ts^- or Hs^- phenotypes of the original Hs^- Ts^- mutant (MATERIALS AND METHODS). Therefore the 5C2 clone contained a high copy suppressor of the RTT102 Ts^- phenotype. The Hs^- phenotype was slightly suppressed.

A 4-kb *Pvu*II fragment of the 5C2 plasmid had com-

TABLE 2

Trehalose and trehalose-6-phosphate levels of different strains grown to stationary phase in YEPGal medium

Strains	Trehalose (nmol/mg wet weight)	Trehalose-6-phosphate (nmol/mg wet weight)
W303a	37.9	0
W102 (*tps2::URA3*)	9.2	14.7
W101 (*tps1::TRP1*)	0	0
W103 (*tps1::TRP1 tps2::URA3*)	0	0
W105 (W102 with *PMU1*)	2.3	0.3

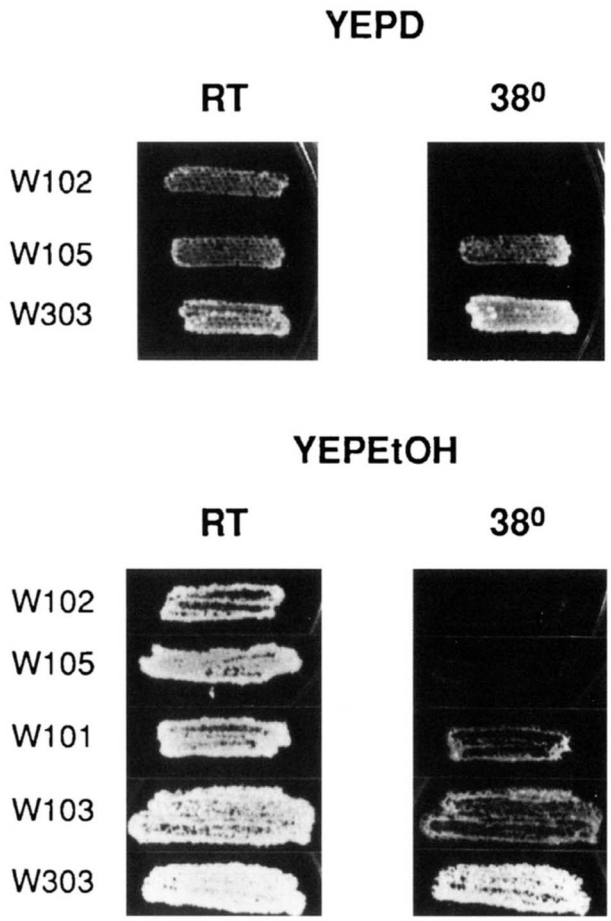


FIGURE 5.—Characterization of the Ts⁻ phenotype of different *tps1* and *tps2* strains. Strains were grown on YEPD (or YEPEtOH) plates at RT, replica plated to YEPD (or YEPEtOH) plates, and incubated at 38° for 2 days. The strains are isogenic with W303, with following mutations: W101 (*tps1::TRP1*), W102 (*tps2::URA3*), and W103 (*tps1::TRP1 tps2::URA3*). W105 strain is W102 with *PMU1* on a high copy plasmid, and W303 is wild type. Note that high copy *PMU1* cannot suppress the Ts⁻ phenotype of *tps2* on ethanol plates.

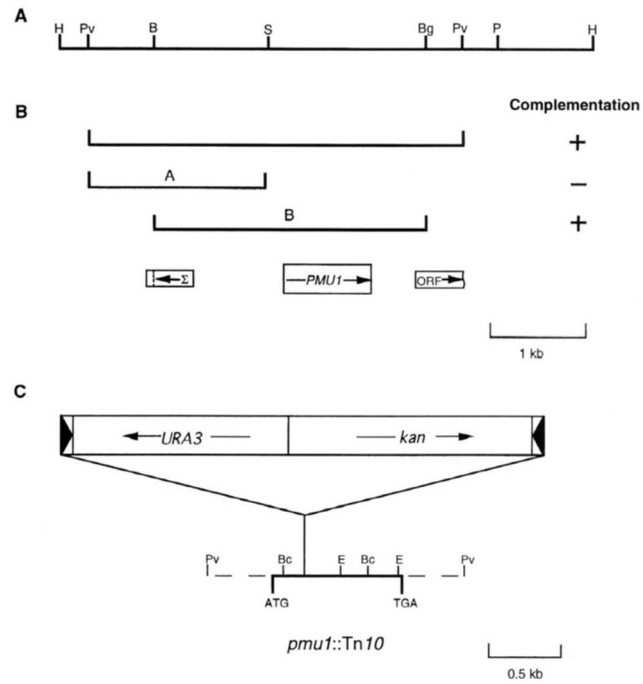


FIGURE 6.—Restriction map, complementation analysis and the transposon-tagged allele of the *PMU1* gene. (A) The restriction map of the 5.7-kb *Hin*dIII insert from the 5C2 plasmid. (B) The complementing 4-kb *Pvu*II fragment. The *PMU1* ORF is shown. A partial ORF that has homology to myosin is located downstream of *PMU1,* and a sigma (Σ) element and a $tRNA^{Lys}$ (small rectangle) is located upstream. Subclone B (a 2.9-kb *Bgl*II fragment from a nested deletion of 5C2 subcloned into YEp213) retains complementing activity, whereas subclone A (a 1.9-kb *Pvu*II fragment from a nested deletion of 5C2 subcloned into pRS425) does not. (C) The mini-Tn10 insertion in *PMU1*. The solid bar represents the *PMU1* ORF with start and stop codons indicated, and the dashed line represents adjacent genomic DNA that is not drawn to scale. Mini-Tn10 consists of two inverted repeats, and *kan* and *URA3* selectable markers. B, *Bgl*I; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eag*I; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sca*I.

plementing activity and was sequenced (MATERIALS AND METHODS; Figure 6). This fragment contained one significant open reading frame 885 bases long that potentially encodes a protein of 295 amino acids with a molecular weight of 33,776 Da (Figure 7A). The codon bias is 0.139, suggesting it is not an abundant protein (BENNETZEN and HALL 1982). The open reading frame is on chromosome *XI,* which was recently sequenced (DUJON *et al.* 1994) and has been given the name EMBL SCYKL128c. Transposon mutagenesis showed that an insertion within this open reading frame eliminated the complementing activity (MATERIALS AND METHODS; Figures 6C and 7A). The encoded protein shows weak overall homology to phosphoglycerate mutases from various organisms and to fructose-2,6-bisphosphatase from one organism (Figure 7B). In particular, the active site of these enzymes, which catalyze phospho group transfer via a phosphohistidine intermediate (PILKIS *et al.* 1987), was highly homologous to a region of this open reading frame. Therefore we named the new gene *PMU1,* for Phospho-Mutase homologue.

High copy *PMU1* fully complements the Ts⁻ defect of *tps2* log phase cells grown in glucose medium (Figure 5). In addition, high copy *PMU1* partially complements the heat shock sensitive defect of *tps2* stationary phase cells (data not shown).

Stationary phase cells of the *tps2* W303 mutant containing high copy *PMU1* (W105) contained much less trehalose-6-phosphate than cells without *PMU1* (Table 2). The most straightforward interpretation of these results is that *PMU1* can somehow remove trehalose-6-phosphate, possibly by transferring the phosphate to some other position or molecule, and this cures the Ts⁻ phenotype, and suppresses the portion of the Hs⁻ phenotype that is due to trehalose-6-phosphate toxicity. However, *PMU1* does not restore trehalose, at least not to wild-type levels, and therefore it cannot fully restore heat shock resistance.

We asked whether *PMU1* is normally involved in the

A

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M S L R A V P G Y F A A Y P S E G F Q G L D S T K Y D H L E L I N H   34
K N W K E L Y H A I P R N T K N R H Y K L L I L A R H G Q G Y H N A   68
A I L R Y G M E K W D A Y W S L L S G D E H G E W L D S K L T P L G   102
K D Q V R R T G S N V L L P M A K Q L G M L P H V F F S S P M R R C   136
L E T F I E S W T P V L A E T Q E L P A G T K I S T R I I E G L R E   170
T L G S H T C D K R V A H S M A V D E Y Q D F S T E S G H T V H W Q   204
Y V P D Y P E D D E L W L P D H R E T C A E M D K R T L N G L F E L   238
F N Q L S S E E K F I S L T C H S G V I Q S V L R N L Q H P P I Y N   272
L D T G K V V A V V V E V P V N T A D R G R L                         295
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B

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Pmu1 (yeast)      LILARHGQGYHN (25) WLDSKLTPLGKDQVRRTGSNV
Gpm1 (yeast)      LVLVRHGQSEWN ( 8) WVDVKLSAKGQQEAARAGELL
Pgm (bacteria)    LILLRHGESEWN ( 8) WVDVNLTPKGEKEATRGGELL
Bpgm (human)      LIMLRHGEGAWN ( 8) WVDQKLNSEGMEEARNCGKQL
Fbp (rat)         SIYLRHGESELN
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FIGURE 7.—The deduced amino acid sequence of *PMU1* and homology to other proteins. (A) The deduced amino acid sequence of *PMU1*. The bold **M** indicates the site of the mini-Tn10 insertion. A strongly hydrophobic region is underlined. (B) Homology of the deduced amino acid sequence of *PMU1* to the active site of various phosphomutases. Gpm1, the phosphoglycerate mutase from *S. cerevisiae* (HEINISCH *et al.* 1991); Pgm, the 3-phosphoglycerate mutase from *Streptomyces coelicolor* (WHITE *et al.* 1992); Bpgm, the 2,3-bisphosphoglycerate mutase from human (JOULIN *et al.* 1988); and Fbp, the fructose-2,6-bisphosphatase from rat (PILKIS *et al.* 1987). The bold **H** indicates the histidine in the active site, which forms a phospho-histidine intermediate during the phospho-transfer reaction (based on studies on Gpm1 and Fbp). Numbers in parentheses indicate the number of amino acids between the indicated blocks of homology.

heat shock response. A heat shock spot assay at 48° was performed on tetrad segregants containing a disrupted *PMU1* allele, *pmu1*::Tn*10* (MATERIALS AND METHODS; Figure 6C). All stationary phase cultures of tetrad segregants had wild-type survival indicating that *PMU1* was not important, *per se*, for heat shock resistance (data not shown). Northern blot analysis of the *PMU1* mRNA from the W303α strain indicated that there was no change in mRNA levels upon a temperature upshift from 22° to 39° (data not shown). These results taken together suggested that *PMU1* may be preventing the accumulation of trehalose-6-phosphate in *tps2* cells, and so preventing trehalose-6-phosphate toxicity.

DISCUSSION

We have found that two previously known mechanisms for heat shock resistance are synergistic with each other, and together account for most of the heat shock resistance of stationary phase cells. One mechanism is provided by Hsp104 that is known to provide thermotolerance to both log phase cells preconditioned to high temperatures (SANCHEZ and LINDQUIST 1990) and to stationary phase cells (SANCHEZ *et al.* 1992). The other mechanism is provided by trehalose that has been increasingly implicated in the heat shock response. The synthesis of trehalose is well correlated with high temperatures (HOTTIGER *et al.* 1987, 1989, 1992; DE VIRGILIO *et al.* 1991; NEVES and FRANÇOIS 1992), and *in vitro* and *in vivo* evidence suggests trehalose is a thermoprotectant (COLAÇO *et al.* 1992; HOTTIGER *et al.* 1994). In addition, Tps1 and Tps2 have been shown to be heat shock proteins (BELL *et al.* 1992; DE VIRGILIO *et al.* 1993; VUORIO *et al.* 1993).

Trehalose and Hsp104 appear to provide distinct biochemical functions. *In vitro* evidence suggests that trehalose acts as a thermoprotectant by stabilizing proteins and preventing heat inactivation (COLAÇO *et al.* 1992; HOTTIGER *et al.* 1994), whereas Hsp104 repairs heat-damaged proteins by resolubilizing the insoluble aggregates (PARSELL *et al.* 1994). Thus the two functions appear to complement one another in the heat shock response, one putatively by protection, and the other by repair. If one of these two mechanisms is missing, stationary phase cells become sensitive to heat shocks above 50°, but are still fairly resistant at more moderate temperatures. However, we now show that double or triple mutants lacking both mechanisms are one to two hundred-fold more heat shock sensitive than any single mutant, and sensitivity is seen even at moderate temperatures where the single mutants display little if any heat shock sensitivity.

We find that in the *tps2* mutant, both the lack of trehalose and the presence of trehalose-6-phosphate contribute to heat shock sensitivity in stationary phase. The *tps2* mutant is more sensitive (four- to sixfold) than either the *tps1* or the *tps1 tps2* mutants. Similarly the *tps2 hsp104* mutant is more sensitive (four- to 10-fold) than either the *tps1 hsp104* or the *tps1 tps2 hsp104* mutant. DE VIRGILIO *et al.* (1994) also observed that *tps1*

and *tps2* stationary phase cells are very sensitive to heat at extremely high temperatures with the *tps2* mutant being more sensitive initially. The Ts⁻ defect of *tps2* mutant in log phase, on the other hand, is due solely to the accumulation of trehalose-6-phosphate. We find that only the *tps2* mutant is Ts⁻, whereas the *tps1* and *tps1 tps2* mutants, which do not accumulate trehalose-6-phosphate or trehalose, can grow at 37°. In addition, we isolated a high copy suppressor of the *tps2* Ts⁻ defect, *PMU1*, which is capable of reducing trehalose-6-phosphate levels, suggesting that trehalose-6-phosphate is responsible for the Ts⁻ defect. A region of Pmu1 is highly homologous to the active site sequence of phosphoglycerate mutases as well as fructose-2,6-bisphosphatase from different organisms, suggesting that Pmu1 may have a function related to the manipulation of phosphate groups on carbohydrates.

Trehalose and Hsp104 together can account for most of the heat shock resistance of stationary phase cells. However, the *tps1 hsp104* mutant is still slightly more resistant in stationary phase than wild-type cells in log phase (Figure 4), and this suggests there is still at least one mechanism of resistance yet to be identified. Perhaps some other hsp is reponsible for this resistance.

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LITERATURE CITED

ALDRIDGE, S., 1995 Trehalose boosts prospects for improved biopharmaCeuticals and vaccines. Genet. Eng. News **15:** 10–11.

BARNES, C. A., G. C. JOHNSTON and R. A. SINGER, 1990 Thermotolerance is independent of induction of the full spectrum of heat shock proteins and of cell cycle blockage in the yeast *Saccharomyces cerevisiae.* J. Bacteriol. **172:** 4352–4358.

BELL, W., P. KLAASSEN, M. OHNACKER, T. BOLLER, M. HERWEIJER *et al.,* 1992 Characterization of the 56-kDa subunit of yeast trehalose-6-phosphate synthase and cloning of its gene reveal its identity with the product of CIF1, a regulator of carbon catabolite inactivation. Eur. J. Biochem. **209:** 951–959.

BENNETZEN, J. L., and B. D. HALL, 1982 Codon selection in yeast. J. Biol. Chem. **257:** 3026–3031.

BLÁZQUEZ, M. A., R. LAGUNAS, C. GANCEDO and J. M. GANCEDO, 1993 Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. FEBS Lett. **329:** 51–54.

BROACH, J. R., and R. J. DESCHENES, 1990 The function of RAS genes in *Saccharomyces cerevisiae.* Adv. Cancer Res. **54:** 79–139.

CABIB, E., and F. L. LELOIR, 1958 The biosynthesis of trehalose-6-phosphate. J. Biol. Chem. **231:** 259–275.

CAMERON, S., L. LEVIN, M. ZOLLER and M. WIGLER, 1988 cAMP-independent control of sporulation, glycogen metabolism, and heat shock resistance in *S. cerevisiae.* Cell **52:** 555–566.

COLAÇO, C., S. SEN, M. THANGAVELU, S. PINDER and R. ROSER, 1992 Extraordinary stability of enzymes dried in trehalose: simplified molecular biology. Biotechnology **10:** 1007–1011.

CROWE, J. H., L. M. CROWE and D. CHAPMAN, 1984 Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science **223:** 701–703.

DE VIRGILIO, C., U. SIMMEN, T. HOTTIGER, T. BOLLER and A. WIEMKEN, 1990 Heat shock induces enzymes of trehalose metabolism, trehalose accumulation, and thermotolerance in *Schizosaccharomyces pombe,* even in the presence of cycloheximide. FEBS Lett. **273:** 107–110.

DE VIRGILIO, C., P. PIPER, T. BOLLER and A. WIEMKEN, 1991 Acquisition of thermotolerance in *Saccharomyces cerevisiae* without heat shock protein hsp104 and in the absence of protein synthesis. FEBS Lett. **288:** 355–358.

DE VIRGILIO, C., N. BURCKERT, W. BELL, P. JENO, T. BOLLER *et al.,* 1993 Disruption of *TPS2,* the gene encoding the 100-kDA subunit of the trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae,* causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity. Eur. J. Biochem. **212:** 315–323.

DE VIRGILIO, C., T. HOTTIGER, J. DOMINGUEZ, T. BOLLER and A. WIEMKEN, 1994 The role of trehalose synthesis for the acquisition of thermotolerance in yeast I. Genetic evidence that trehalose is a thermoprotectant. Eur. J. Biochem. **219:** 179–186.

DUJON, B., D. ALEXANDRAKI, B. ANDRE, W. ANSORGE, V. BALADRON *et al.,* 1994 Complete DNA sequence of yeast chromosome XI. Nature **369:** 371–378.

ELBIEN, A. D., 1974 The metabolism of α-α trehalose. Adv. Carbohydr. Chem. Biochem. **30:** 227–256.

HEINISCH, J., R. C. VON BORSTEL and R. RODICO, 1991 Sequence and localization of the gene encoding yeast phosphoglycerate mutase. Curr. Genet. **20:** 167–171.

HENGGE-ARONIS, R., W. KLEIN, R. LANGE, M. RIMMELE and W. BOOS, 1991 Trehalose synthesis genes are controlled by the putative sigma factor encoded by rpoS and are involved in stationary-phase thermotolerance in *Escherichia coli.* J. Bacteriol. **173:** 7918–7924.

HOHMANN, S., M. J. NEVES, W. DE KONING, R. ALIJO, J. RAMOS *et al.,* 1993 The growth and signalling defects of the ggs1 (fdp1/byp1) deletion mutant on glucose are suppressed by a deletion of the gene encoding hexokinase PII. Curr. Genet. **23:** 281–289.

HOTTIGER, T., T. BOLLER and A. WIEMKEN, 1987 Rapid changes of heat and desiccation tolerance correlated with changes of trehalose content in *Saccharomyces cerevisiae* cells subjected to temperature shifts. FEBS Lett. **220:** 113–115.

HOTTIGER, T., T. BOLLER and A. WIEMKEN, 1989 Correlation of trehalose content and heat resistance in yeast mutants altered in the RAS/adenylate cyclase pathway: is trehalose a thermoprotectant? FEBS Lett. **255:** 431–434.

HOTTIGER, T., C. DE VIRGILIO, W. BELL, T. BOLLER and A. WIEMKEN, 1992 The 70-kilodalton heat-shock proteins of the SSA subfamily negatively modulate heat-shock-induced accumulation of trehalose and promote recovery from heat stress in the yeast *Saccharomyces cerevisiae.* Eur. J. Biochem. **210:** 125–132.

HOTTIGER, T., C. DE VIRGILIO, M. N. HALL, T. BOLLER and A. WIEMKEN, 1994 The role of trehalose synthesis for the acquisition of thermotolerance in yeast II. Physiological concentrations of trehalose increase the thermal stability of proteins *in vitro.* Eur. J. Biochem. **219:** 187–193.

JOULIN, V., M.-C. GAREL, P. LE BOULCH, C. VALENTIN, R. ROSA *et al.,* 1988 Isolation and characterization of the human 2,3-bisphosphoglycerate mutase gene. J. Biol. Chem. **262:** 15785–15790.

KILLICK, K. A., and B. E. WRIGHT, 1972 Trehalose synthesis during differentiation in Dictyostelium discoideum. 3. *In vitro* unmasking of trehalose-6-phosphatase synthetase. J. Biol. Chem. **247:** 2967–2969.

KLECKNER, N., J. BENDER and S. GOTTESMAN, 1991 Uses of transposons with emphasis on Tn10. Methods Enzymol. **204:** 139–179.

LILLIE, S. H., and J. R. PRINGLE, 1980 Reserve carbohydrate metabolism in *Saccharomyces cerevisiae:* responses to nutrient limitation. J. Bacteriol. **143:** 1384–1394.

LINDQUIST, S. L., and E. A. CRAIG, 1988 The heat shock proteins. Annu. Rev. Genet. **22:** 631–677.

NEVES, M.-J., and J. FRANÇOIS, 1992 On the mechanism by which a heat shock induces trehalose accumulation in *Saccharomyces cerevisiae.* J. Biochem. **288:** 859–864.

NEVES, M.-J., J. A. JORGE, J. M. FRANÇOIS and H. F. TERENZI, 1991 Effects of heat shock on the level of trehalose and glycogen, and the induction of thermotolerance in *Neurospora crassa.* FEBS Lett. **283:** 19–22.

OLSON, M. V., J. E. DUTCHIK, M. Y. GRAHAM, G. M. BRODEUR, C. M. HELMS *et al.,* 1986 Random-clone strategy for genomic restriction mapping in yeast. Proc. Natl. Acad. Sci. USA **83:** 7826–7830.

PARSELL, D. A., A. S. KOWAL, M. A. SINGER and S. LINDQUIST, 1994 Protein disaggregation mediated by heat shock protein Hsp104. Nature **372:** 475–478.

PILKIS, S. J., M. O. LIVELY and M. R. EL-MAGHRABI, 1987 Active site sequence of hepatic fructose-2,6-bisphosphatase. J. Biol. Chem. **262:** 12672–12675.

PIPER, P. W., and A. LOCKHEART, 1988 A temperature-sensitive mutant of *Saccharomyces cerevisiae* defective in the specific phosphatase of trehalose biosynthesis. FEMS Microbiol. Lett. **49:** 245–250.

PRINGLE, J. R., and L. H. HARTWELL, 1981 The *Saccharomyces cerevisiae* cell cycle, pp. 97–142 in *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance,* edited by J. N. STRATHERN, E. W. JONES and J. R. BROACH. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SANCHEZ, Y., and S. LINDQUIST, 1990 HSP104 required for induced thermotolerance. Science **248:** 1112–1115.

SANCHEZ, Y., J. TAULIEN, K. A. BORKOVICH and S. LINDQUIST, 1992 Hsp104 is required for tolerance to many forms of stress. EMBO J. **11:** 2357–2364.

SANGER, F., S. NICKLEN and A. R. COULSON, 1977 DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.

SCHENBERG-FRASCINO, A., and E. MOUSTACCHI, 1972 Lethal and mutagenic effects of elevated temperature on haploid yeast. Mol. Gen. Genet. **115:** 243–257.

SCHULZE, C., 1895 Die anwendung des pasteurisirens gegen nachgährungen der weine auf den flaschen. Landwirtsch. Jahrb. **24:** 403–433.

SHERMAN, F., 1956 The heat inactivation and production of cytochrome deficiency in yeast. Exp. Cell Res. **11:** 659–660.

SHERMAN, F., G. R. FINK and J. B. HICKS, 1986 *Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SMITH, B. J., and M. P. YAFFE, 1991 Uncoupling thermotolerance from the induction of heat shock proteins. Proc. Natl. Acad. Sci. USA **88:** 11091–11094.

SUR, I. P., Z. LOBO and P. K. MAITRA, 1994 Analysis of PFK3—a gene involved in particulate phosphofructokinase synthesis reveals additional functions of *TPS2* in *Saccharomyces cerevisiae.* Yeast **10:** 199–209.

THEVELEIN, J. M., and S. HOHMANN, 1995 Trehalose synthase: guard to the gate of glycolysis in yeast? Trends Biochem. Sci. **20:** 3–10.

TODA, T., S. CAMERON, P. SASS, M. ZOLLER and M. WIGLER, 1987 Three different genes in *S. cerevisiae* encode the catalytic subunit of cAMP-dependent protein kinase. Cell **50:** 277–287.

TOKIWA, G., M. TYERS, T. VOLPE and B. FUTCHER, 1994 Inhibition of G1 cyclin activity by the RAS/cAMP pathway in yeast. Nature **371:** 342–345.

VUORIO, O., N. KALKKINEN and J. LONDESBOROUGH, 1993 Cloning of two related genes encoding the 56 kDa and 123 kDa subunits of trehalose synthase from the yeast *Saccharomyces cerevisiae.* Eur. J. Biochem. **216:** 849–861.

WERNER-WASHBURNE, M., D. E. STONE and E. A. CRAIG, 1987 Complex interactions among members of an essential subfamily of Hsp70 genes in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **7:** 2568–2577.

WHITE, P. J., J. NAIRN, N. C. PRICE, H. G. NIMMO, J. R. COGGINS *et al.,* 1992 Phosphoglycerate mutase from Streptomyces coelicolor A3(2): purification and characterization of the enzyme and cloning and sequence analysis of the gene. J. Bacteriol. **174:** 434–440.

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