TREHALOSE METABOLISM AND ITS ROLE IN THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*

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Maria de Jesus de Souza Ribeiro

aus Niterói-Rio de Janeiro, Brasilien

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GLOSSARY OF ABBREVIATIONS

ADP	adenosine 5' –diphosphate
AMP	ampiciline
APS	ammonium persulfate
ATP	adenosine 5' –triphosphate
BSA	bovine serum albumin
bp	base pairs
bZIP	basic-leucine zipper
cAMP	adenosine 3'–5'–cyclic monophosphate
cAPK	cAMP-dependent protein kinase
cDNA	complementary desoxyribonucleic acid
CHI	cycloheximide
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxid
DNA	desoxyribonucleic acid
dNTPs	2'-deoxynucleoside 5'-triphosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EMBL	european molecular biology laboratory
FPLC	fast protein liquid chromatography
Gal	galactose
G6P	glucose-6 phosphate
G1P	glucose-1 phosphate
GOD	glucose oxidase detection
HPLC	high pressure liquid chromatography
HSE	heat-shock element
HSF	heat-shock factor
hsp(s)	heat-shock protein(s)
kb	kilo bases (i.e. 1000 nucleotides)
kDa	kiloDalton
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MOPS	3-morpholinopropanesulfonic acid
mRNA	messenger ribonucleic acid

ME	malt extract
NADH	nicotinamide adenine dinucleotide, reduced form
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol 4000
PEP	phosphoenolpyruvic acid
Pi	inorganic phosphate
PMSF	phenylmethylsulfonylfluoride
RACE	rapid amplification of 5'/3' cDNA ends
Raff	raffinose
RNA	ribonucleic acid
SAPK	stress-activated protein kinase
SDS	sodium dodecylsulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
STRE	stress-response element
TCA	trichloroacetic acid
TEMED	N,N,N'N'-tetramethylethylenediamine
Tre6P	trehalose-6-phosphate
Tricine	N-tris(hydroxymethil)-methylglycine
TRIS	tris(hydroxymethyl)-aminomethane
UDP	uridine 5'–diphosphate
UDPG	UDP-glucose
UV	Ultraviolet
YES	yeast extract, dextrose, supplements

SUMMARY

One of the best characterized stress responses in microorganisms is that to heat shock. This response enables mildly heat shocked cells to survive a subsequent severe heat stress that would be lethal in the absence of the prior mild heat shock. An introduction describing various aspects of the stress response followed by a specific review of the heat-shock response in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, emphasizing particularly the differences between these two yeast species, are presented in **chapter 1**.

One important aspect of the heat-shock response is the accumulation of the non-reducing disaccharide trehalose, which is widely distributed in nature. Trehalose was found to accumulate not only in *S. cerevisiae* under heat-shock conditions, but also under other adverse environmental conditions such as during nutrient depletion and dehydration, suggesting that trehalose may possess two functions: as an energy reserve and as a stress protectant. A detailed description of the occurrence, biosynthesis, degradation, physiological roles, and potential biological and commercial applications of trehalose is given in **chapter 1**. Heat-shock proteins (hsps) are also known to be synthesized during a mild heat shock and have been generally assumed to play a major role in the development of induced thermotolerance. Genetic experiments to determine the specific role of trehalose in heat-induced thermotolerance in *S. cerevisiae* have been hampered mainly by the finding that deletion of the *TPS1* gene coding for one of the key enzymes in trehalose formation, trehalose-6-phosphate (Tre6P) synthase, causes several pleiotropic effects including the inability to grow on glucose. A most unexpected finding in research on trehalose metabolism in the fission yeast *S. pombe* was the recent discovery that deletion of the homologous gene, *tps1*$^{+}$, did not lead to the pleiotropic effects observed in *S. cerevisiae* and that these cells were able to grow on glucose. Moreover, heat-induced increase in trehalose accumulation was found to be totally independent of protein synthesis in *S. pombe*, while it is partially dependent on protein synthesis in *S. cerevisiae*. These differences make *S. pombe* a particularly interesting model organism for studies on trehalose metabolism and its role in the stress response.

In order to determine the potentially different roles of hsp synthesis and trehalose synthesis for development of induced thermotolerance, the effects of both the protein translation inhibitor cycloheximide and a deletion of the *S. pombe tps1*$^{+}$ gene were studied. The conclusions of these experiments, presented in **chapter 3,** are that the relative importance of hsp and trehalose synthesis in the acquisition of thermotolerance is strongly dependent on the temperature used for the mild heat shock. Trehalose accumulation and hsp synthesis have different temperature optima for maximal induction. Mild heat shocks at temperatures around 37.5°C cause a high level of hsp synthesis that seems to be more important than trehalose accumulation at this temperature. However, mild heat shocks at temperatures higher than 42.5°C prevent hsp synthesis, while

trehalose accumulation is enhanced. Mild heat shocks at temperatures between 37.5 and 42.5°C result in partial activation of both responses. Evidence for additional hsp- and trehalose-independent post-translationally regulated factors involved in thermotolerance acquisition is provided in this chapter. Based on the fact that a set of heat-shock genes, including the *S. cerevisiae TPS1*, is under the negative control of a cAMP-dependent protein kinase (cAPK), thermotolerance in mutants carrying a deletion in the *S. pombe* cAPK, Pka1, was also studied (**chapter 3**). During the challenging heat shock (8 min at 50°C), *pka1*$^-$ strains, contrary to wild-type strains, accumulated trehalose and were shown to be extremely thermotolerant. Deletion of the *tps1*$^+$ gene in a *pka1*$^-$ background caused a partial decrease in the acquired thermotolerance. It is therefore obvious that trehalose synthesis is an important element of acquired thermotolerance even during the challenging heat shock. However, additional, yet unidentified, rapid, adaptive responses triggered by the challenging heat shock may also contribute significantly to the levels of thermotolerance. **Chapter 3** has been published in *Molecular Microbiology* **25:** 571-581 (Ribeiro *et al.*, 1997).

Based on the knowledge gained in **chapter 3**, it was important to elucidate the formation of trehalose particularly under heat-shock conditions. In *S. pombe*, *tps1*$^+$ was the only gene involved in trehalose synthesis so far characterized. **Chapter 4** describes the cloning and characterization of the *S. pombe TPS2* homologue, *tps2*$^+$. Deletion of *tps2*$^+$ caused loss of Tre6P phosphatase activity and Tre6P accumulation under conditions in which wild-type cells accumulate trehalose, indicating that *tps2*$^+$ codes for the Tre6P phosphatase in *S. pombe*. As in *S. cerevisiae*, heat shock induced an increase in Tre6P phosphatase activity, which was preceded by an accumulation of *tps2*$^+$ mRNA indicating that *tps2*$^+$ is transcriptionally activated during heat shock. Due to the European *Schizosaccharomyces* Genome Sequencing Project, two additional genes, *tps3*$^+$ and *tps4*$^+$, which share homology to *S. cerevisiae TPS3* and *TPS2*, respectively, were identified. Single *tps3*$^+$ and *tps4*$^+$ deletion mutants, but not a single *tps2*$^+$ deletion mutant, had lower *in vitro* activities of Tre6P synthase, indicating that both Tps3 and Tps4, but not Tps2, are needed for optimal Tre6P synthase activity. These results, including the construction of various single and double mutants, provided the basis for a more detailed analyses, presented in **chapter 5**, of the structural composition of the Tre6P synthase complex in *S. pombe*. Based on size exclusion gel filtration analysis, it is shown that a multimeric complex, comprising Tps1 exists in *S. pombe*. The most striking observation is that Tps2, Tps3, and Tps4 do not seem to be part of the Tre6P synthase complex, suggesting that they may be only weakly, if at all, associated with Tps1, and that Tps1 may be associated with further yet unidentified proteins, or that Tps1 may form a homomeric complex. The implications of these findings for the role of trehalose synthesis in *S. pombe* are discussed in detail in **chapter 6**.

CONTENTS

ACKNOWLEDGEMENTS I
GLOSSARY OF ABBREVIATIONS II
SUMMARY IV
CONTENTS VI

CHAPTER 1: GENERAL INTRODUCTION 1

THE STRESS RESPONSE 1
THE STRESS RESPONSE IN YEAST 2
Saccharomyces cerevisiae 2
Schizosaccharomyces pombe 7
STRESS RESPONSE INDUCES DIVERSE PROTECTIVE MECHANISMS 11
TREHALOSE METABOLISM 13
Occurrence 13
Biosynthesis 14
Tre6P synthase and Tre6P phosphatase in *S. cerevisiae* – Biochemical properties 16
Regulation of Tre6P synthase and Tre6P phosphatase 18
Catabolism in *S. cerevisiae* 21
PHYSIOLOGICAL ROLES OF TREHALOSE 23
As a storage carbohydrate: comparison with glycogen 23
Trehalose as stress protectant 25
POTENTIAL BIOLOGICAL AND COMMERCIAL APPLICATIONS OF TREHALOSE 30
PARTICULAR ASPECTS OF TREHALOSE METABOLISM IN *S. POMBE* 30
OUTLINE OF THE THESIS 32

CHAPTER 2: MATERIALS AND METHODS 34

A brief note concerning nomenclature 34
Media 34
Yeast strains and growth conditions 36
Bacterial strains and growth conditions 36
Tetrad analysis 36
Sporulation analysis 37

Colony PCR 42
Screening for an *S. pombe* Tre6P phosphatase cDNA by functional complementation of an *S. cerevisiae tps2* mutant 42
RACE experiments 42
DNA sequencing and sequence analysis 44
Plasmid constructions 45
Preparation of genomic or plasmid DNA from yeast 48
Yeast transformation 48
PCR amplification of fragments for transformation 49
PCR-screening of transformants for correct integration of the deletion cassettes 51
Southern blot analysis 52
RNA isolation from yeast 53
Northern blot analysis 54
Generation times of growth on SD or YES media 54
Heat shock conditions 55
Osmotic shock conditions 55
Thermotolerance 55
Enzyme assays 55
Trehalose and Tre6P quantification 57
Protein quantification 57
Effect of pH and temperature on the activity of Tre6P phosphatase 57
FPLC experiments 58
Electrophoresis 58
Autoradiography 59

CHAPTER 3: TREHALOSE SYNTHESIS IS IMPORTANT FOR THE ACQUISITION OF THERMOTOLERANCE IN *SCHIZOSACCHAROMYCES POMBE* 60

SUMMARY 61
NTRODUCTION 61
ESULTS 62
SCUSSION 67
PERIMENTAL PROCEDURES 69
ERENCES 69

CHAPTER 4: DELETION OF THE *SCHIZOSACCHAROMYCES POMBE tps2⁺*, *tps3⁺*, AND *tps4⁺* GENES AND THEIR ROLES IN TREHALOSE METABOLISM UNDER HEAT-SHOCK CONDITIONS 72

INTRODUCTION 73
RESULTS 75
DISCUSSION 97

CHAPTER 5: STRUCTURAL COMPOSITION OF THE TRE6P SYNTHASE COMPLEX IN *SCHIZOSACCHAROMYCES POMBE* 99

INTRODUCTION 100
RESULTS 101
DISCUSSION 108

CHAPTER 6: GENERAL DISCUSSION 110

CHAPTER 7: REFERENCES 116

CURRICULUM VITAE 139

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CHAPTER 1

GENERAL INTRODUCTION

Several aspects of trehalose metabolism, including the synthesis of trehalose, its regulation, and the role of trehalose have been successfully approached in the yeast *Saccharomyces cerevisiae* in previous theses (Thomas Hottiger, 1988; Walter Bell, 1992; Claudio De Virgilio, 1993, and Anke Reinders, 1998). However, this yeast model has also some important drawbacks. *S. cerevisiae* cells that are unable to synthesize trehalose due to the deletion of *TPS1*, the gene encoding trehalose-6-phosphate (Tre6P) synthase, show pleiotropic defects, including the inability to grow on fermentable sugars such as glucose and fructose, the loss of many regulatory responses, and deficiency for sporulation. Because of the pleiotropic effects of the *TPS1* deletion, approaches to determine the specific role of trehalose in the acquisition of heat-induced thermotolerance in *S. cerevisiae* cells have been hampered. Here, I chose *Schizosaccharomyces pombe* as a model system to study trehalose metabolism, and its role under heat shock conditions. At variance with the situation in *S. cerevisiae*, deletion of *tps1*$^{+}$, which encodes Tre6P synthase in *S. pombe*, does not cause the pleiotropic phenotype observed in *S. cerevisiae*. Thus, *S. pombe* is a valuable system to study trehalose metabolism and trehalose's role as a thermoprotectant. Furthermore, *S. pombe* which belongs to the ascomycetes and which is only distantly related to the budding yeast *S. cerevisiae*, shares many molecular, genetic, and biochemical features with cells from multicellular organisms. It is therefore a particularly useful model system for higher eukaryotes.

In this introduction, aspects of the stress response common to microorganisms are outlined, followed by a more detailed description of the stress response in yeast. Trehalose occurrence, distribution, biosynthesis, catabolism and possible regulatory mechanisms will be briefly introduced. The role of trehalose as a stress protectant will be described with emphasis on the role of trehalose as a thermoprotectant. The last part of the general introduction deals with some aspects of trehalose metabolism in *S. pombe*, followed by an outline of the thesis.

THE STRESS RESPONSE

The survival of cells is dependent on their ability to sense changes in the environment and to respond appropriately to new situations. What constitutes stress is difficult to define and seems to be specific for every organism. In a simple form, stress could be defined as a factor that causes growth reduction and in severe cases leads to a disruption of the cell system and consequently causes cell death.

Of the eukaryotes, yeast cells are very tolerant to stress conditions, being found in a vast range of habitats including plants and their fruits, and sugar-containing juices. In *Saccharomyces cerevisiae*, environmental stress activates signalling pathways that trigger

specific responses, including the transcriptional induction of stress-regulated genes (Hill and Treisman, 1995). The stress response mechanisms lead to an enhanced protection of cells, by adjusting cellular functions to the new status, and by conferring an enhanced tolerance against subsequent stress. Factors and conditions that induce the stress response may be nutrient starvation, heat, freezing, heavy metal ions, oxygen and oxygen metabolites, high and low osmolarity, desiccation, high ethanol concentrations, low or high pH and DNA-damaging agents (Ruis and Schüller, 1995).

The best-characterized stress response is probably that to heat shock. Studies on heat-shock response were initiated in 1962 (Ritossa, 1962, cited in Lindquist, 1986) with a report describing a dramatic change on the salivary gland chromosomes of a fruit fly, *Drosophila busckii* (the appearance of new "puffs"), induced by heat, dinitrophenol (DNP), or sodium salicylate. Only 12 years later, the molecular analysis of the heat-shock response started with the observation that the induction of these "puffs" coincided with the synthesis of a small set of new proteins (Tissière *et al.*, 1974, cited in Lindquist, 1986). In the following years, similar responses to heat (including also other types of stress) were reported for a variety of eukaryotes and prokaryotes, suggesting that the heat-shock response represents a conserved mechanism which is probably beneficial for the living cell (Mager and Ferreira, 1993).

S. cerevisiae and the distantly related yeast *Schizosaccharomyces pombe* are commonly used systems to study cellular functions of eukaryotic cells. As unicellular microorganisms, *S. cerevisiae* and *S. pombe* offer the advantages of bacterial systems with regard to the ease of manipulation and growth conditions. A wide range of techniques in genetics, cell biology, and biochemistry is available for both yeasts. Genes can be directly deleted or replaced via homologous recombination at high efficiencies in both yeasts and some other fungi, but not yet in other eukaryotic organism. Furthermore, the recent completion of the Genome Sequencing Project in *S. cerevisiae* and in *S. pombe* has fostered considerable progress in our understanding of cellular stress response mechanisms. Based on these advantages of *S. cerevisiae* and *S. pombe*, the most profound knowledge on stress response that we have today stems from these fungi. Therefore, aspects of the stress response in yeast will be introduced in the following.

THE STRESS RESPONSE IN YEAST

SACCHAROMYCES CEREVISIAE

HSE/STRE

When *S. cerevisiae* cells are exposed to a mild heat shock (a shift from 23°C to 36°C), they rapidly adjust gene expression to maximize synthesis of a distinct group of highly conserved proteins known as heat-shock proteins (hsps) or molecular chaperones (Piper, 1993). This adaptive response has also been defined as acquisition of thermotolerance (Lindquist,

1986) since it increases the ability of cells to survive otherwise lethal high temperatures. Stresses like heat cause proteins to denature and aggregate. The production of hsps is beneficial since they facilitate survival by preventing protein aggregation, by promoting the degradation of denatured or unfolded proteins, and by preventing oxidative damage (Ang *et al.*, 1991; Parsell *et al.*, 1994; Wieser *et al.*, 1991). The elevation of temperature induces the transcription of such heat-shock genes via at least two different types of upstream activating sequence (UAS) elements. The heat-shock elements (HSE) consist of a variable number of inverted nGAAn motifs (Pelham, 1982; Amin *et al.*, 1988) in promoter regions to which the heat-activated heat-shock transcription factor Hsf1 specifically binds. In the yeasts *S. cerevisiae* and *Kluyveromyces lactis*, Hsf1 is constitutively bound to its target DNA (HSE) in the absence of a heat shock (Sorger *et al.*, 1988; Jakobson *et al.*, 1991), but a heat shock is required to fully activate the Hsf1. The strong activation of the Hsf1 by heat shock correlates with hyperphosphorylation of Hsf1.

Utilization of STREs (stress response elements) is an alternative, Hsf1-independent mechanism to induce the transcription of heat-shock genes in *S. cerevisiae*. STREs consist of an AGGGG or CCCCT core (Kobayashi and McEntee, 1990; Vuorio *et al.*, 1993) and were identified in the promoter regions of a broad spectrum of genes such as the DNA damage-responsive gene *DDR2*, the cytosolic catalase T gene *CTT1*, the heat-shock gene *HSP12* and genes involved in carbon storage (Mager and Kruijff, 1995; Ruis and Schüller, 1995). In contrast to HSEs, STREs mediate the response not only to heat shock but also to other stresses such as nitrogen starvation, osmotic stress and oxidative stress, as well as ethanol and low pH. Recently, STRE-dependent gene expression has been shown to require the transcription trans-activators Msn2 and Msn4, which bind to STREs *in vitro* (Martinez-Pastor *et al.*, 1996; Boy-Marcotte *et al.*, 1998). In response to various stresses, the Msn2/Msn4 transcription factors translocate into the nucleus and activate transcription of target genes by binding to STREs (Martinez-Pastor *et al.*, 1996; Görner *et al.*, 1998). A single yeast heat-shock gene can apparently be either under HSE control, under STRE control, or controlled by both HSE and STRE. Whether a heat-shock gene is under HSE or STRE control in turn influences its stress-induction pattern. Based on two-dimensional gel electrophoresis analysis, it was recently shown that Msn2 and Msn4 control the expression of most of the carbon metabolic enzymes and antioxidant defence proteins during the heat-shock response, whereas Hsf1 is specific for induction of the chaperones and chaperone-associated heat-shock proteins. This shows that the two regulons have distinct physiological functions in the heat-shock response (Boy-Marcotte *et al.*, 1999). In addition, STREs differ from HSEs in that their heat induction is very strongly influenced by cAMP-dependent protein kinase (cAPK) activity (Marchler *et al.*, 1993; Görner *et al.*, 1998).

In the following section I will summarize the signalling pathways which play important roles in the control and modulation of the stress response in *S. cerevisiae*. The pathways include the HOG pathway, the PKC pathway, and the Ras/cAMP pathway.

Signalling pathways which modulate the stress response in *S. cerevisiae*

Multiple mitogen-activated protein kinase (MAPK) cascades exist in eukaryotic cells and mediate appropriate cellular responses to diverse environmental stimuli. The MAPK cascades are ancient and conserved signalling cassettes comprising a series of three or more protein kinases, each phosphorylating and thereby activating the next in line. The core unit comprises a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). Several MAPK cascades can exist in a single cell and operate in parallel, each mediating a specific signal (Herskowitz, 1995). In *S. cerevisiae*, at least five MAPKs exist. One of these cascades operates in cells undergoing meiosis and regulates spore formation. The other four cascades operate in vegetatively growing cells. Two out of these cascades control developmental events-mating and filamentation, whereas the remaining two cascades control the response to solute concentration: the HOG (high osmolarity glycerol-response) pathway, orchestrating the response to high osmolarity, and the PKC (protein kinase C) pathway mediating the response to low osmolarity (for reviews, see Herskowitz, 1995; Madhani and Fink, 1998).

The osmosensing HOG pathway, was first identified through a mutation in the *HOG1* gene that conferred sensitivity to high external osmolarity (Brewster *et al.*, 1993; Schüller *et al.*, 1994). The HOG pathway consists of Sho1 (an osmosensor) Ste11 (MAPKKK), Pbs2 (MAPKK), and Hog1 (MAPK). As well as by Sho1, the HOG pathway is also activated by another integral membrane protein, Sln1, in combination with the response regulator Ssk1 (Maeda *et al.*, 1994a). Ssk1 activates two redundant MAPKKKs, Ssk2 and Ssk22, which subsequently activate Pbs2, the MAPKK for Hog1 (Posas and Saito, 1998). Pbs2 has recently been proposed to act as a scaffold protein because it binds several components of the HOG MAPK cascade: Sho1, Ste11 and Hog1 (Posas and Saito, 1997). In high osmotic conditions, yeast cells accumulate the osmolyte glycerol, to counteract the osmotic imbalance between the inside of the cell and the external environment (Blomberg and Adler, 1992). Glycerol formation involves two enzymes, each encoded by two highly homologous genes: *GPD1* and *GPD2* encoding glycerol-3-phosphate dehydrogenase, and *GPP1* and *GPP2* encoding glycerol-3-phosphatase (Albertyn *et al.*, 1994; Larsson *et al.*, 1993). These genes were shown to be differently regulated in respect to growth conditions. The HOG pathway has been recently shown to contribute significantly to the control of *GPD1* expression (Rep *et al.*, 1999). STREs have been found in the promoter region of the *GPD1* gene. However, control of *GPD1* expression seems not to involve Msn2/Msn4 transcription factors, since their deletion did not affect the osmotically-induced expression of *GPD1* (Rep *et al.*, 1999). *GPD1* can be induced by heat shock, although there is no evidence for heat-shock elements in the *GPD1* promoter, suggesting that STREs may be able to function in conjunction with transcription factors other than Msn2/Msn4 (Rep *et al.*, 1999; Boy-Marcotte *et al.*, 1999).

Response to extracellular signals is also regulated by PKC. The product of the *PKC1* gene is the sole Pkc in *S. cerevisiae*, and is a homologue of mammalian Ca^{2+}-stimulated PKC isoforms. Pkc1 is essential for all growth conditions, and is required for maintaining cell

integrity. It is stimulated by hypotonic shock and by heat stress (Kamada *et al.*, 1995). Pkc1 controls a MAPK cascade composed of the MAPKKK, Bck1, two redundant threonine/tyrosine MAPKKs, Mkk1 and Mkk2, and the MAPK Slt2. Deletion of any gene encoding a component of this pathway produces several phenotypes, including a tendency to lyse at higher temperatures (37°C) (Lussier *et al*, 1997). Due to its key role in maintaining cell integrity, it has been asserted that the Pkc pathway must act on other cellular processes, such as morphological changes that occur during cell growth, and mating, and in regulation of the cell cycle (for a review see Heinisch *et al.*, 1999).

The Ras/cAMP signalling pathway is likely to play an important part in regulating the general stress response, particularly via the control of expression of genes that are induced by heat, hyperosmotic stress, and nutrient starvation. As mentioned before, the expression of many stress-induced genes is dependent on the transcription factors Msn2 and Msn4. Both transcription factors seem to be translocated to the nucleus upon exposure to stress (Görner *et al.*, 1998). Phosphorylation of Msn2 and Msn4 by cAPK may cause the nuclear export of these transcription factors since mutations in cAPK consensus sites of Msn2 cause constitutive nuclear localization of Msn2 under non-stressed conditions. Furthermore, conditions causing increased cAPK activity lead to cytoplasmic localization of Msn2 (Görner *et al.*, 1998). An elaborate regulatory pathway, which is depicted in Figure 1, controls cAMP metabolism in *S. cerevisiae* (for reviews, see Thevelein, 1992 and 1994; Colombo *et al.*, 1998; Xue *et al.*, 1998; Kraakman *et al.*, 1999; Vanhalewyn *et al.*, 1999; Thevelein and de Winde, 1999). As in other eukaryotes, cAMP is synthesized by adenylate cyclase, encoded by the *CYR1/CDC35* gene (Matsumoto *et al.*, 1985). Adenylate cyclase is activated by the products of the *RAS1* and *RAS2* genes (Toda *et al.*, 1985). Ras1,2 are small GTP-binding protein, which display extensive homology to mammalian Ras. Deletion of one of the *RAS* genes does not affect viability, whereas deletion of both *RAS* genes is lethal (Tatchell *et al.*, 1984). In *S. cerevisiae*, Ras is converted to the GTP-bound form by GTP-exchange factors (GEF) encoded by *CDC25* and *SDC25*. Ras, in its GTP-bound form is active, whereas the GDP-bound form is inactive (Robinson *et al.*, 1987). Ras-GAPs (GTPase-activating protein), encoded by the *IRA1* and *IRA2* genes, act negatively by stimulation of the intrinsic Ras-GTPase activity (Tanaka *et al.*, 1990), converting Ras to the GDP-bound, inactive form. The Ras proteins are also required for maintenance of a basal adenylate cyclase activity to compensate for the hydrolysis of cAMP by phosphodiesterase activity. The *PDE1* and *PDE2* genes encode the low- and high-affinity phosphodiesterases, respectively (Nikawa *et al.*, 1987). Previous reports indicated a role for Pde2 in several processes controlled by cAPK, whereas the function of Pde1 remained enigmatic (Nikawa *et al.*, 1987). However, a recent report has shown that Pde1 has a specific role in controlling agonist-induced cAMP signal caused by glucose and intracellular acidification (Ma *et al.*, 1999). The *S. cerevisiae* cAPK is a tetrameric enzyme composed of two catalytic subunits, e.ncoded by the *TPK* genes (*TPK1*, *TPK2* and *TPK3*), and of two regulatory subunits, encoded by *BCY1*. Deletion of all three *TPK* genes is lethal. Mutations in

BCY1 bypass the requirement for adenylate cyclase activity (Toda *et al.*, 1987a; Toda *et al.*, 1987b).

As in other eukaryotic cells, in *S. cerevisiae* cAMP activates cAPK by binding to the regulatory subunit (*BCY1*), thereby causing its dissociation and thus activation of the catalytic subunits. The activated catalytic subunits of cAPK can activate or inhibit several target proteins by phosphorylation. These target proteins are involved in different regulatory pathways required for the shift from gluconeogenic to fermentative growth, in stress resistance, in growth control and in the breakdown of storage carbohydrates. In response to glucose availability, adenylate cyclase can also be stimulated transiently via a Ras-independent pathway, involving the Gpr1 G protein-coupled receptor (GPCR) and the Gpa2 protein (α subunit of G protein), causing a transient increase in cAPK activity (Colombo *et al.*, 1998; Xue *et al.*, 1998; Kraakman *et al.*, 1999). A second pathway, also stimulated by addition of glucose and complex growth medium to derepressed cells, seems to account for sustained cAPK activity (Thevelein, 1991; Kraakman *et al.*, 1999). This pathway called the fermentable-growth-medium (FGM) induced pathway, requires a fermentable carbon source and all other nutrients required for growth for activation (Thevelein, 1994). One component of the FGM pathway is the protein kinase Sch9 (Crauwels *et al.*, 1997). Sch9 shares homology with the catalytic subunits of cAPK (Toda *et al.*, 1988). Whereas the components of the Ras/cAMP pathway have been studied in detail, little is known about the signals and mechanisms that activate the pathway, and about the direct targets of cAPK. Recently, the Rim 15 protein kinase has been identified as a downstream target of cAPK that acts as an activator of STRE-controlled gene expression upon entry into stationary phase (Reinders *et al.*, 1998).

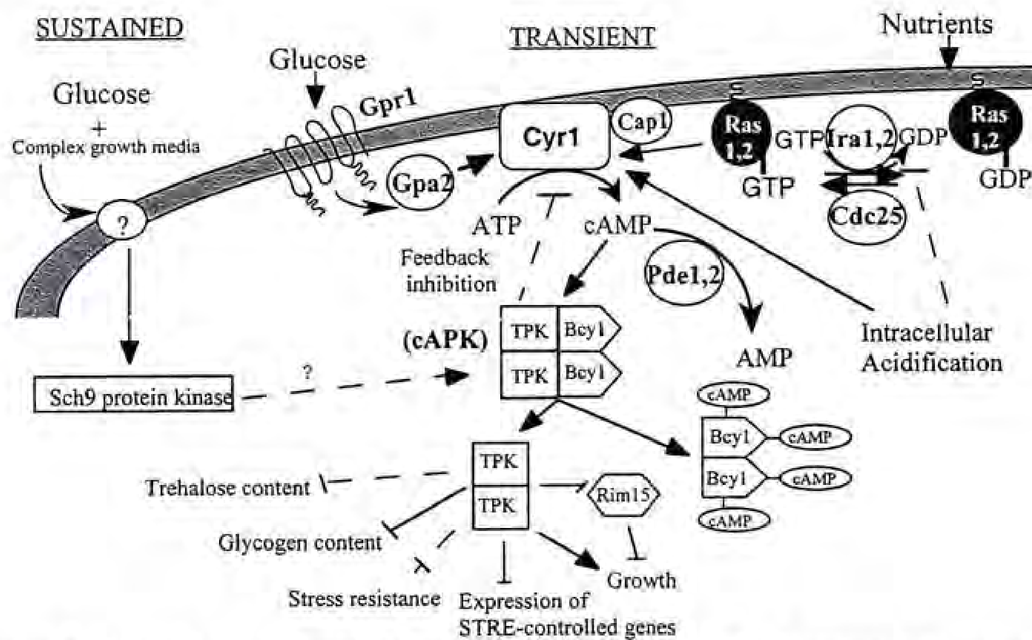

Figure 1. Model of the Ras/cAMP and the FGM pathways in *S. cerevisiae*. Glucose activates both the Gpr1-Gpa2 GPCR system and adenylate cyclase via Ras proteins. This stimulation leads to a transient activation of cAMP synthesis and consenquently of cAPK. cAPK regulates several intracellular processes. The FGM pathway utilizes a yet to-be-determined glucose-sensing system. In the FGM pathway, Sch9 is responsible for the sustained activation of cAPK targets during growth on glucose. The Rim15 protein kinase is inhibited by cAPK and is required for the induction of a range of stationary-phase-associated characteristics. Dashed arrows and bars refer to potential interactions. (Arrow) Positive interaction; (bar) negative interactions.

SCHIZOSACCHAROMYCES POMBE

Hsf/Atf1

Much less is known about the heat-shock response in *S. pombe*. Like *S. cerevisiae*, *S. pombe* cells must cope with adverse environmental conditions. The mechanisms required for adaptation to environmental changes may also modulate the activity of stress-regulated transcription factors and thus result in changes in gene transcription. As previously described, a major aspect of the heat-shock response is the transcriptional induction of genes that encode heat-shock proteins. A major mediator of this induction is the transcription factor Hsf1, whose DNA binding site, the HSE, is similar in all studied eukaryotes. In *S. cerevisiae*, Hsf1 has been shown to be constitutively bound to HSE even in the absence of the heat stress, however a heat shock is required to fully activate it. *S. pombe* cells possess only one gene coding for an HSF, *hsf$^+$*. No constitutive binding of Hsf to the HSE seems to occur in *S. pombe*, and Hsf binding is strongly increased by heat shock (Gallo *et al.*, 1991). The C-terminus of the *S. pombe* Hsf contains transcriptional activation domains as do HSFs from other organisms (Green *et al.*, 1995; Saltsman *et al.*, 1999). One of these domains has recently been shown to be required for the sustained response to heat shock (Saltsman *et al.*, 1999). In addition to a requirement during heat shock, the HSFs of *S. pombe* and *S. cerevisiae*, have been shown to be necessary for cell

growth at normal temperature (Gallo *et al.*, 1993; Sorger *et al.*, 1988). This is not surprising, given the fact that HSF is required for basal expression of some essential heat-shock genes.

Another transcription factor that plays an important role in the response of *S. pombe* cells to adverse environmental conditions has been described. The *atf1*$^{+}$ gene encodes the Atf1 transcription factor that contains a basic-leucine zipper (bZIP) domain at its C-terminus, and shows homology to members of the ATF-2 family of mammalian transcription factors (Takeda *et al.*, 1995). Furthermore, Atf1 has a DNA-binding specificity that is similar to ATF-2 (TG/TACGTC/AC/A) and can activate transcription from a reporter containing ATF binding sites within its promoter. At variance with Hsf, Atf1 is not required for normal growth, but is required for growth at low temperatures and it is important in nutrient-deprived cells to enter either G_0 state or to undergo meiosis and spore formation. Loss of Atf1 causes rapid cell death in stationary phase as well as failure in the initiation of sexual differentiation (Takeda *et al.*, 1995). As previously mentioned, cAMP is a general second messenger for external signals. In *S. cerevisiae*, the Ras/cAMP pathway mediates the signal for the availability of carbon sources and plays a critical role in controlling the mitotic cell cycle and stationary phase entry. Similar roles for the Ras/cAMP pathway have been described in *S. pombe* (Maeda *et al.*, 1990). However, Atf1 activity was not significantly changed under conditions which result in changes of cAMP levels or under conditions where cAPK is down- or up-regulated suggesting that Atf1 is not under the regulation of the Ras/cAMP pathway (Takeda *et al.*, 1995). Atf1 is, however, a downstream element of the Wis1/Spc1 MAPK pathway (see below). Atf1 binds specifically to, and is phosphorylated by, the Spc1 MAPK (Wilkinson *et al.*, 1996). Recent studies in *S. pombe* have further uncovered another set of transcription factors of the bZIP family that also appear to function downstream of the Spc1 MAPK cascade (Shiozaki and Russell, 1996). One such transcription factor Pcr1 (also known as Mts2) (Watanabe and Yamamoto, 1996) may form a heterodimer with Atf1. Cells lacking Pcr1 display similar phenotypes to cells lacking Atf1.

Signalling pathways which modulate the stress response in *S. pombe*

cAPK and the protein kinases in the MAPK-pathways are examples of stress stimulated kinases connecting stress signals with the transcription machinery (Shiozaki and Russell, 1995; Görner *et al.*, 1998; Kronstad *et al.*, 1998; Samejima *et al.*, 1997). For a long time, only one MAP kinase cascade was known in *S. pombe*, the Spk1 MAPK including the Byr1 MAPK kinase (MAPPK), Byr2 MAPKK kinase (MAPKKK) and the small GTPase Ras1 (Wang *et al.*, 1991; Gotoh *et al.*, 1993), which are involved in the transduction of the mating pheromone signal. Lately, three additional MAPK cascades have been identified. Interestingly, several stress conditions including heat shock, osmotic and oxidative stress, nutrient deprivation and ultraviolet (UV) radiation lead to the activation of the same MAPK cascade, the Wis1/Spc1 cascade (Degols *et al.*, 1996). This MAPK pathway is composed of Spc1 (MAPK; also known

as Sty1 and Phh1), Wis1 (MAPKK) and two MAPKKKs encoded by the *win1*$^+$ and *wis4*$^+$ genes (Samejima *et al.*, 1998; Shieh *et al.*, 1998). The Spc1 MAPK is homologous to Hog1 from *S. cerevisiae*. Loss of either Wis1 or Spc1 causes sensitivity to heat and osmotic stress, and sterility indicating that signalling through this pathway is also important for sexual differentiation. The activation of the Wis1/Spc1 MAPK cascade is depicted in Figure 2. Mcs4 (mitotic catastrophe suppressor) is an upstream regulator that mediates activation of the Wis1/Spc1 MAPK upon exposure of cells to several environmental stresses. Furthermore, it has been demonstrated that Mcs4 is also necessary for a correct onset of mitosis, a function independent of the Spc1 MAPK (Shieh *et al.*, 1997). Upon adverse environmental conditions, Mcs4 is activated and promotes activation of the Wis1/Spc1 cascade by phosphorylation of the MAPKK Wis1, which in turn phosphorylates Spc1 at threonine-171 and tyrosine-173 (Nguyen and Shiozaki, 1999). The phosphorylation of Spc1 causes its translocation into the nucleus via nuclear transport factors (Gaits and Russell, 1999). In the nucleus, activated Spc1 phosphorylates and thereby activates the transcription factor Atf1 (Gaits *et al.*, 1998). Activation of Atf1 causes the transcriptional induction of a set of genes required for stress adaptation, such as *gpd1*$^+$(the gene encoding for glycerol-3-phosphate dehydrogenase) whose product is important in the production of glycerol, *tps1*$^+$(the gene encoding for trehalose-6-phosphate synthase), thereby promoting trehalose formation, *ste11*$^+$, the product of which is required for initiation of meiosis, and the tyrosine- and serine-threonine phosphatases genes, *pyp2*$^+$ and *ptc1*$^+$ (Degols *et al.*, 1996; Gaits and Russell, 1999). These phosphatases negatively regulate the Wis1/Spc1 MAPK cascade, possibly via direct dephosphorylation of Spc1 and/or Wis1. Dissociation of Spc1 from phosphorylated Atf1 allows Spc1 to form a complex with the exportin Crm1, in order to be translocated back into the cytoplasm (Toone *et al.*, 1998; Gaits *et al*, 1998; Gaits and Russell, 1999). After export, Spc1 is dephosphorylated by Pyp2 and re-associates with the Wis1 MAPKK. In summary, the stress-activated Wis1/Spc1 protein kinase cascade integrates nutritional stress, heat stress, and osmotic stress by activating a specific transcription factor, Atf1 (Shiozaki and Russell, 1996).

The Ras/cAMP pathway in *S. pombe* differs profoundly from the Ras/cAMP pathway in *S. cerevisiae* (see Figure 3). In contrast to the situation in *S. cerevisiae*, Ras in *S. pombe* (encoded by *ras1*$^+$) does not influence adenylate cyclase (encoded by *cyr1*$^+$; Yamawaki-Kataoka *et al.*, 1989; Young *et al.*, 1989). The single Ras protein in *S. pombe*, Ras1, rather acts with the Gα-subunit encoded by *gpa1*$^+$, to control transduction of the mating pheromone signal via a MAPK cascade (Xu *et al.*, 1994). Adenylate cyclase is activated by another Gα-subunit, the *gpa2*$^+$ product (Isshiki *et al.*, 1992). A G-protein-coupled receptor (GPCR) that could activate Gpa2 has not yet been reported. The gene *cgs2*$^+$ encodes phosphodiesterase (De Voti *et al.*, 1991). As in *S. cerevisiae* cells, cAPK has catalytic and regulatory subunits. The gene for the regulatory subunit of cAPK, *cgs1*$^+$, (De Voti *et al.*, 1991) has been identified. The catalytic subunit of cAPK is encoded by only one gene, *pka1*$^+$ (Maeda *et al.*, 1994b). Deletion of *pka1*$^+$

is not lethal, but results in slow growth, shortened cells, and alterations in gene expression. Mutations in *pka1⁺* can be suppressed by overexpression of the *sck1⁺* and *sck2⁺* genes (suppressor of loss of cAMP-dependent protein kinase), which are homologous to the *S. cerevisiae SCH9* (Toda *et al.*, 1988; Jin *et al.*, 1995; Fujita and Yamamoto, 1998). The Sck1 and Sck2 kinases possibly share substrates with cAPK (Fujita and Yamamoto, 1998).

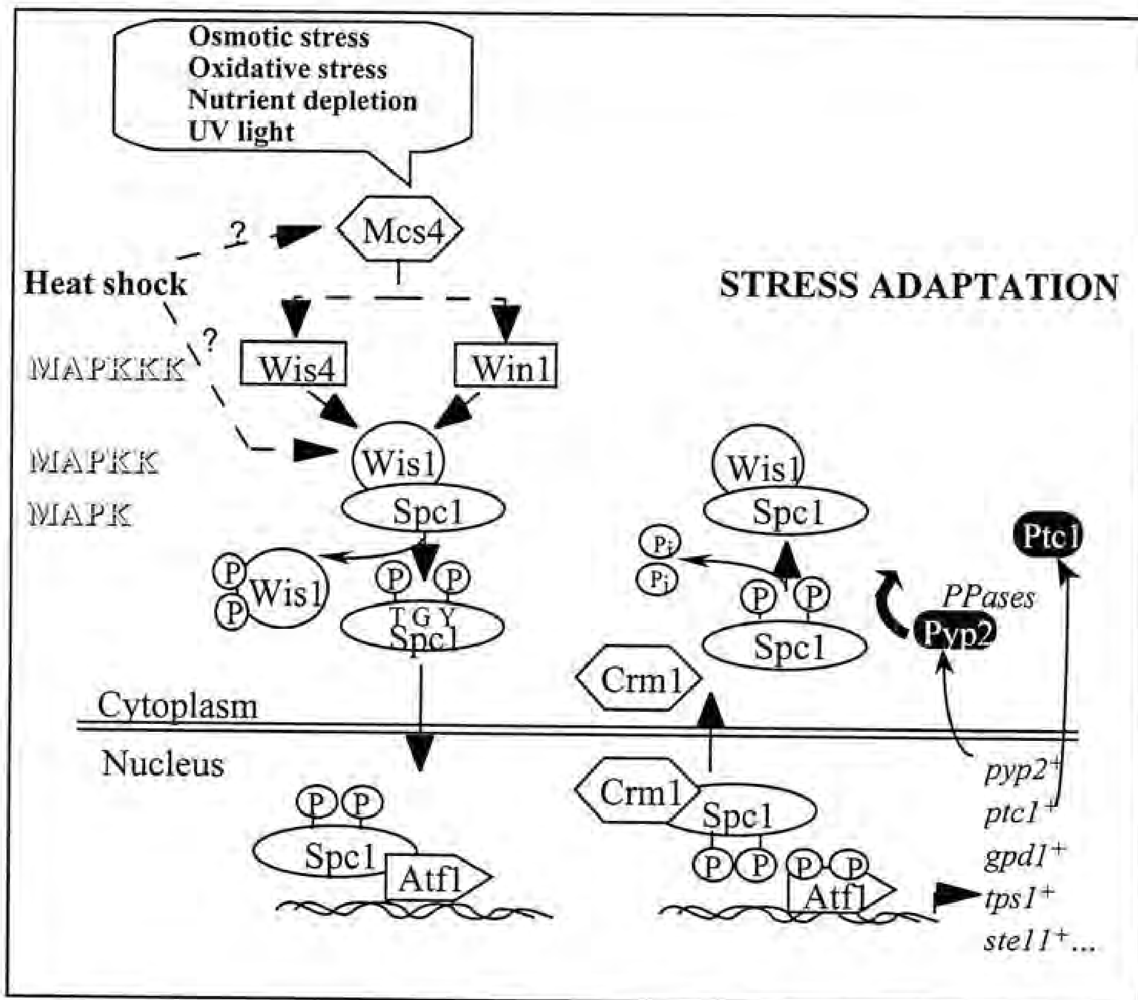

Figure 2. Activation of the Wis1/Spc1 MAPK pathway in *S. pombe*. Several external signals trigger activation of Msc4, which in turn activates the MAPKKKs Wis4 and Win1. The MAPKKK phosphorylates Wis1, which is necessary for a strong activation of the Spc1 MAPK through phosphorylation of tyrosine and threonine residues. Phosphorylated Spc1 is translocated to the nucleus. In the nucleus, activated Spc1 promotes phosphorylation of its substrate Atf1, which activates transcription of genes required for stress adaptation such as the phosphatase encoding *pyp2⁺*. The dissociation of Spc1 from Atf1 allows Spc1 to form a complex with the exportin Crm1, resulting in translocation back to the cytoplasm. In the cytoplasm, Spc1 is inactivated by the action of specific phosphatases, among them the Pyp2. The non-phosphorylated form of Spc1 associates again with Wis1.

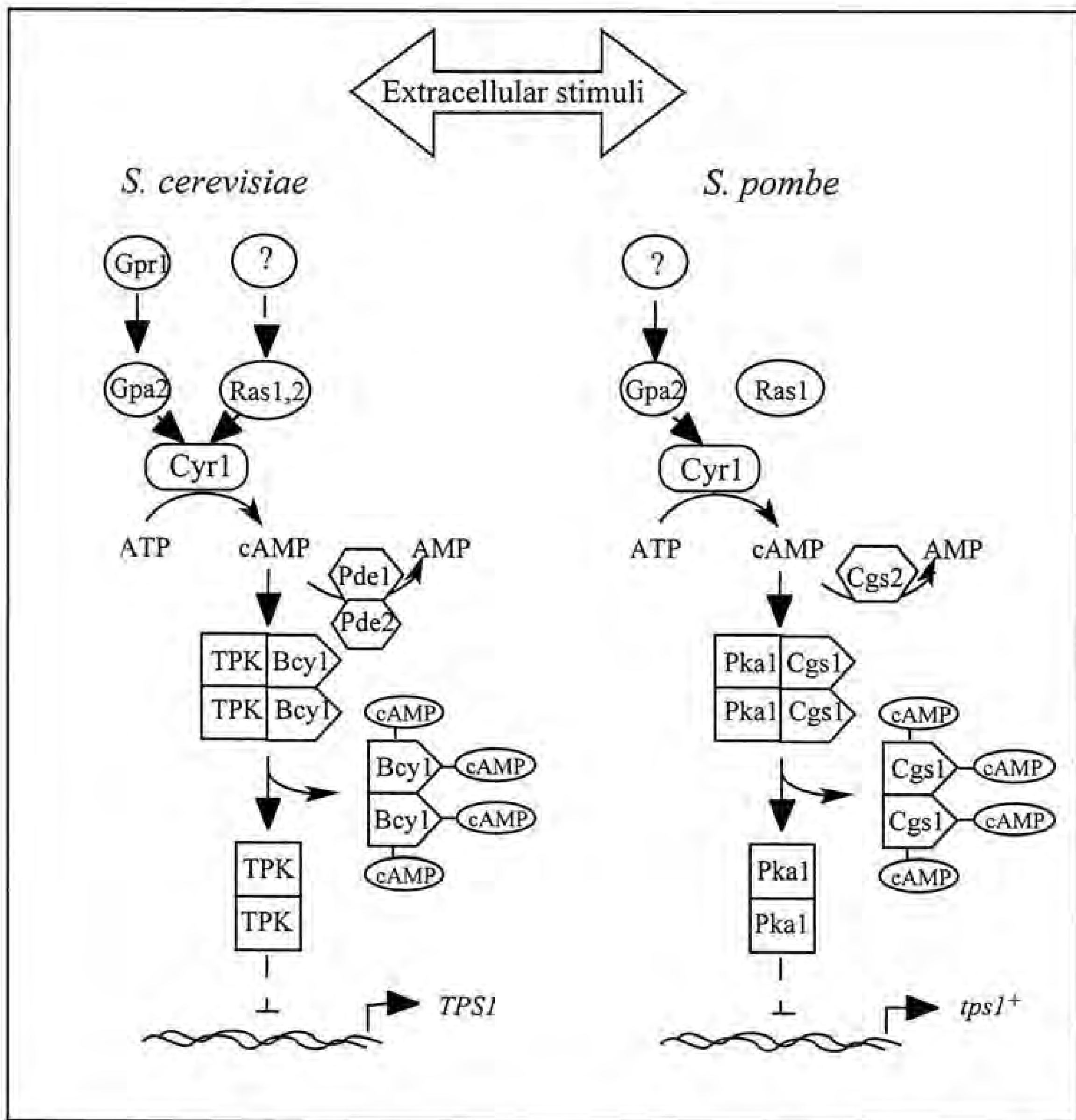

Figure 3. Ras/cAMP pathway schemes in *S. cerevisiae* and in *S. pombe*. (Arrow) positive interactions; (bar) negative interaction. Dashed arrows and bars refer to potential interactions. For further details, see text.

STRESS RESPONSE INDUCES DIVERSE PROTECTIVE MECHANISMS

In *S. cerevisiae*, a sudden temperature shift (a shift from 23°C to 36°C) generates transient alterations in the pattern of protein synthesis. However, not all proteins have their expression enhanced. For the majority of yeast genes the mild heat shock leads to a decrease in expression. Only a minority of genes (generally the heat-shock genes) are induced strongly after a mild heat shock. Initially, four classes of hsps were observed to have their expression strongly enhanced in response to high temperatures: Hsp70, Hsp90, Hsp 100, and the small

hsps (Hsp26 and Hsp12). Later, Hsp60 was also found to be an important hsp (Lindquist and Craig, 1988). Over the past decade, it has become evident that heat-shock proteins are key determinants governing the balance between aggregation and proper folding of proteins damaged by stress (Ellis, 1987; Ang *et al.*, 1991).

Hsp70s, Hsp60, and Hsp90s are molecular chaperones. This class of hsps generally participates in protein folding. Among these hsp families, the Hsp70 family has been studied in detail and its members are among the best-conserved proteins in the cell. The Hsp70 protein family is a diverse and multifunctional group involved in protein translocation, folding and degradation. *S. cerevisiae* possesses two important Hsp70 sub-families the Ssa (Ssa1-4) and Ssb (Ssb1,2) sub-families, which seem to execute different functions (James *et al.*, 1997; Morano *et al.*, 1998). Whereas the Ssa sub-family of Hsp70 proteins is required for protein translocation into both the endoplasmic reticulum (ER) and mitochondria, members of the Ssb sub-family are primarily found in association with actively translating polysome complexes. Expression of *SSA* and *SSB* genes is also differently regulated. Among the *SSA* gene family, three out of four genes are induced by heat shock, while the *SSB* genes are repressed by heat shock (Parsell and Lindquist, 1993). Regulation of *SSA3* expression by heat shock is mediated exclusively by Hsf1 and does not require Msn2 or Msn4 (Treger *et al.*, 1998). In general, it seems that induction of the expression of hsps which function as molecular chaperones is mediated by Hsf1, whereas Msn2 and Msn4 control the expression of genes involved in oxidative and heat stress (Boy-Marcotte *et al.*, 1999).

One member of the Hsp100 family is Hsp104. Hsp104 is not present in *S. cerevisiae* cells growing exponentially on glucose, but its synthesis is strongly induced in stationary-phase cells and during heat-shock conditions. Although not essential for cell viability, Hsp104 is required for heat shock-induced acquisition of thermotolerance (Sanchez and Lindquist, 1990). The role of Hsp104 as a thermoprotectant will be discussed later (see physiological roles of trehalose).

Apart from the classical hsps discussed above, other proteins such as ubiquitin show an enhanced synthesis upon exposure to heat shock. Ubiquitin is a small protein that becomes covalently attached to lysine residues of target proteins, thereby acting as a tag for ATP-dependent proteolysis. In *S. cerevisiae* ubiquitin is encoded either as a polypeptide or as a fusion protein by *UBI1*, *UBI2*, *UBI3* and *UBI4*. Among these genes, *UBI4* seems to be the only gene with a different regulation. *UBI4* transcription is enhanced by heat shock and its transcript accumulates during the diauxic shift and in cells entering stationary phase (Finley *et al.*, 1987). The promoter region of the *UBI4* gene contains both HSEs and STREs. Interestingly, *UBI4* expression is still enhanced in *S. cerevisiae* strains deleted in all three transcription factors genes, *HSF1*, *MSN2*, and *MSN4* (Simon *et al.*, 1999).

The synthesis of hsp is only one important aspect of the stress response. When *S. cerevisiae* cells face adverse environmental conditions, many types of cellular damage may result. Freezing stress, for example, involves damage of membranes, enzymes, and DNA. Consequently, stress tolerance requires protection from or repair of a number of different

damages, and it seems unlikely that this protection can be provided by only one mechanism. Other factors may therefore contribute to the survival of yeast cells that are exposed to stress. Trehalose is considered to be a good candidate for protection of cells upon general stress, first because the genes involved in its formation, namely *TPS1*, *TPS2*, *TPS3*, and *TSL1* contain STREs motifs in their promoter region, and secondly because its synthesis seems to be the immediate response to most stress conditions in *S. cerevisiae* and, as shown more recently, also in *S. pombe* (Wiemken, 1990; Piper, 1993; De Virgilio *et al*, 1991a; Winderickx *et al.*, 1996; Ribeiro *et al.*, 1997; Fernàndez *et al.*, 1997; Soto *et al.*, 1999).

TREHALOSE METABOLISM

OCCURRENCE

Known as mycose or mushroom sugar, α,α-trehalose (α-D-glucopyranosyl 1-1 α-D-glucopyraniside) is a naturally occurring non-reducing disaccharide composed of two glucose residues. It was first isolated by Wiggers, 1832 (cited in Panek, 1995), in the fungal disease ergot of rye as an unknown sugar. A quarter-century later, the French chemist Berthelot found the same sugar in cocoons of the beetle *Larinus*. These cocoons were called "trehala", inspiring Berthelot to name the sugar "trehalose".

Since then, trehalose has been demonstrated to occur in algae, bacteria, fungi, insects, invertebrates and yeasts (Elbein, 1974), as well as in some lower vascular plants such as *Selaginella lepidophylla* (Rose of Jericho). In insects, trehalose is exclusively synthesized in the fat body, is present in high concentrations and constitutes the major hemolymph (blood) sugar (Becker *et al.*, 1996). In higher plants, trehalose has only been found in leaves of one desiccation-tolerant angiosperm, namely *Myrothamnus flabellifolia* from southwestern Africa (Bianchi, *et al.*, 1993; Drennan *et al.*, 1993; Müller *et al.*, 1995). A most unexpected finding in research on plant carbohydrate metabolism is the recent discovery that *Arabidopsis thaliana* encodes genes whose products are involved in trehalose metabolism (Vogel *et al.*, 1998; Blázquez *et al.*, 1998; for a review see Müller *et al.*, 1999). This discovery could indicate that trehalose is even more widely distributed in the plant kingdom.

In algae, trehalose has been particularly described in the group of unicellular cyanophytes and in a number of rhodophytes (Berthelot, 1838 cited in Elbein, 1974). In bacteria, trehalose isolation has been correlated to those so-called "higher bacteria" which are more closely related to the fungi. Thus, free trehalose has been described not only in the mycelia but also in the spores of some streptomycetes (Elbein, 1974). Trehalose is very common in fungi, occurring both in the spores and mycelia, although the levels may vary significantly with the stage of development. In yeast cells the presence of trehalose was first shown by Koch and Koch (1925, cited in Panek, 1995) and Tanret (1931, cited in Panek, 1995). Depending on the environmental conditions and the stage of the yeast life cycle, trehalose can

represent less than 1% or more than 23% of the dry weight of the cells (Küenzi and Fiechter, 1972).

BIOSYNTHESIS

Classical trehalose formation in fungi

The enzymes involved in trehalose biosynthesis in the yeast *S. cerevisiae* are localized in the cytosol where production of trehalose occurs mainly in a two-step process (Cabib and Leloir, 1958; Elander, 1968). In the first step, trehalose-6 phosphate (Tre6P) synthase (EC 2.4.1.15) transfers the glucosyl residue of uridine 5'-diphosphoglucose (UDPG) to glucose-6P (G6P) to yield Tre6P. The second step is catalyzed by a specific Tre6P phosphatase (EC 3.1.3.12) which cleaves off the organic phosphate from Tre6P producing trehalose and inorganic phosphate (P_i).

The same mode of trehalose biosynthesis has also been demonstrated in several other organisms, such as bacteria (Giæver *et al.*, 1988; Kaassen *et al.*, 1992), filamentous fungi (Borgia *et al.*, 1996; Wolschek and Kubicek, 1997), *Dictyostelium sp.* (Roth and Sussman, 1966), *Neurospora crassa*, and even in some insects (Becker *et al.*, 1996 and references cited therein). In addition, other yeasts such as *Candida utilis*, *Kluyveromyces lactis*, *S. pombe*, *Candida albicans*, and *Hansenula polymorpha* are probably able to synthesize trehalose in a two-step process (Vincent-Soler *et al.*, 1989; Luyten *et al.*, 1993; Blázquez *et al.*, 1994; Zaragoza *et al.*, 1998; Reinders *et al.*, 1999).

The *S. cerevisiae TPS1* gene, encoding Tre6P synthase was cloned by screening a yeast cDNA expression library with monoclonal antibodies raised against the purified protein (Bell *et al.*, 1992). The Tps1 protein consists of 495 amino acids and has a molecular weight of 56 kDa (Bell *et al.*, 1992; Vuorio *et al.*, 1993). Several findings suggest that Tps1 may provide the catalytic activity of Tre6P synthase: (i) deletion mutants have no detectable trehalose or Tre6P synthase activity (Bell *et al.*, 1992); (ii) the *TPS1* gene complements the trehalose-synthesis defect of an *E. coli otsA* mutant (McDougall *et al.*, 1993); and (iii) transgenic tobacco plants are able to synthesize trehalose when *TPS1* is expressed in these plants (Holmström, 1996). Deletion of *TPS1* causes pleiotropic defects including a deficiency to grow on fructose and glucose, a lack of specific glucose-induced signalling events, and poor sporulation (Thevelein and Hohmann, 1995, see chapter 5 and chapter 6).

The *TPS2* gene encodes Tre6P phosphatase since its disruption in *S. cerevisiae* cells causes loss of the Tre6P phosphatase activity and accumulation of Tre6P instead of trehalose at temperatures above 38.6°C. Tre6P accumulation is associated with the inability of such strains to grow at high temperature (De Virgilio *et al.*, 1993, see chapter 4). Tps2 is a protein of 896 amino acids with a predicted molecular weight of 102.8 kDa (De Virgilio *et al.*, 1993).

Trehalose formation in plants

Trehalose biosynthesis in plants has not been studied in detail. Unpublished data obtained by PCR screening with tobacco, potato, and sunflower using degenerate primer-sets derived from the sequence of Tre6P synthase and Tre6P phosphatase genes of *S. cerevisiae* suggest the presence of homologous genes in these plants (Goddijn and Smeekens, 1998). Furthermore, genes from *Arabidopsis thaliana* encoding proteins with homology to Tre6P synthase and Tre6P phosphatase had been demonstrated to functionally complement *S. cerevisiae* mutants with defects in trehalose biosynthesis. These findings suggest that a two-step trehalose biosynthesis may be conserved between plants and *S. cerevisiae* (Blázquez *et al.*, 1998; Vogel *et al.*, 1998; for a review see Müller *et al.*, 1999). Further strong evidence for this assumption is the recent isolation and molecular characterization of a full-length cDNA encoding Tre6P synthase (*SlTPS1*) from the resurrection plant *Selaginella lepidophylla*. Expression of *SlTPS1* cDNA in an *S. cerevisiae tps1* mutant restores growth on glucose as well as osmotolerance and thermotolerance (Zentella *et al.*, 1999).

Biosynthesis by a trehalose phosphorylase

An alternative mechanism for trehalose synthesis by the enzyme trehalose phosphorylase (α,α-trehalose: orthophosphate D-glucosyl-transferase; EC 2.1.4.64) was demonstrated initially in the green algae *Euglena gracilis* (Maréchal and Belocopitow, 1972). This mechanism was subsequently reported to be more widely distributed in nature, and to be employed also by soil bacteria (Kizawa *et al.*, 1995), some yeasts (Schick *et al.*, 1995) such as the basidiomycetes *Flammulina velutipes* and *Schizophyllum commune* (Kitamoto *et al.*, 1988, cited in Eis *et al.*, 1998), and the actinomycete *Catellatospora ferruginea* (Aisaka and Masuda, 1995). Recently, trehalose phosphorylase has also been described in the commercial mushroom *Agaricus bisporus* (Wannet *et al.*, 1998). Trehalose synthesis occurs by transfer of the glucosyl residue of the substrate α- or β-glucose-1-phosphate (G1P) to glucose yielding exclusively α,α-trehalose. Purification of the trehalose phosphorylase from *A. bisporus* revealed a homotetrameric structure with 61 kDa subunits and a native molecular weight of 240 kDa (Wannet *et al.*, 1998). Interestingly, different physiological roles have been proposed for the trehalose phosphorylases from several organisms. While in the green algae *E. gracilis* trehalose phosphorylase can perform both synthesis and degradation of trehalose, in *F. velutipes* the enzyme seems to be active only in phosphorolysis. In *C. ferruginea* some physiological conditions favours the reaction towards trehalose synthesis (Maréchal and Belocopitow, 1972; Kitamoto *et al.*, 1988, cited in Eis *et al.*, 1998; Aisaka and Masuda, 1995).

Trehalose formation from maltooligosaccharides: the roles of maltooligosyl trehalose synthase and trehalose synthase

It seems that several bacteria also possess alternative systems to produce trehalose which are independent of Tre6P synthase (Tps1) and Tre6P phosphatase (Tps2) enzymes. One mechanism is based on trehalose formation from maltodextrins by the combination of a maltooligosyl trehalose synthase (MTSase, TreY) with a maltooligosyl trehalose trehalohydrolase (MTHase, TreZ). MTSase catalyzes the production of maltooligosyl trehaloses by forming α,α-1,1-glucosidic linkage via an intramolecular transglucosylation. MTHase cleaves the product of the MTSase into the maltooligosyl group and trehalose (Maruta *et al.*, 1995; Nakada *et al.*, 1995; Maruta *et al.*, 1996). MTSase and MTHase proteins are not homologous to Tps1 and Tps2, but rather to α-amylolytic enzymes belonging to the α-amylase family (Maruta *et al.*, 1996). Some other bacteria such as *Pseudomonas putida*, *Pimelobacter* sp., and *Thermus aquaticus* ATCC33923 can only produce trehalose from maltose but not from other maltooligosaccharides (Nishimoto *et al.*, 1995; Tsusaki *et al.*, 1997). In this case, the enzyme has been named trehalose synthase (TreS). Interestingly, both mechanisms are phosphate-independent pathways, different from the classical Tre6P synthase/Tre6P phosphatase, which occurs in the presence of Tre6P (Giæver *et al.*, 1988), or from the system using trehalose phosphorylase, which occurs in the presence of G1P.

TRE6P SYNTHASE AND TRE6P PHOSPHATASE IN *S. CEREVISIAE* – BIOCHEMICAL PROPERTIES

Three decades after the discovery of trehalose in yeast cells in 1925 (Koch and Koch, 1925, cited in Panek, 1995) trehalose formation was evaluated and the first data on the biochemical properties of Tre6P synthase were provided (Cabib and Leloir, 1958). Tre6P synthase was purified 15 to 20 fold from brewer's yeast and had maximal activity at pH 6.6 in the presence of 25 mM magnesium ions. Later, Panek (1991) suggested that the activation of Tre6P synthase by magnesium ions is, in fact, due to the formation of a G6P-Mg^{2+} complex, which is likely to be a better substrate for Tre6P synthase than the free G6P. However, higher concentrations of magnesium ions are also proposed to provoke an inhibitory effect on the Tre6P synthase activity (Cabib and Leloir, 1958). An inhibition of 50% of the Tre6P synthase activity was also caused by the addition of 33 mM phosphate. Throughout the years, several groups focused their research on further characterization of the kinetic and regulatory properties of Tre6P synthase and Tre6P phosphatase in *S. cerevisiae* (Elander, 1968; Panek *et al.*, 1987; Vandercammen *et al.*, 1989; Londesborough and Vuorio, 1991; Vuorio *et al.*, 1992; Bell *et al.*, 1992; Vuorio *et al.*, 1993; De Virgilio *et al.*, 1993). The kinetic properties emerging from these studies revealed approximate K_m values for the Tre6P synthase of 3.5 mM for G6P

and 0.5 mM for UDPG. UDP and UTP were reported to be competitive inhibitors of the Tre6P synthase with respect to the substrate UDPG. A comparison of the K_m values for G6P and UDPG with the intracellular concentration of G6P and UDPG in *S. cerevisiae* cells demonstrated that, while the K_m value for UDPG is close to its intracellular concentration, the K_m value for G6P is at least threefold higher than its intracellular concentration in cells growing exponentially on glucose. In stationary-phase cells, the K_m value for G6P is even tenfold higher than the intracellular concentration (Gancedo and Gancedo, 1973; Lillie and Pringle, 1980). Consequently, G6P may be the limiting factor for biosynthesis of trehalose in *S. cerevisiae* (Vandercammen *et al*., 1989).

An overall picture that also emerged from these studies is that both Tre6P synthase and Tre6P phosphatase are part of a multimeric protein complex. The complex is composed of at least four different subunits with an approximate molecular mass of 600 to 800 kDa (Bell *et al*., 1992; Vuorio *et al*., 1993; De Virgilio *et al*., 1993; Reinders *et al*., 1997; Bell *et al*., 1998). Besides Tre6P synthase (Tps1) and Tre6P phosphatase (Tps2), two further proteins (encoded by *TSL1* and *TPS3*) with a potential regulatory and/or structural role have been identified as subunits of the complex (Bell *et al*., 1992; Vuorio *et al*., 1993; De Virgilio *et al*., 1993; Table 1). One of these polypeptide was further characterized and showed a molecular weight of 123 kDa. It was named Tsl1 for trehalose synthase long chain (Bell *et al*., 1992; Londesborough and Vuorio, 1993; Vuorio *et al*., 1993). The Tsl1 protein consists of 1098 amino acids with a predicted molecular weight of 123 kDa (Vuorio *et al*., 1993). Tps3 has a predicted length of 1055 amino acids with a molecular weight of 115 kDa (EMBL M88172) and is highly homologous to Tsl1 (Table 1).

The characterization of Tre6P phosphatase was neglected for a long time mainly because its substrate, Tre6P, was not commercially available. In 1989, Vandercammen and coworkers partially purified Tre6P phosphatase enzyme from baker's yeast and used this extract to determine the kinetics of Tre6P hydrolysis. The kinetic properties of Tre6P phosphatase, which emerged from these studies, were an approximate K_m value of 0.2 mM for Tre6P, activation of Tre6P phosphatase by phosphate ions, dependency on magnesium ions, and optimal activity at pH 6.0. Tre6P phosphatase seems also to share some kinetic properties with similar enzymes in other organisms, however, the pH optimum of 6.0 differs from those observed for Tre6P phosphatase from insects and bacteria, which is around pH 7.0 (Friedman, 1960; Matula *et al*., 1971, cited in Vandercammen *et al*., 1989). After the cloning of the *S. cerevisiae TPS2* gene by De Virgilio and coworkers (1993), Tps2 was shown to be important for proper sporulation in *S. cerevisiae* cells (Neves *et al*., 1995) and was found to be necessary for chitin formation in *Aspergillus nidulans* at elevated temperatures (Borgia *et al*., 1996). *TPS2* is allelic to *HOG2*, a gene whose mutation confers an osmosensitive phenotype, and to *PFK3*, a gene required for the synthesis of a particulate phosphofructokinase. In addition, Tps2 is essential for growth at elevated temperatures and for normal response of yeast cells to

nutrient stress (Brewster *et al.*, 1993; Sur *et al.*, 1994). Gounalaki and Thireos (1994) also showed that a wild-type *TPS2* gene is required for multidrug resistance and for the acquisition of tolerance to a variety of stresses.

Table 1. Biosynthesis of trehalose in *Saccharomyces cerevisiae* Overview

	Properties
Tps1	Enzyme responsible for Tre6P synthesis from UDPG and G6P. Is expressed constitutively, but further induced by heat. *tps1* mutants are not able to grow on glucose and have impaired thermotolerance.
Tps2	Catalyzes the cleavage of the phosphate from Tre6P, thus producing trehalose; Is also expressed constitutively and is further induced by heat. *tps2* mutants show low thermotolerance and are not able to grow at elevated temperatures.
Tps3	Regulator of trehalose biosynthesis. Is constitutively expressed in exponential and stationary phase.
Tsl1	Regulator of trehalose biosynthesis. Its expression increases during stationary phase.

Adapted from Singer and Lindquist, 1998b.

REGULATION OF TRE6P SYNTHASE AND TRE6P PHOSPHATASE

Dependent on the growth conditions, trehalose levels can vary significantly in *S. cerevisiae*. A strong correlation between growth rate and trehalose accumulation has been described. Yeast cells growing on fermentable sugars such as glucose or fructose contain only traces of trehalose, but have the highest growth rate. Stationary-phase cells contain the highest trehalose levels while cells growing on nonfermentable carbon sources have a low growth rate and intermediate trehalose levels (Lillie and Pringle, 1980; Thevelein, 1984). One plausible explanation for the differences in trehalose levels lies in the control of the enzymes involved in its metabolism. Several models have been discussed and they include both transcriptional and post-translational control mechanisms.

Post-translational mechanisms

Magnesium ions activate Tre6P synthase (Cabib and Leloir, 1958; Panek, 1991; Vuorio *et al.*, 1992), while P_i inhibits Tre6P synthase activity but stimulates Tre6P phosphatase activity (Vandercammen *et al.*, 1989; Londesborough and Vuorio, 1991; Vuorio *et al.*, 1992). In fact, P_i is a potential effector of Tre6P synthase acting as a non-competitive inhibitor with a K_i in the range of 2 to 5 mM (Cabib and Leloir, 1958; Elander, 1968; Vandercammen *et al.*, 1989; Londesborough and Vuorio, 1991). Importantly, P_i is also a stimulator of two key enzymes involved in glycolysis, namely phosphofructokinase 1 and 2, and it is a potent inhibitor of glycogen synthase (Banuelos *et al.*, 1977; François *et al.*, 1987). Therefore, it is plausible to assume that P_i fulfils an important role in the control of carbohydrate metabolism, and especially in the regulation of trehalose levels. High concentrations of P_i would favour glycolysis, while low concentrations of P_i would favour the synthesis of reserve carbohydrates such as trehalose. Inorganic phosphate seems also to be required to ensure the glycolytic flux through glyceraldehyde-3-phosphate dehydrogenase (Hohmann *et al.*, 1996). It is worth mentioning that trehalose biosynthesis has recently been suggested to serve as a kind of metabolic buffer system during exponential growth on glucose (Thevelein and Hohmann, 1995; see chapter 5 and chapter 6). When glucose is added to derepressed *S. cerevisiae* cells, the influx of glucose is extremely fast, therefore a large amount of G6P is formed. The excess of G6P could be diverted to trehalose synthesis, thereby enhancing the recovery of P_i, which would relieve a bottleneck in glycolysis at glyceraldehyde-3-phosphate dehydrogenase. The link between trehalose formation and glycolysis will be discussed in detail in chapter 5 and chapter 6 (for a review, see Thevelein and Hohmann, 1995). Another powerful effector of Tre6P synthase is fructose-6-phosphate (F6P). F6P is a strong activator of Tre6P synthase, (Vuorio *et al.*, 1992; Londesborough and Vuorio, 1993), but does not influence the activity of Tre6P phosphatase (Londesborough and Vuorio, 1993).

Temperature also plays a role at the post-translational level in the regulation of Tre6P synthase activity. At 50°C, the inhibition of Tre6P synthase by P_i and its stimulation by F6P, *in vitro*, are negligible, but at lower temperatures, such as 30°C, both effects are most pronounced (Londesborough and Vuorio, 1993). Along the same lines, it may be mentioned that the level of substrate availability is also temperature-dependent. As described above, availability of G6P seems to be the limiting factor in the formation of trehalose in *S. cerevisiae* cells. This assumption is further supported by the findings that during the initial phase of a mild heat shock (a shift from 27°C to 40°C), the concentrations of G6P and UDPG, the substrates of Tre6P synthase, were found to increase 5 to 10 times (Winkler *et al.*, 1991; Ribeiro *et al.*, 1994). The increased availability of these substrates is due to a differential effect of temperature on glucose influx and glycolysis, causing upstream substrates to accumulate.

Partial proteolysis of the Tre6P synthase/phosphatase complex can result in an increase in Tre6P synthase, but not in Tre6P phosphatase activity. Prolonged storage of the purified complex at low temperatures or storage in the absence of the serine protease inhibitor PMSF

(phenylmethylsulfonyl fluoride) leads to an activation of Tre6P synthase (Londesborough and Vuorio, 1991). Such partially digested Tre6P synthase/phosphatase extracts were no longer activated by F6P or inhibited by phosphate, even at 30°C when the influence of F6P and phosphate are greatest with respect to the stimulation and inhibition of the intact Tre6P synthase activity, respectively. It was suggested that the active site of Tre6P synthase could be partially occluded by a flap of protein from one end of the complex. The active site could be made accessible by either binding of F6P, the product of the "Tre6P synthase activator", phosphoglucoisomerase, or by limited proteolysis (Londesborough and Vuorio, 1991). A more detailed analysis revealed that, in fact, the protein affected by proteolysis was Tsl1 and that this subunit was most likely responsible for holding together the intact trehalose synthase complex and conferring sensitivity to F6P (Vuorio *et al.*, 1993). Activation by proteolysis *in vitro*, was also shown for Tre6P synthase from *Candida utilis* (Vicent-Soler *et al.*, 1991).

Several studies investigated trehalose mobilization in *S. cerevisiae* cells. The main conclusion emerging from these studies was that trehalose mobilization is regulated by cAMP-dependent phosphorylation of the neutral trehalase, the key enzyme of trehalose degradation (Van der Plaat, 1974; Uno *et al.*, 1983; Thevelein, 1984; Dellamora-Ortiz *et al.*, 1986; Thevelein, 1988). Indeed, addition of glucose to stationary-phase cells causes a transient increase in cAMP levels and activation of trehalase (Van der Plaat, 1974; François *et al.*, 1984). Along the same lines, Panek and coworkers (1987) proposed a reversible inactivation of Tre6P synthase by phosphorylation. However, this assumption was severely criticized by other groups because of the lack of reliability of the experimental procedure used for the enzyme determinations (Vandercammen *et al.*, 1989). Vandercammen and coworkers (1989) could not find evidence for a phosphorylation-mediated inactivation of the Tre6P synthase either *in vivo* or *in vitro*. Regulation of Tre6P synthase by phosphorylation remains uncertain, but yeast mutants with increased or decreased activity of cAPK had lower and higher activity of Tre6P synthase, respectively (Panek *et al.*, 1987; François *et al.*, 1991).

Transcriptional mechanisms

S. cerevisiae cells growing exponentially on glucose have low Tre6P synthase and Tre6P phosphatase activities. However, a strong induction in both Tre6P synthase and Tre6P phosphatase activities is observed during the diauxic shift (*i.e.*, transition from fermentative growth to respiratory growth) and upon nitrogen starvation (François *et al.*, 1991). Panek and coworkers (1987) also reported that addition of glucose to derepressed cells induced inactivation of Tre6P synthase. Based on reversibility studies and experiments using the protein translation inhibitor cycloheximide, François and coworkers (1991) confirmed the inactivation and repression of Tre6P synthase and Tre6P phosphatase by glucose. This study further revealed that the observed inactivation is due to proteolysis. The effect of high protein kinase A

activity is probably exerted at the transcriptional level rather than at the post-translational level, as suggested by Panek and coworkers (1987).

During a mild heat shock (a shift from 27° to 40°C), *S. cerevisiae* cells rapidly accumulate trehalose. Its synthesis is a result of the balance between the enzymes involved in its synthesis and degradation. Both Tre6P synthase and Tre6P phosphatase activities are strongly induced under heat-shock conditions (Hottiger *et al.*, 1987a; De Virgilio *et al.*, 1990; De Virgilio *et al.*, 1993; Ribeiro *et al.*, 1994). This increase in activity is preceded by an accumulation of *TPS1* and *TPS2* mRNAs. The rapid accumulation of both *TPS1* and *TPS2* mRNAs resembles the pattern described for members of the heat-shock gene family (Werner-Washburne *et al.*, 1989). Similar to the promoters of heat-shock genes, several STRE motifs are present in the promoter region of both *TPS1* and *TPS2* genes (Winderickx *et al.*, 1996; Boy-Marcotte *et al.*, 1999). STRE-dependent transcriptional induction of *TPS1* and *TPS2* is triggered by a variety of stress conditions such as heat shock, high osmolarity stress, oxidative stress and nitrogen starvation and is negatively controlled by cAPK (Marchler *et al.*, 1993; Schüller *et al.*, 1994; Gounalaki and Thireos, 1994; Görner *et al.*, 1998). Interestingly, despite the presence of STREs in *TPS1* and *TPS2* promoters, their gene expression differs substantially from the expression of typical STRE-controlled genes such as *CTT1* and *SSA3* (Winderickx *et al.*, 1996). Transcription of *TPS1* and *TPS2* genes was less downregulated in the presence of glucose, which may be explained by the role of trehalose biosynthesis in the control of glycolysis (Thevelein and Hohmann, 1995; see also chapter 5 and chapter 6). STREs are also present in multiple copies in the promoter sequences of the genes encoding the other subunits of the Tre6P synthase complex, *i.e.*, in the promoter region of *TPS3* and *TSL1* (Winderickx *et al.*, 1996). Deletion of two of the four STREs present in the promoter region of *TPS2* results in diminished stress induction of the gene (Gounalaki and Thireos, 1994). The STRE motifs present in the *TPS1* promoter region were recently shown to be specifically activated by the Msn2 and Msn4 transcription factors (Boy-Marcotte *et al.*, 1999).

CATABOLISM IN *S. CEREVISIAE*

In fungi, trehalose degradation is performed by trehalase (α,α-trehalose 1-D-glucohydrolase; EC 3.2.1.28), a glycosidase that hydrolyzes α,α-trehalose to produce two D-glucose molecules. Trehalase activity in fungi was first demonstrated by Bourquelot in 1893 (cited in Nwaka and Holzer, 1998). Two years later, Fischer (1895, cited in Nwaka and Holzer, 1998) described the presence of trehalase in yeast cells. Since then, trehalase has been found in a large variety of organisms, including some that have not yet been shown to store trehalose, *e.g.*, higher plants (for a review see Müller *et al.*, 1999). So far, three means of cleavage of trehalose have been described in the literature in a variety of organisms: (i) hydrolysis by trehalase; (ii) phosphorolytic cleavage by a phosphorylase to yield G1P and glucose; and (iii)

cleavage of Tre6P by a phosphotrehalase forming glucose and G6P. Phosphotrehalase has been described in *Bacillus popilliae* (Bhumiratana *et al.*, 1974; cited in Thevelein, 1996). In practically all fungal species studied so far, trehalose degradation is carried out by trehalases. *Pichia fermentans* is so far the only known yeast species to possess trehalose phosphorylase activity (Schick *et al.*, 1995). From an energetic point of view, phosphorolysis of trehalose liberating glucose and G1P is more favourable than hydrolysis of trehalose into two glucose molecules by trehalase, because formation of the phosphorylated sugar means that no additional expenditure of ATP is needed (Wannet *et al.*, 1998).

Fungal trehalases are nowadays grouped into two main classes according to their pH optima and regulatory properties: the acid or non-regulatory trehalases which are extracellular or vacuolar glycoproteins with high heat stability, and the neutral or regulatory trehalases which are unglycosylated cytosolic proteins with low heat stability.

Acid trehalase

Acid trehalase shows a widespread occurrence in fungi. In *S. cerevisiae*, it is a vacuolar protein, which strongly binds to Concanavalin A, suggesting that it is a glycosylated protein (Londesborough and Varimo, 1984). The *S. cerevisiae ATH1* gene was recently cloned and it codes for an acid trehalase of 117 kDa (Destruelle *et al.*, 1995). The acid trehalase is not activated by phosphorylation and is termed non-regulatory trehalase. Instead, it seems to be controlled transcriptionally by catabolic inactivation (San Miguel and Argüelles, 1994), repression (Mittenbühler and Holzer, 1988) and by trehalose itself (Nwaka *et al.*, 1996).

Acid trehalase in *S. cerevisiae* is found in stationary-phase cells, but not in exponentially growing cells (San Miguel and Argüelles, 1994). The enzyme's activity level does not increase significantly with elevated temperature (Nwaka *et al.*, 1995a). As trehalose is cytosolic and the Ath1 protein is vacuolar, the mechanism by which Ath1 influences cytosolic trehalose levels is not clearly understood. The *ath1* mutants are not able to grow on trehalose, unlike wild-type cells, suggesting that extracellular trehalose reaches the vacuoles by membrane transport (Nwaka *et al.*, 1996; Panek, 1995). A trehalose transporter has been reported to promote trehalose uptake in *S. cerevisiae* cells (Eleutherio *et al.*, 1993).

Neutral trehalase

Despite also being detected in some other fungi, neutral trehalase seems to be more widespread in zygomycetes and in certain yeast genera more closely related to zygomycetes (Thevelein, 1984). The *S. cerevisiae* neutral trehalase is a cytosolic enzyme and early experiments have provided evidence for a tight regulation by cAMP (Van de Plaat, 1974; Van Soligen and Van der Plaat, 1975).

Neutral trehalase, encoded by *NTH1*, has an N-terminal domain with two consensus sites for cAMP-dependent protein phosphorylation (Amaral *et al.*, 1997; Nwaka *et al.*, 1995a), and a putative Ca^{2+}-binding sequence (Amaral *et al.*, 1997). In contrast to acid trehalase, the activity of neutral trehalase is high in exponentially-growing cells and drops in stationary phase (San Miguel and Argüelles, 1994). Thus, neutral trehalase activity has been correlated with mobilization of trehalose during recovery from heat shock or exit from stationary phase (Kopp *et al.*, 1993; Nwaka *et al.*, 1994, 1995a). Upon heat shock, exponentially growing, wild-type yeast cells are able to accumulate trehalose, which is rapidly degraded by activation of neutral trehalase upon shift of the cells to a lower temperature (De Virgilio *et al.*, 1991c). Cells deleted in *NTH1* are not able to degrade the trehalose accumulated during heat shock when returned to a lower temperature (De Virgilio *et al.*, 1991c; Nwaka *et al.*, 1995b). They also exhibit a delayed growth recovery. In contrast to *ath1* deletion mutants, *nth1* deletion mutants grow normally on trehalose, suggesting that neutral trehalase is not required for hydrolysis of extracellular trehalose (Nwaka *et al.*, 1996).

Recently, a second gene, *NTH2*, with 77% identity to the *NTH1* gene has been identified by the *S. cerevisiae* Genome Sequencing Project (Nwaka *et al.*, 1995b). Its function is still unknown since its disruption or overexpression does not affect trehalose levels and mutants do not display a loss of trehalase activity (Nwaka *et al.*, 1995a, 1995b).

PHYSIOLOGICAL ROLES OF TREHALOSE

Trehalose as a storage carbohydrate: comparison with glycogen

Storage carbohydrates play an important role in the metabolism of yeast cells. Generally, organisms accumulate endogenous reserves upon depletion of nutrients and energy sources (Wiemken, 1990). Glycogen has been recognized as a storage polysaccharide in many organisms and the metabolism of glycogen in yeast cells has been investigated in detail (Rothman and Cabib, 1969, François and Hers, 1988, Roach, 1990). Glycogen is a highly branched molecule with a molecular weight of about 10^7 consisting of numerous chains of α-(1-4)-linked D-glucose residues with (1-6)-inter-chain linkages.

Originally, trehalose was thought to function together with glycogen as a storage compound. The fact that yeast cells can accumulate during their life cycles two different storage carbohydrates, trehalose and glycogen, intrigued researchers for many years. The question remained, however, why yeast requires a second energy store in addition to glycogen. Careful analysis of glycogen and trehalose accumulation profiles in *S. cerevisiae* sparked a dramatic change in the perception of the function of trehalose in yeast as presented in the following.

The metabolism of trehalose and glycogen is regulated by several endogenous and environmental factors. Under certain environmental conditions, and depending on the stage of

the yeast life cycle, each of these carbohydrates can represent less than 1% or more than 23% of the dry weight of the cells (Küenzi and Fiechter, 1972). The accumulation of trehalose in other fungi appears to be associated in general with periods of reduced growth rate (Elbein, 1974). In reproductive stages, such as spores, and survival forms, such as sclerotia, the levels of trehalose can be very high, much higher than other carbohydrates like glycogen or sugar alcohols.

Yeast cells growing on glucose obtain the energy needed for growth exclusively through glycolysis, whereas other pathways for energy production are repressed. On rich, glucose-based medium, yeast cells have low levels of glycogen (Kuenzi and Fiechter, 1972, Grba *et al.*, 1975 and 1979). Glycogen levels increase when approximately half of the initial glucose has been consumed, with peak concentrations occurring at the diauxic shift (Lillie and Pringle, 1980). During the diauxic lag phase, glycogen synthesis ceases and glycogen is even partially degraded. Further respiratory growth seems to allow the restoration of the glycogen pool. Trehalose accumulation does not display a pattern expected for a mere storage carbohydrate. Trehalose synthesis is observed only when glucose is exhausted and cells begin to enter stationary phase. Trehalose accumulation continues even in the absence of extracellular glucose. This coincides with a shift in glycogen metabolism from synthesis to degradation (Lillie and Pringle, 1980). It has also been claimed that trehalose synthesis in the diauxic lag phase is possibly driven at the expense of glycogen breakdown (Lillie and Pringle, 1980, Francois *et al.*, 1991), so that it does not result in a net increase in reserve carbohydrate. During prolonged incubation in stationary phase, glycogen is mobilized in preference to trehalose, displaying a half-life of about 20 days. Trehalose degradation is a very slow process (half-life of about 100 days), but trehalose is ultimately very quickly metabolized, possibly as a desperate measure to avoid starvation: trehalose degradation coincides with the onset of cell death (Wiemken, 1990; Lillie and Pringle, 1980).

The role of storage carbohydrates during sporulation and germination processes has also been investigated. In *S. cerevisiae*, only glycogen breakdown coincides with the appearance of mature spores; trehalose is not metabolized and the bulk of trehalose is accumulated during the last phase of sporulation and localized to the mature spores. Again, its synthesis is proposed to be driven at the expense of glycogen breakdown (Kane and Roth, 1974). This energetically costly transformation would likely not take place if glycogen and trehalose were equivalent in function. From an energetic point of view, trehalose is a less efficient reserve than glycogen because more energy is required for biosynthesis of trehalose per unit of glucose stored, and less energy is released upon metabolization (hydrolysis versus phosphorolysis; Wiemken, 1990). Trehalose degradation in fungal spores also displays some properties not expected from a reserve compound. Yeast spores in the presence of a fermentable carbon source such as glucose, are induced to rapidly metabolize their accumulated trehalose. However, it seems that in the germinating spores, the mobilized trehalose is not used as an energy source. Instead, trehalose is cleaved and glucose is secreted into the medium (Thevelein, 1984; Van Laere, 1989; Wiemken, 1990).

In summary, the profile of trehalose and glycogen accumulation and utilization during the yeast life cycle suggests that these molecules play distinct roles in cellular economy and physiology (Wiemken, 1990). The presence of high amounts of trehalose in resting cells, and in survival forms such as spores, conidia, sclerotia, and in anhydrobiotic organisms points towards an additional function for trehalose in increasing the resistance of such cells to adverse environmental conditions.

Trehalose as stress protectant

Dehydration

Water is usually thought to be necessary for the living state, but several organisms are nevertheless capable of surviving essentially complete dehydration without dying. These organisms commonly survive in this state, which is known as anhydrobiosis, even when more than 99% of their body water is removed. The dry but viable tissues contain as little as 0.1% water, a condition that would normally be thought not to be consistent with life (Crowe *et al.*, 1992).

In addition to more familiar organisms, such as yeast cells and fungal spores, microscopic animals such as nematodes, tardigrades, cysts of some crustacean embryos, but also the so called "resurrection" plants, *Selaginella lepidophylla* and *Myrothamus flabellifolia*, can remain in the anhydrobiotic state for decades. In the desiccated state, these organisms are metabolically inactive and highly resistant towards adverse environmental conditions such as heat and freezing. Survival of dehydration by some of these organisms was found to be correlated with high trehalose accumulation (Crowe *et al.*, 1985; Martin *et al.*, 1986). When water becomes available again, the desiccated cells quickly swell and resume active life. This "resurrection" is generally accompanied by a rapid degradation of the accumulated trehalose (Wiemken, 1990).

In order to retain the viability and activity of pressed baker's yeast, it is important that the yeast cells contain sufficient amounts of trehalose (Suomalainen and Pfäffli, 1961, cited in Thevelein, 1996). These findings were extremely valuable for the companies producing baker's yeast, particularly in connection with the production of "Instant Active Dry Yeast". This commercial baker's yeast does not require rehydration prior to mixing with flour and remains active in the dried state (Trivedi and Jacobson, 1986, cited in Thevelein, 1996).

Evidence for trehalose as a stress protectant has been accumulating at a fast pace in recent years. In a clear-cut report by Crowe and coworkers (1984) a water-replacement hypothesis was proposed to explain the protective effect of trehalose in anhydrobiotic organisms. This hypothesis proposed that trehalose interacts directly via hydrogen-bonds with the polar head-groups of phospholipids in the membrane, thereby effectively replacing the water molecules around these charged groups and thus preventing damaging phase transitions

(Crowe *et al.*, 1984; Crowe e*t al.*, 1988). Hydrogen bonds with the multiple hydroxyl groups of trehalose would result in a better stabilizing effect under adverse physical conditions, (*e.g.*, such as dehydration, heat, and freezing) when compared to hydrogen bonds with water molecules. The role of trehalose as stress protectant has been reviewed in several papers (Van Laere, 1989; Wiemken, 1990; Crowe *et al.*, 1992). Some studies have shown the very specific protective effects of trehalose during desiccation of biological structures at ambient temperatures (Crowe *et al.*, 1990; Roser, 1991, cited in Thevelein, 1996; Colaço *et al.*, 1992). Due to these protective properties, trehalose has been used a cryoprotectant for freeze storage of intact cells and organisms (Coutinho *et al.*, 1988; De Antoni *et al.*, 1989).

Freezing

Freezing and dehydration have been assumed to exert similar stresses on living cells because both conditions result in the removal of water from the system. However, Crowe and coworkers (1990) showed that proline effectively stabilizes proteins and phospholipids bilayers during freezing, but not during complete dehydration, and concluded that freezing and dehydration are not equivalent phenomena. The parallels between freezing and dehydration damage ignored detailed investigation on the relation between freezing tolerance and cellular trehalose content.

Several studies have shown a positive correlation between trehalose content and freezing tolerance in yeast (Oda *et al.*, 1986; Coutinho *et al.*, 1988; Hino *et al.*, 1990). The finding that disruption of the *ATH1* gene, which in turn leads to high levels of trehalose, confers high freeze-thaw tolerance to yeast corroborates this suggestion (Kim *et al.*, 1996). The loss of tolerance to freezing by yeast cells has been correlated with the rapid depletion of trehalose upon contact with rich media containing fermentable sugars (Oda *et al.*, 1986). These findings have influenced the industrial preparation of yeast doughs (Sugihara and Kline, 1968, cited in Thevelein, 1996). Further evidence for trehalose acting as a protectant against freezing stress has been demonstrated in *S. pombe* cells, *Phycomyces* and in *Neurospora* spores (Soto *et al.*, 1999; Van Laere, 1989).

Some *in vitro* observations point towards an outstanding property of trehalose to protect freeze-dried proteins. During the freeze-drying process, it is thought necessary that the stabilizing compound is hydrogen-bonded to the dried protein, thus serving as a "water substitute" in the dry state. A second hypothesis is based on the tendency of trehalose solutions to undergo glass rather than crystal formation upon desiccation, which would result in the establishment of a physical state particularly protective for embedded macromolecules. The glass capsule around macromolecules would preserve their native state and, in this way, would also prevent any distortion of their structure during dehydration (Thevelein, 1996). While trehalose shares some structural features with other molecules such as glucose, maltose, and sucrose (Colaço *et al.*, 1992), it possesses a number of physical properties, which give it a

unique functionality. This was nicely demonstrated when the tetramer phosphofructokinase was freeze-dried in the presence of different sugar molecules (Carpenter *et al.*, 1987): trehalose displays better protective properties than other sugars.

Osmotic stress

Water plays a central role in life, not only as a solvent, but also for creation of turgor and hence, for the determination of size and shape of cells and entire organisms. Therefore, it seems fundamental for uni- and multicellular organisms to possess mechanisms to adapt to alterations in osmolarity of the surrounding growth medium.

In its natural habitat on fruits and in sugar-containing juices, yeast cells need to respond to changes in osmolarity. One response to osmotic stress is the accumulation of solutes that do not affect the physical and biochemical processes within the cell. Such solutes are accumulated to increase the internal osmolarity and thereby enable the cells to absorb water more efficiently from the environment. These solutes include certain ions such as potassium, sugars, sugar alcohols, amino acids, and their derivatives (Yancey *et al.*, 1982). Furthermore, an important role of trehalose as osmoprotectant has also been described in *E. coli* (for a review, see Strøm and Kaasen, 1993) and in the green algae *Euglena gracilis* (Takenaka *et al.*, 1997). Whether trehalose functions as an osmoprotectant in yeast cells is uncertain. However, there is some evidence for a role of trehalose under osmotic stress in *S. cerevisiae*, in *S. pombe*, and also in some yeasts species isolated from tropical habitats (Mackenzie *et al.*, 1988; D'Amore *et al.*, 1991; Soto *et al.*, 1999; Ribeiro *et al.*, 1999).

Glycerol is another important solute in the osmotic stress response. *S. cerevisiae* cells respond to a shift to higher osmolarity by increasing the intracellular levels of the osmolyte glycerol, which seems to be important in order to balance the osmotic pressure across the cell membrane (Blomberg and Adler, 1992). An osmotic shock leads to an increased expression of genes involved in the production of glycerol. Strains with a reduced ability to produce glycerol are sensitive to hyperosmotic stress (Larsoon *et al.*, 1993; Albertyn *et al.*, 1994; Ansell *et al.*, 1997). Osmotic induction of the *GPD1* gene, which encodes glycerol-3-phosphate dehydrogenase, has recently been monitored in time course experiments. Conclusions that emerged from this study are that the induction of *GPD1* expression by osmotic stress is transient and that under severe osmotic stress a progressive delay of its induction was observed (Rep *et al.*, 1999). Thus, the profile of osmotic induction of *GPD1* expression is dependent on osmolyte concentration (Rep *et al.*, 1999). Hounsa and coworkers (1998) proposed a synergistic function for trehalose and glycerol in *S. cerevisiae* cells during osmotic stress. It was asserted that the intracellular amount of trehalose is directly related to the survival when cells undergo severe osmotic stress in the absence of growth. Under such conditions, no glycerol is produced (Albertyn *et al.*, 1994). Under conditions of moderate osmotic stress,

trehalose does not appear to be required for protection and glycerol production seems to be more important (Hounsa *et al.*, 1998).

Heat stress: heat-shock proteins *versus* trehalose

The heat-shock response is a molecular reaction to adverse environmental conditions and is conserved in all organisms. As described previously, when organisms or cells are exposed to a mild heat shock, they rapidly redirect gene expression to maximize hsp synthesis (Piper, 1993).

Hsp104 has been found to be important for heat-induced and stationary phase induced thermotolerance (Sanchez and Lindquist, 1990; Sanchez *et al.*, 1992). Unlike many other hsps, Hsp104 does not prevent the aggregation of thermally denatured proteins, but promotes the resolubilization and reactivation of proteins that have already begun to aggregate (Parsell *et al.*, 1994). Several groups have questioned the requirement of hsp synthesis for the acquisition of thermotolerance. For example, *S. cerevisiae* cells with a mutation in the *HSF1* gene show only a low-level of hsp gene transcription, but can still acquire thermotolerance (Smith and Yaffe, 1991a,b). In addition, acquisition of thermotolerance in *S. cerevisiae* was shown to be induced in the presence of cycloheximide, which prevented hsp synthesis (De Virgilio *et al*, 1991b; Gross and Watson, 1996). Upon downshift of heat-shocked yeast cells or bacterial cells to 23°C, acquired thermotolerance is lost within 1 to 2 h, even though the levels of hsps remain high (Cavicchioli and Watson, 1986). Moreover, in *E. coli* cells it was shown that the induction of hsp synthesis in the absence of a mild heat shock was insufficient to induce thermotolerance (VanBogelen *et al.*, 1987).

In the light of these findings it seems likely that acquisition of thermotolerance is driven by more than one protective mechanism and therefore involves alternative factors that can act in the absence of, or in parallel to, hsp synthesis. Another thermoprotectant candidate is trehalose. Its role in the preservation of dried organisms provoked a series of investigations (see above; Crowe *et al.*, 1992). In 1987, two independent studies reported the accumulation of high amounts of trehalose upon heat shock (Attfield, 1987; Hottiger *et al.*, 1987a), which then yielded support for a role of trehalose as a stress protectant during heat conditions. Since then, several studies were performed to investigate the role of trehalose in the general stress response (Van Laere, 1989; Wiemken, 1990; Piper, 1993).

Like Hsp 104, trehalose is present in trace amounts in log-phase cells, but in high amounts in stationary-phase cells and spores (Kane and Roth, 1974; Lillie and Pringle, 1980). Both these physiological states, during which trehalose levels are elevated, are highly thermotolerant (Sanchez *et al.*, 1992; De Virgilio *et al.*, 1991b). Observations in other fungi and prokaryotes supported the correlation in *S. cerevisiae* that physiological circumstances leading to high amounts of trehalose coincide with increased tolerance to heat (Van Laere, 1989; Wiemken, 1990). In addition, several conditions that induce the heat-shock response in *S.*

cerevisiae have also been found to cause an increase in trehalose levels, partially due to the stimulation of *de novo* synthesis of enzymes involved in its biosynthesis (Hottiger *et al.*, 1987a, 1989, 1992; De Virgilio *et al.*, 1990).

Strong evidence for a protective role of trehalose during heat shock emerged from work with Tps1. Absence of Tps1 prevents trehalose formation and causes impaired thermotolerance (De Virgilio *et al.*, 1993). However, a subsequent report indicated that *tps1* mutant cells are also impaired in hsp synthesis (Hazell *et al.*, 1995). Other studies generated doubts as to whether trehalose plays a role as a thermoprotectant. The original assumption that trehalose may have a protective role in the acquisition of thermotolerance arose from the correlative findings that, like hsps, trehalose is also produced in response to stress. After the stress has passed, trehalose degradation is far more rapid than that of hsps (Hottiger *et al.*, 1987a,b). However, the *nth1* deletion mutant, which lacks neutral trehalase, retains high levels of trehalose upon a temperature downshift following a prior heat shock despite the fact that thermotolerance decreased at the same time (Nwaka *et al.*, 1995a; Nwaka and Holzer, 1998). Corroborating the early findings by De Virgilio and coworkers (1991b), a synergistic function of trehalose and molecular chaperones, especially Hsp104, in enhancement of thermotolerance in stationary yeast cells was suggested by Elliot and coworkers (1996). Further, *in vivo* NMR analysis demonstrated that trehalose, but not Hsp104, increased the membrane fluidity of *S. cerevisiae* cells during heat shock. This suggested that trehalose may have a function as a protectant of membrane structures *in vivo* (Iwahashi *et al.*, 1995). Indeed, *in vivo* experiments using two different temperature-sensitive reporter proteins further supported the hypothesis that trehalose protects cell from heat by stabilizing proteins at high temperatures (Hottiger *et al.*, 1994; Singer and Lindquist, 1998a). An additional role of trehalose may be the ability to suppress the aggregation of proteins that have already been denatured (Singer and Lindquist, 1998a). This property could also explain a need for rapid degradation of trehalose after heat shock. Its degradation would be necessary to provide an efficient refolding of the proteins by the molecular chaperones (Singer and Lindquist, 1998b).

An integrative model for trehalose and hsps in the development of thermotolerance was proposed by Singer and Lindquist (1998b). Elevated temperatures lead to protein denaturation and aggregation. In the presence of trehalose, unfolding of proteins and aggregation is decreased. Trehalose levels must be rapidly reduced after heat shock in order to facilitate the binding of molecular chaperones to the unfolded substrates, and to promote proper refolding. The persistence of high amounts of trehalose can interfere with the reactivation of denatured substrates and can impede the ability of hsps to resolve heat-induced damage to cellular proteins. Such findings explain both the need to degrade trehalose upon downshift of heat-shocked cells and the thermosensitivity of mutants unable to do so (Singer and Lindquist, 1998b).

POTENTIAL BIOLOGICAL AND COMMERCIAL APPLICATIONS OF TREHALOSE

The mechanism by which trehalose protects freeze-dried proteins and stabilizes biological molecules during desiccation is still under debate. However, the stability, nontoxicity, and chemical inertness of trehalose has made it an ideal preservative for biological materials such as vaccines, restriction enzymes, sera, conjugated proteins, microorganisms, flavours, fragrances, and intact mammalian cells (Panek, 1995). The Quadrant Research Foundation showed that the characteristic colour, aroma, and flavour of many foods, especially dehydrated fresh fruits and herbs, were much better conserved in the presence of trehalose. However, the price of commercial trehalose is too high and its application is restricted to high-value products such as pharmaceuticals.

An increase in intracellular trehalose levels by genetic manipulation of the trehalose degrading enzymes in yeast would contribute substantially to various aspects of the food, baking, and brewing industries. As mentioned before, high levels of trehalose are correlated with enhanced freezing tolerance. Yeast strains used in the preparation of frozen doughs are routinely optimized for maximal trehalose content. However, the trehalose pool is rapidly mobilized when a rich medium with fermentable sugar is supplied. Disruption of the trehalase genes may circumvent this problem. These yeast cells could then also be utilized for trehalose production.

Trehalose accumulation as an element of the heat-shock response has been shown to protect tissues against a variety of toxic conditions, including anoxia and reperfusion injury. These properties have attracted appreciable interest in medicine (Lee and Goldberg, 1998).

PARTICULAR ASPECTS OF THE TREHALOSE METABOLISM IN *S. POMBE*

Little is known about trehalose metabolism, and consequently the stress response, in *S. pombe* cells. Participating in trehalose metabolism so far, three enzymes have been described: an acidic trehalase, a neutral trehalase, and a Tre6P synthase (Inoue and Shimoda, 1981a,b, De Virgilio *et al.*, 1991a; Blázquez *et al.*, 1994). Heat shock conditions induce trehalose formation in *S. pombe*, as in *S. cerevisiae*, and lead to the activation of the Tre6P synthase (Tps1) and the constitutive neutral trehalase (Ntp1) (De Virgilio *et al.*, 1990; De Virgilio *et al.*, 1991a; Blázquez *et al.*, 1994; Ribeiro *et al.*, 1997; see chapter 3). Furthermore, acquisition of thermotolerance in *S. pombe* has been correlated with the accumulated trehalose, since heat-shock conditions in the presence of cycloheximide did not reduced either trehalose accumulation, or the development of thermotolerance (De Virgilio *et al.*, 1990; Ribeiro *et al.*, 1997; see chapter 3).

The *tps1*$^{+}$ gene encodes a protein with a predicted molecular weight of 54.7 kDa and 479 amino acids. Furthermore, it has homology to Tre6P synthase from other organisms and is 65% identical to Tps1 of *S. cerevisiae* (Blázquez *et al.*, 1994). The *tps1*$^{+}$ function was shown to be necessary for germination of spores in *S. pombe* cells (Blázquez *et al.*, 1994). The most

striking finding concerning trehalose formation in *S. pombe* cells is the fact that deletion of *tps1*$^{+}$ does not have a noticeable effect with respect to growth on glucose and does not cause the pleiotropic phenotype observed in *S. cerevisiae tps1* mutants (see chapter 3). Possible models to explain the pleiotropic effects observed in *S. cerevisiae* by *TPS1* deletion will be introduced and discussed in chapter 5 and chapter 6. It seems quite clear that trehalose formation in these two yeast species fulfils overlapping, but not identical roles, which makes *S. pombe* a particularly attractive model organism for studies on trehalose metabolism. Further details concerning trehalose metabolism in *S. pombe* will be introduced in the following chapters.

OUTLINE OF THE THESIS

Genetic approaches to determine the specific role of trehalose in heat-induced thermotolerance in *Saccharomyces cerevisiae* cells, as well as in mutants with attenuated cAMP-dependent protein kinase activity (cAPK), have been hampered by the finding that deletion of the *TPS1* gene, coding for trehalose-6-phosphate (Tre6P) synthase, causes pleiotropic effects and one of these effects is the inability to grow on glucose. The fission yeast *Schizosaccharomyces pombe* is an excellent model to evaluate the role of trehalose as an element of the heat-shock response, because the *S. pombe TPS1* homologue, *tps1*$^{+}$, is essential for Tre6P synthesis, but is dispensable for growth on glucose and does not cause the pleiotropic phenotype observed in *S. cerevisiae*. In addition, trehalose accumulation in *S. pombe* is also induced by a heat shock, but unlike in *S. cerevisiae*, it is completely independent of protein synthesis. These observations suggest that trehalose metabolism could play different roles in these two yeast species.

The main goal of the experiments described in **chapter 3** is the evaluation of the relative contributions of the heat-induced synthesis of heat-shock proteins (hsp) and the accumulation of trehalose for the acquisition of thermotolerance in the fission yeast *S. pombe*. In order to address this issue, two approaches were followed: use of an *S. pombe tps1*$^{-}$ strain and use of the protein translation inhibitor cycloheximide. Initially, the upper temperature limit for hsp synthesis was assessed in a wild-type background, in order to characterize the optimum temperature for induction of trehalose and hsp synthesis. Acquisition of thermotolerance and subsequent survival after a challenging heat shock (8 min at 50°C) were investigated. The results presented in **chapter 3** clearly show that thermotolerance in *S. pombe* may be acquired in the complete absence of hsp synthesis, mainly during heat shocks at temperatures between 42.5°C and 47.5°C. Thermotolerance was investigated with respect to cAPK, since the Ras/cAMP pathway in *S. pombe*, as in *S. cerevisiae*, also negatively regulates thermotolerance. In **chapter 3,** I will present data which show that rapid, trehalose-independent, post-translationally controlled mechanisms are triggered during the challenging heat shock, and that these mechanisms contribute substantially to the development of the induced thermotolerance.

In order to gain further insight into the physiological and biochemical mechanisms governing the regulation of trehalose formation in heat shocked *S. pombe* cells, the second part of this work is dedicated to the construction of specific mutants with defects in the synthesis of trehalose. The only gene known, at the time when this project began, that was involved in trehalose synthesis was the *tps1*$^{+}$ gene coding for the Tre6P synthase. An *S. pombe* cDNA library was used to screen for transformants able to complement the growth defect of a *tps2* mutant in *S. cerevisiae*. The isolation and characterization of the *S. pombe tps2*$^{+}$ identified in this manner, is presented in **chapter 4**. Deletion of the *tps2*$^{+}$ gene causes loss of Tre6P phosphatase activity and, therefore, accumulation of Tre6P, suggesting that it codes for the *S. pombe* Tre6P phosphatase. In addition, due to the European *Schizosaccharomyces* Genome Sequencing Project, two new genes, *tps3*$^{+}$ and *tps4*$^{+}$, which share homology to the *S. cerevisiae*

TPS3 and *TPS2* genes, respectively, were identified. Detailed study of the *S. pombe tps3⁻* and *tps4⁻* mutants showed that deletion of the *tps3⁺* gene caused a strong reduction in the Tre6P synthase activity *in vitro*, but did not affect the *in vivo* accumulation of trehalose under heat-shock conditions. In addition, deletion of the *tps4⁺* gene did neither cause Tre6P formation, nor loss of the Tre6P phosphatase activity, but a notable decrease in the *in vitro* Tre6P synthase activity and in the *in vivo* accumulation of trehalose under the same conditions. These findings suggest that *tps3⁺* and *tps4⁺* genes may be required for Tre6P synthase activity. These results will be discussed in **chapter 4**.

The results presented in **chapter 4** provide an important basis to unravel the structural composition of trehalose formation in *S. pombe*, which is the aim of the last part of the present thesis. Deletion mutants with defects in different genes acting in the trehalose formation pathway were used to determine, by size-exclusion gel filtration analysis, whether a structural multimeric protein complex is also present in *S. pombe* cells. In **chapter 5** it is shown for the first time that a multimeric complex, comprising Tre6P synthase activity, exists in *S. pombe*. Strikingly, however, this complex comprises Tre6P synthase activity, but Tps2, Tps3, and Tps4 do not seem to be part of this *S. pombe* Tre6P synthase complex. Taken together, these findings give further support for the suggestion that trehalose formation in *S. pombe* and *S. cerevisiae* serve different roles.

The conclusions obtained from each chapter will be discussed in more detail in a general discussion presented in **chapter 6**. The literature referred to throughout this thesis is summarized at the end of this work in **chapter 7**.

CHAPTER 2

MATERIALS AND METHODS

A Brief Note Concerning Nomenclature

A generally accepted system for genetic nomenclature in *Schizosaccharomyces pombe* has been published by Kohli (1987) and is used throughout this thesis. *S. pombe* proteins are simply indicated in nonitalicized, lowercase letters. Genes symbols are designated by three italicized lowercase letters with a superscript "plus" for the wild-type genes. For example, the protein trehalose-6-phosphate synthase and its gene are indicated by Tps1 and *tps1*$^{+}$, respectively. Gene deletions are denoted by lowercase letters representing the deleted gene followed by two colons and the wild-type gene marker used for deletion, such as *tps4::ura4*$^{+}$. In the text and figures, gene deletions are abbreviated by the gene followed by a " $^{-}$ ", such as *tps2*$^{-}$.

Media

- <u>YES</u>
  - 0.5% Yeast extract
  - 3.0% Glucose
  - 100 mg/l auxotrophic requirements

- <u>SD</u>
  - 0.67% Yeast Nitrogen Base (YNB) without amino acids
  - 2.0% Glucose
  - 100 mg/l auxotrophic requirements

- <u>SGal/Raff</u>
  - 0.67% YNB without amino acids
  - 2.0% Galactose
  - 1.0% Raffinose
  - 100 mg/l auxotrophic requirements

- <u>ME</u>
  - 3.0% Malt extract
  - 100 mg/l auxotrophic requirements

- YPD

  1.0% Yeast extract
  2.0% Bacto-peptone
  2.0% Glucose

- EMMA

  Stock solutions used for preparation:

20× K-hydrogenphtalate	6.0 g/100 ml
20× $MgCl_2 \times 6H_2O$	2.14 g/100 ml
20× $Na_2HPO_4 \times 12H_2O$	9.06 g/100 ml
20× KCl	2.0 g/100 ml
20× NH_4Cl	0.856 g/100 ml
200× $CaCl_2 \times 2H_2O$	0.24 g/100 ml
200× Na_2SO_4	0.80 g/100 ml

Stock solution of trace elements:

H_3BO_4	500 mg/l
$MnSO_4 \times H_2O$	530 mg/l
$CuSO_4 \times 5H_2O$	40 mg/l
$ZnSO_4 \times 7H_2O$	400 mg/l
KI	100 mg/l
$(NH_4)_6Mo_7O_{24} \times 4H_2O$	1000 mg/l
$FeCl_3 \times 6H_2O$	200 mg/l

Vitamines and carbon sources in stock solutions:

Inositol (sterile filtrate)	1 g/l
Ca-Panthotenate (sterile filtrate)	1 g/l
Nicotine acid (sterile filtrate)	1 g/l
Biotin (in 50%EtOH)	10 mg/l
Glucose (autoclaved)	20%

For 1l EMMA medium:

H_2O	677.5 ml
20× salts	50 ml each
200× salts	5 ml each
trace elements	0.5 ml

After autoclaving, the following reagents were added:

20% glucose	50 ml
Inositol (10 mg/ml)	10 ml
Ca-Panthothenat	1 ml
Nicotine acid	1 ml
Biotin	1 ml

Agar-plates for the different media were prepared by adding 2% Bacto-agar prior to autoclaving.

Yeast strains and growth conditions

The yeast strains used in this study are listed in Table 1. They were grown at 27°C/140 rpm until mid-log phase (6 to 10×10^6 cells/ml) or stationary-phase cells (7 to 10×10^7 cells/ml). Cell numbers were determined by the use of a hematocytometer. For experiments with stationary-phase cells, the cultures were grown on media containing half the amount of carbon source (*i.e.*, 1% glucose or 1% galactose and 0.5% raffinose) and fivefold excess of auxotrophic requirements to ensure that the carbon source was the limiting factor. The presence of glucose in the media was routinely monitored using glucose indicator strips (Diabur-Test, Boehringer Mannheim).

Bacterial strains and growth conditions

E. coli DH5α (Gibco BRL) was used for the amplification of plasmids and of the *S. pombe* cDNA library. *E. coli* media were prepared according to standard recipes (Sambrook *et al.*, 1989).

Tetrad analysis

S. pombe cells from opposite mating type, freshly grown on YES plates, were mixed together with a drop of water to form a patch on an ME plate and incubated at 30°C for two to

three days. The mature crosses were performed as described by Alfa and coworkers (1993). By the use of a micromanipulator, the four spores from a complete tetrad were placed at distinct positions on very thin YES plates and incubated at 30°C for two to three days. The new colonies were tested for segregation of the markers, trehalose and Tre6P accumulation, sporulation efficiency, growth at elevated temperatures, and Tre6P synthase and Tre6P phosphatase activities as described below.

Sporulation analysis

Strains of opposite mating type were grown separately for 24 h in 10 ml of EMMA medium at 30°C. From this preculture, 5×10^6 cells/ml were transferred to new Erlenmeyer flasks containing 10 ml of fresh EMMA media and incubated further at 30°C for 24 h. Then, 5×10^6 cells/ml of each mating type were mixed in 10 ml of the same medium and incubated at 30°C to allow mating. The numbers of vegetative cells (v), asci (a) and zygotes (z) were counted with a hematocytometer after additional 24 h. The percentage of sporulation was determined with the following calculation (Schweingruber and Edenharter, 1990):

$$\% \text{ sporulation} = \frac{(2a+2z) \times 100}{v+2a+2z}$$

Table 1: Yeast strains used in this study

Strain	Genotype		Source (reference)
972	*h*$^-$		Schweingruber, Bern
PB004	*h*$^-$	*ade6-M216 leu1-32 ura4-D18*	Blázquez *et al.* (1994)
PB003	*h*$^+$	*ade6-M216 leu1-32 ura4-D18*	Blázquez *et al.* (1994)
PBL-17	*h*$^+$	*ade6-M216 leu1-32 ura4-D18 tps1::LEU2*	Blázquez *et al.* (1994)
CHP421	*h*$^-$	*his7-366 leu1-32 ura4-D18*	Jin *et al.* (1995)
CHP453	*h*$^-$	*his7-366 leu1-32 ura4-D18 pka1::ura4*$^+$	Jin *et al.* (1995)
MRP-3A	*h*$^+$	*his7-366 leu1-32 ura4-D18 pka1::ura4*$^+$ *tps1::LEU2*	This study
MRP-7A	*h*$^+$	*leu1-32 ura4-D18 pka1::ura4*$^+$	This study
MRP-7B	*h*$^-$	*ade6-M216 leu1-32 ura4-D18 tps1::LEU2*	This study
MRP-7C	*h*$^-$	*his7-366 leu1-32 ura4-D18*	This study
MRP-7D	*h*$^-$	*ade6-M216 his7-366 leu1-32 ura4-D18 pka1::ura4*$^+$ *tps1::LEU2*	This study
MRP-9C	*h*$^-$	*ade6-M216 his7-366 leu1-32 ura4-D18*	This study
MRP-9D	*h*$^+$	*leu1-32 ura4-D18 pka1::ura4*$^+$ *tps1::LEU2*	This study
MRP3	*h*$^-$	*ade6-M216 leu1-32 ura4-D18 tps2::kanMX2*	This study
MRP4	*h*$^-$	*ade6-M216 leu1-32 ura4-D18 tps4::ura4*$^+$	This study
MRP5	*h*$^-$	*ade6-M216 leu1-32 ura4-D18 tps2::kanMX2 tps4::ura4*$^+$	This study
MRP9	*h*$^+$	*ade6-M216 leu1-32 ura4-D18 tps2::kanMX2*	This study
MRP10	*h*$^+$	*ade6-M216 leu1-32 ura4-D18 tps4::ura4*$^+$	This study
MRP11	*h*$^+$	*ade6-M216 leu1-32 ura4-D18 tps3::kanMX2*	This study
YSH6.106.3A	*MAT* α	*his3-11, ura3-1, ade2-1, leu2, trp1-1, can1-100*	Reinders *et al.* (1997)
YSH6.106.8C	*MAT* α	*his3-11,15, ura3-1, ade2-1, leu2, trp1-1, can1-100, tps2::LEU2*	Reinders *et al.* (1997)

Table 2: Primers used in this study

Number	Description	Orientation	Length	Sequence
	ADH1	F	22	5'-CCTCGTCATTGTTCTCGTTCCC-3'
34	*tps2*$^+$-Northern	F	24	5'-TTACTGCTACTTCTGCTATGCTAC-3'
35	*tps2*$^+$-RACE	F	25	5'-TTGGTTTGCTCTAAGTATGATGTTG-3'
174	CP2-RACE	F	24	5'-CAAGCATCCCAAGCTATTAAGCAG-3'
175	SP6-RACE	R	24	5'-ATTGTTCCTCGACGTGATGTACGG-3'
176	SP5-RACE	R	24	5'-AATAATGCATCGGCACGAGTGAGC-3'
177	SP4-RACE	R	24	5'-TTACTGACACCTTCGCGTATGCTG-3'
16159	pGEM-T vector	R	25	5'-CACACAGGAAACAGCTATGACCATG-3'
16160	pGEM-T vector	F	25	5'-CGACGTTGTAAAACGACGGCCAGTG-3'
16421	*kanMX2*	R	26	5'-GCATGGTTACTCACCACTGCGATCCC-3'
21215	*tps2*$^+$ deletion	R	121	5'-CTGAACTAAGATCCAGGATTGTGTGTCTC AATAAATAAATCTTTGTAAAGAACAAGATG AATCGGGCATCTTCGTTGAAATAGTTAGTTT GTCATTCGCATAGGCCACTAGTGGATCTG-3'
21249	SP2-RACE	R	24	5'-GAGCATGCTCTGGTGTTAATGTAG-3'
21258	CP1-RACE	F	24	5'-GGTTACCACCCATACCATGCAGTC-3'
21259	SP3-RACE	R	24	5'-CTTTGTGGCTGCTAGCTTGTTTGC-3'
21260	SP1-RACE	R	24	5'-CTTTGGATCGGCTGCAAGTGTTGC-3'
22272	*tps2*$^+$-RACE	R	24	5'-GAGTACCTCGTTGTTGCACCTCGG-3'
22273	*tps2*$^+$-RACE	F	24	5'-GCGTCAGTGGTCGCATTATATACG-3'
22274	*tps2*$^+$ (*Pst*I, *Xba*I and *Not*I) cloning into pUR19 and pIRT2	R	55	5'-AGCTACCTGCAGTCTAGAGCGGCCGCT AGACCATGTATGCCGAAGTGCATTAACG-3'
22276	*tps2*$^+$ (*Pst*I, *Sal*I and *Sac*I)/ Southern and cloning into pUR19 and pIRT2	F	57	5'-ATCGGATGAACTGCAGTCGACGAGCTCA CTGCGAAGAGAACAGTCCGACATTTTATC-3'
22533	*tps2*$^+$-RACE/Southern	R	24	5'-TGCTGAGAGCGTGTCTCATTAATG-3'
22534	*tps2*$^+$-RACE	F	25	5'-CGCTGGTTAGCCTATGCCGAAAAT G-3'

Table 2. -continued

Number	Description	Orientation	Length	Sequence
22535	*tps2*$^+$-RACE	F	27	5'-CCTTGACCTTGAAATTGGTCATGATTG-3'
22536	*tps2*$^+$-RACE	R	23	5'-GGACTCGAGAACAGCAGCTGATG-3'
22537	*tps2*$^+$-screening	F	25	5'-GCTGCTGTTCCTTCAAAGAAAC-3'
22538	*tps2*$^+$-screening/Northern	R	24	5'-TCCTCCTTTGTTGATACTGGAAGG-3'
22539	*tps2*$^+$-screening	F	25	5'-CGTATCCAGAAGATTCACTTCCTTC-3'
22540	*tps2*$^+$-screening	R	24	5'-AGTGGAAGTAGTTCAATTACGAAG-3'
22541	*tps2*$^+$-RACE	F	24	5'-CACAAATCTACACTCTTCCATTAC-3'
22894	*tps2*$^+$-RACE	F	24	5'-TGCTAGAATCTAGTAGAGAAGTTG-3'
22895	*tps2*$^+$-RACE	F	23	5'-ATTGCACGTTGCTTTCCTCGTCG-3'
22896	*tps2*$^+$-RACE	F	24	5'-AACTGATGAAGATATGTTCCGCTC-3'
23812	*tps4*$^+$ deletion	F	100	5'-CTTATTGTAGTGATTCATTGGTTTTATTCT CTATATTTCTCGTCACTTCTCCTCGGTGAAGT TAAGTGCTCTAGAACGCCAGGGTTTTCCCAG TCACGAC-3'
23813	*tps4*$^+$ (*Sac* I) cloning into YCpIF5	R	45	5'-GGTCCAAAAAGAGCTCTGAATTGGCTGA CTAGAGGGACACAAACC-3'
23814	*tps4*$^+$ deletion	R	100	5'-AGATGATAGTTCAAAAAAGGTCATTGAA AAAAAAAACACGTTACTTTCATAAATATACG TTATTATCCCGGTTATTAGCGGATAACAATTT CACACAGGA-3'
23815	*tps4*$^+$(*BamH*I) cloning into pIRT2	F	43	5'-CAGAGTTCTCGGATCCAAATTTCGATTCA AATGCTTACGTTTC-3'
24330	*tps4*$^+$-Northern	F	28	5'-TTCTCCTCGGTGAAGTTAAGTGCTCTAG-3'
24331	*tps4*$^+$-Northern	R	24	5'-CATAAATATACGTTATTATCCCGG-3'
24362	*tps4*$^+$-Northern	R	28	5'-CGCGGAATCCATCAGAAGTATCTATATG-3'
24729	*tps4*$^+$ (*Cla*I) cloning into YCpIF5	F	42	5'-GTTAAGTGCTATCGATATGCCTTCAGGCG CGCAAGGAAATAC-3'
24730	*tps2*$^+$ (*Spe*I) cloning into YCpIF5	R	42	5'-CCGAAGTGCAACTAGTGATGCACAACCT ATAACTTATTGCTC-3'
24731	*tps2*$^+$ (*Sal*I) cloning into YCpIF5	F	42	5'-CGCTTATAATGTCGACATGAGTGTGTAC GGAAAAATCCCGTC-3'

Table 2. -continued

Number	Description	Orientation	Length	Sequence
24770	*tps2*$^{+}$ deletion	F	118	5'-TTTGCTAGAATCTAGTAGAGAAGTTGCC AATAGTTTATGGAAATCATAAGCTTAAAACT GTAGTTTACTGTTTTGTCAATTTCGCTTATAA TACATCACAGCTGAAGCTTCGTACGC-3'
24993	*tps3*$^{+}$ deletion	F	115	5'-GCTCTTTCAAACTTGCCCTTTACCAACC ACTCCATTCCCAAGTTTTTGGATATTCAGCAC TGGTTATAGACGCACTTGTTTAAGTAATCGGT TTACCAGCTGAAGCTTCGTACGC-3'
24995	*tps3*$^{+}$ deletion	R	118	5'-CAGGATTTCACAAATCACGGTGTTTTGTA AAATCAATACATAAAACCAAAAAAAAAATAA CACGTAAACTGCTCATAAATTGCAGACCATT ATAATTGCATAGGCCACTAGTGGATCTG-3'
25229	*tps4*$^{+}$ (*Not*I) cloning into YCpIF5	R	40	5'-ATAAAAACCTGCGGCCGCCATATCGGT TTATGAACGCAAC-3'
25317	*tps3*$^{+}$	R	27	5'-GCCTCTCGACTTGAACAAGTCATTGAG-3'
25321	*ura4*$^{+}$	R	30	5'-GCCATACAGTGCCAGGCGAGGGTATTAT AC-3'
25323	*tps3*$^{+}$ (*Sal*I site)	F	41	5'-TGCAATTGCCGTCGACGAACTTTAGAA GGACAACGAAATCC-3'
25634	*tps4*$^{+}$ (*BamH*I) cloning in pIRT2	R	47	5'-TAAGCATTTAGGATCCCGCTCATTAACA ATTTACCTTGATTCCATAC-3'

Primer numbers correspond to the identification numbers given by the FMI oligonucleotide synthesis lab. Orientation refers to the primers being either forward (F) or reverse (R). The length of the primers is given as number of nucleotides. Underlined sequences of primers 21215, 24770, 24993, and 24995 denote the part of the primer which is complementary to the *KanMX2* module. Underlined sequences of primers 23812 and 23814 denote the part of the primer which is complementary to the *ura4*$^{+}$ marker (see text for further explanation).

Colony PCR

Colony PCR was used as a rapid test to check for the correct cloning of DNA fragments after transformation into *E. coli* cells. To this end, colonies of transformed *E. coli* cells were resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) with 0.1% Tween-20. The suspensions were then heated to 95°C for 5 min and subsequently cooled on ice. The PCR contained 1 × PCR buffer, 0.03 mM of dATP, dCTP, dGTP and dTTP, 0.22 µM of each primer, 0.5 U of *Taq* polymerase (Pharmacia) and 2 µl of the bacterial cell suspension as template. The program used was: 26 cycles of 25 s at 94°C (DNA denaturation), 30 s at 62°C (annealing), and 1 min 15 s at 72°C (extension). An additional 7 min of extension at 72°C and cooling at 4°C were applied. The annealing temperature varied in accordance with the melting points of the primers used.

Screening for an *S. pombe* Tre6P phosphatase cDNA by functional complementation of an *S. cerevisiae tps2* mutant

In order to isolate the *S. pombe* $tps2^+$ gene, an *S. cerevisiae tps2* mutant was transformed with an *S. pombe* cDNA library. This library was cloned into vector pDB20 (Becker *et al.*, 1991), where *S. pombe* cDNAs were expressed under the control of the *S. cerevisiae ADH1* promoter. Approximately 37×10^6 independent transformants were obtained on minimal glucose media (SD) at 27°C. The clones were then replica plated on glucose plates, incubated at 38.6°C, and screened for complementation of the growth defect of the *S. cerevisiae tps2* mutant. After 5 to 10 days at 38.6°C, 53 positive colonies were obtained. These colonies were re-screened on fresh plates. A total of 40 clones still grew at 38.6°C and were assayed for *in vivo* production of trehalose and Tre6P. Of these 40 clones, 15 had higher levels of trehalose and lower levels of Tre6P when compared with the *S. cerevisiae tps2* mutant after a mild heat shock at 40°C. Library plasmids of these 15 clones were isolated and analyzed by restriction enzyme digestion. The largest insert found was 1.5 kb long and coded only for part of the full length Tps2 protein (see also below). In order to get the full length $tps2^+$ cDNA sequence we used the 5' RACE (rapid amplification of 5'/3' cDNA ends) PCR method.

RACE experiments

The RACE PCR was performed according to the kit manual from Boehringer Mannheim (Rotkreuz, Switzerland). All the primers used for sequencing $tps2^+$ on both strands are listed in Table 2. The RACE-PCR steps are summarized below in Figure 1.

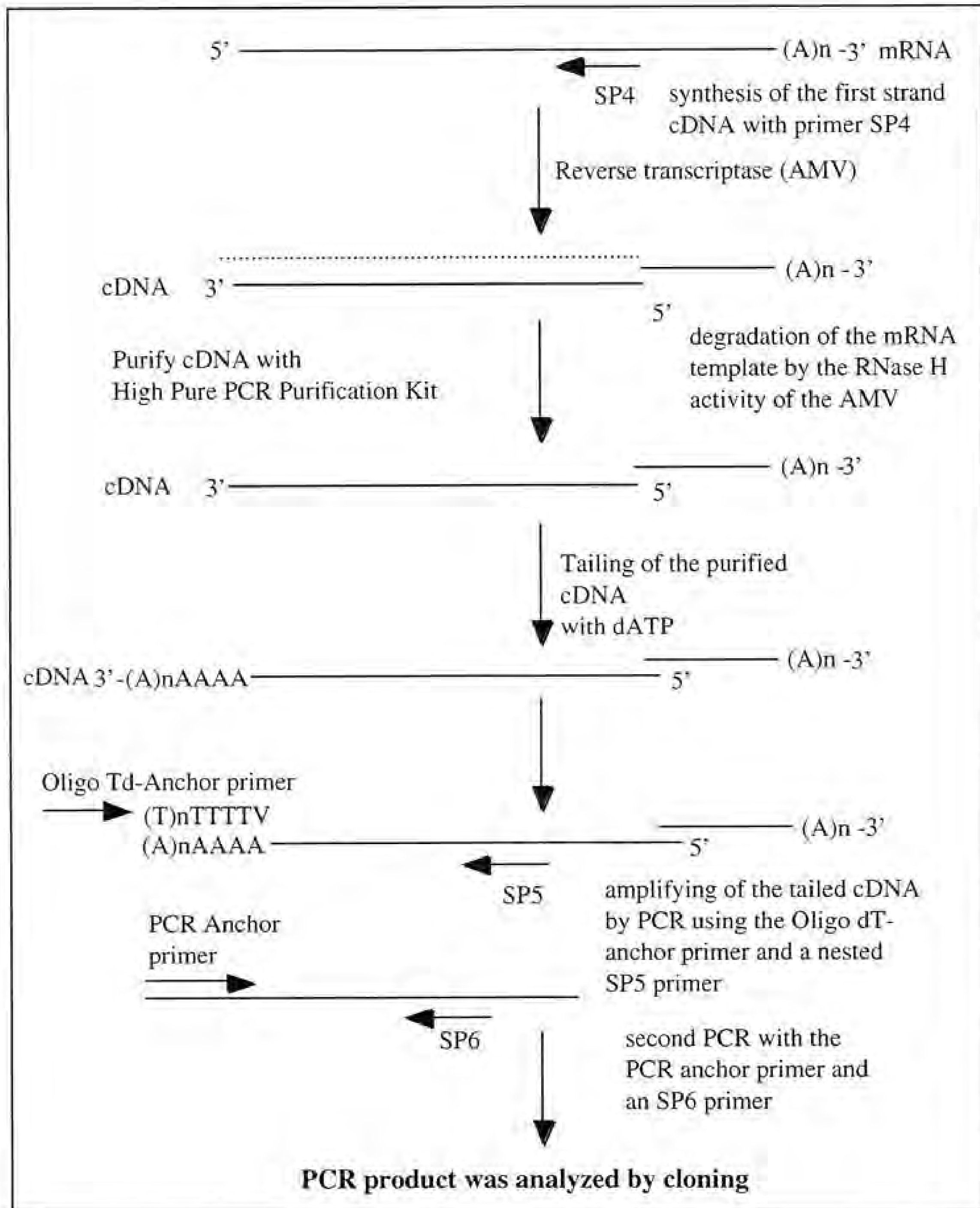

Figure 1. Schematic overview of the RACE procedure

Initially, we used the cDNA library from *S. pombe* as a template in a specific PCR using a forward primer in the *ADH1* promoter and either the SP1 (21260), SP2 (21249) or SP3 (21259) reverse primer which anneal 645, 798 or 828 bp, respectively, upstream of the stop codon of the partial Tre6P phosphatase sequence obtained in the complementation screen. A pattern of specific bands varying from 200 bp to 800 bp was identified by agarose

electrophoresis gel. The PCR products in the range of 700 to 1000 bp were cut out from the gel and cleaned with GENECLEAN II kit (BIO 101 Inc.). The supernatant obtained after this cleaning step was used as template in a new PCR using the forward primer *ADH1* and the SP3 reverse primer. A pattern of bands in the range of 400 bp to 800 bp were observed followed agarose gel electrophoresis. A strong 600 bp band was obtained, cut from the gel, cleaned, and ligated into pGEM-T vector from Promega (Catalys AG, Wallisellen, Switzerland). Subsequently, *E. coli* cells were transformed with the ligation mixture and selected for blue/white colour on LB-AMP plates containing 40 μl XGal (20 mg/ml) and 4 μl IPTG (200 mg/ml). White colonies were tested for insert size by colony PCR using primers no. 16159 and no. 16169 in the pGEM-T vector from Promega. The largest inserts were around 800 bp. Specificity was further tested by colony PCR using primer CP1 (21258) together with either primer no. 16159 or no. 16160. Four inserts, yielded a specific signal and sequencing confirmed that they contained an additional group of the *S. pombe* $tps2^{+}$ amino-terminal sequence. Based on this sequence, new primers SP4 (177), SP5 (176), and SP6 (175) were designed which anneal 1106, 1136 and 1185 bp, respectively, upstream of the stop codon of the partial Tre6P phosphatase sequence. A new round of PCR using the cDNA library as template yielded no further specific bands. It is possible, therefore, that the cDNA library used does not contain a full length *S. pombe* cDNA clone. As a consequence, we chose to extract total cellular RNA from *S. pombe* strain 972 (h^{-}) as described previously (Piper, 1994), and to extract PolyA-mRNA from the total RNA using the Oligotex poly A' mRNA Kit from Qiagen to perform 5' RACE-PCR experiments as described by Vogel and coworkers (1998). The primer SP4 was used for new cDNA synthesis using the isolated mRNA as template. Primer SP5 was used for the first round of nested PCR, and primer SP6 for the second round of PCR. The PCR products in the range of 1000-1800 bp from the 5' RACE-PCR were isolated after agarose gel electrophoresis, cleaned using the GENECLEAN II kit and ligated into pGEM-T vector. *E. coli* cells transformed with the ligation mixture were selected on LB-AMP plates as described above. The plasmids from the white colonies were tested for their insert size and for specificity by the colony PCR method described above. The gene-specific forward primer CP2 (number 21258) was used together with either primer no. 16159 or no. 16160 in the pGEM-T vector to test the new sequence obtained as described in Vogel and coworkers (1998). The longest 5' Race product (1210 bp) was sequenced in both strands.

DNA sequencing and sequence analysis

Positive cDNA clones isolated by complementation of the growth defect at 38.6°C of an *S. cerevisiae tps2* mutant and clones obtained from the RACE experiments were either sequenced by the Sanger dideoxynucleotide method (Sanger *et al.*, 1977) using ΔT^{th} DNA polymerase (Toyobo, Japan) and [α-^{33}P] dATP (Hartmann Analytic, Braunschweig, Germany), or using a cycle sequencing program and an automated sequencer (ABI 301, Perkin Elmer). For

the latter purpose, 1 µl DNA (300 –500 ng) was mixed with 1 µl primer (0.5 µM), 4 µl reaction mix (Perkin Elmer) and 4 µl water. Cycle sequencing was performed in a Techne GENIUS PCR machine. The program used was the following: 27 cycles of 30 s at 96°C, 15 s at 50°C, and 4 min at 60°C. Subsequently, 10 µl of water were added to the reaction, the DNA was precipitated with 2 µl 3 M sodium acetate (pH 5.2) and 50 µl ethanol (100%). After centrifugation, the pellet was washed with 70% ice-cold ethanol, dried, resuspended in 25 µl template suppressing reagent (TSR, Perkin Elmer), heated for 2 min at 95°C, and then applied to the automated sequencer. Sequence comparisons and alignments were performed with the GAP, PILEUP, and PRETTY programs of the Genetics Computer Group (GCG) of the University of Wisconsin.

Plasmid constructions

- **YCpIF5-derived plasmids**

For complementation analysis of the *S. cerevisiae tps2* mutant, *S. pombe* $tps2^+$ and $tps4^+$ genes were cloned into the *E. coli-S. cerevisiae* shuttle vector YCpIF5, which allows galactose-inducible expression of these genes (Foreman and Davis, 1994). The full-length ORF of $tps2^+$ including 386 bp of the 3' flanking region was amplified using the Expand Long Template PCR System (Boehringer Mannheim), primers 24731 and 24730 (Table 2), and genomic DNA of strain 972h (h^-). Primer 24731 was designed with a *Sal*I site immediately upstream of the start codon and primer 24730 was designed with an *Spe*I site 386 bp downstream of the stop codon. The PCR conditions were the following: 2 min at 92°C, then 5 cycles of 15 s at 92°C, 30 s at 54°C, and 2 min at 68°C. Afterwards, 24 cycles of 15 s at 92°C, 30 s at 76°C, and 75 s at 68°C with an extension of 20 s per cycle. Subsequently, the reaction was incubated at 68°C for 7 min and cooled at 4°C. The PCR-product obtained was purified by phenol extraction and then precipitated with ethanol. The precipitated DNA was resuspended in an appropriate amount of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0), digested with *Sal*I and *Spe*I, and ligated at the corresponding restriction sites of YCpIF5 (Fig. 2A). The resulting plasmid was named YCpIF5-$tps2^+$. The full-length $tps4^+$ ORF including 313 bp of the 3' flanking region was amplified as described above for the $tps2^+$ ORF using primers 24729 (designed with a *Cla*I site immediately upstream the start codon) and primer 25229 (designed with *Not*I site 313 bp downstream of the stop codon). The PCR-product was digested with *Cla*I and *Not*I and ligated at the corresponding restriction sites of YCpIF5 (Figure 2A). The resulting plasmid was named YCpIF5-$tps4^+$.

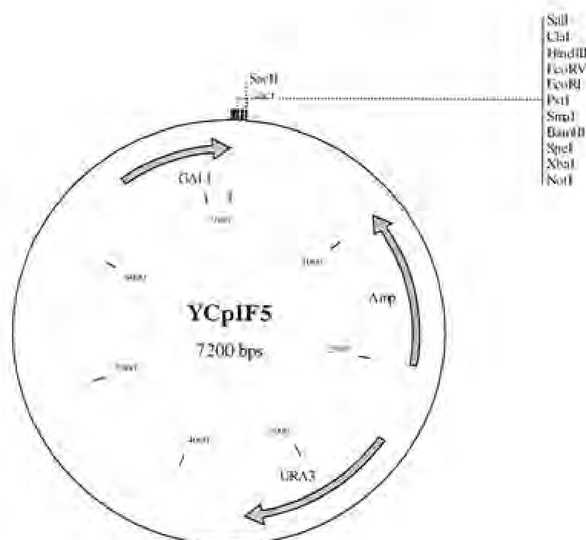

Figure 2A. *E. coli-S. cerevisiae* shuttle vector YCpIF5.

- **pUR19- and pIRT2-derived plasmids**

The *S. pombe tps2⁺* gene under control of its own promoter was amplified using the Expand Long Template PCR System (Boehringer Mannheim) as described in the manual. The PCR reaction mixture contained genomic DNA of strain 972 (h^-) as template, primers 22276 (designed with additional *Pst*I, *Sal*I, and *Sac*I restriction sites) and 22274 (designed with additional *Pst*I, *Xba*I, and *Not*I sites). The PCR conditions were the following: 2 min at 92°C, then 5 cycles of 15 s at 92°C, 30 s at 54°C, and 2 min at 68°C. Afterwards, 24 cycles of 15 s at 92°C, 30 s at 76°C, and 75 s at 68°C with an extension of 20 s per cycle. Subsequently, the reaction was incubated at 68°C for 7 min and cooled at 4°C. The PCR-product obtained was purified by phenol extraction and then precipitated with ethanol. The precipitated DNA was resuspended in an appropriate amount of TE buffer. The PCR product (3157 bp), containing the full length *tps2⁺* ORF including 321 nucleotides upstream of the start codon and 385 nucleotides downstream the stop codon, was either cut with *Pst*I and ligated at the corresponding site of plasmid pUR19 (Barbet *et al.*, 1992) to obtain pUR19-*tps2⁺* (Figure 2B), or cut with *Sac*I and *Pst*I and ligated at the corresponding restriction sites of plasmid pIRT2 (Hindley *et al.*, 1987) to obtain pIRT2-*tps2⁺* (Figure 2C).

The full length *S. pombe tps4⁺* gene was amplified as described above for the full length *tps2⁺* gene. The resulting PCR-product (3538 bp) contained the full ORF including 744 nucleotides upstream of the start codon and 274 nucleotides downstream the stop codon. Primers 23815 and 25634 (both designed with a *Bam*HI site) were used. pIRT2-*tps4⁺* was

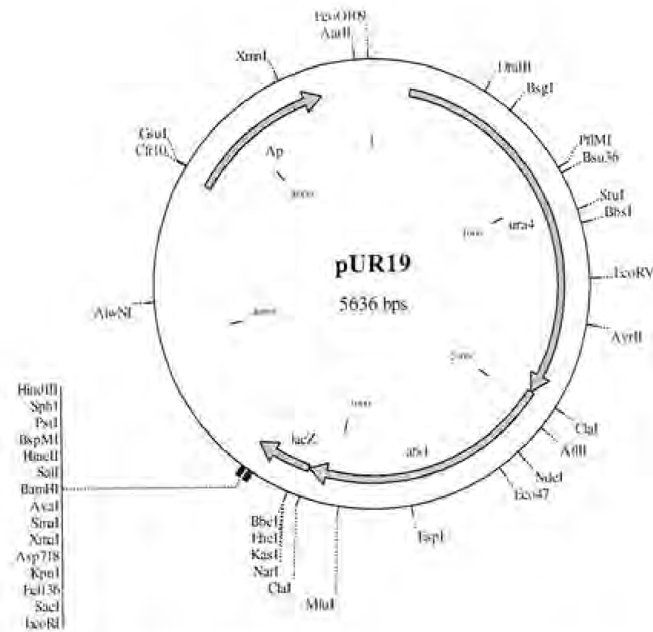

Figure 2B. *E. coli-S. pombe* shuttle vector pUR19

obtained by digestion of the PCR-product with *Bam*HI and subsequent ligation at the corresponding restriction site of plasmid pIRT2 (Figure 2C). For complementation analysis of the *S. pombe* $tps2^+$ deletion mutant by the *S. cerevisiae TPS2* gene, the *S. cerevisiae TPS2* coding sequence including its own promoter was cut from vector YCplac111-*TPS2* by digestion with *Sac*I. The excised fragment was then ligated at the corresponding *Sac*I restriction site of plasmid pUR19 (Figure 2B) to produce pUR19-*TPS2*.

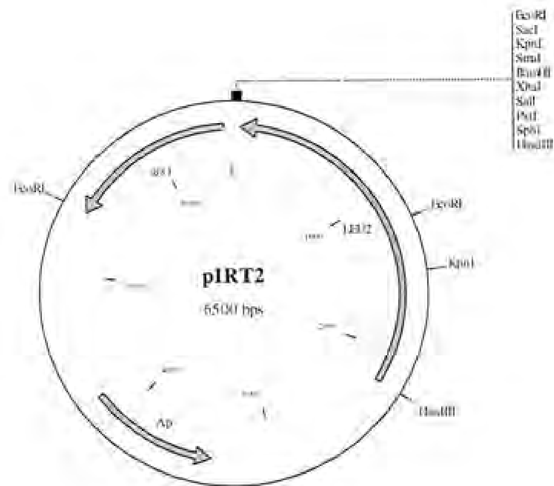

Figure 2C. *E. coli-S. pombe* shuttle vector pIRT2.

Preparation of genomic or plasmid DNA from yeast

Genomic or plasmid DNA was obtained according to the procedure described by Hoffman and Winston (1987). Yeast cells were grown to stationary phase in 5 ml YES at 27°C, centrifuged, resuspended in 0.5 ml of sterile water, and transferred to Eppendorf tubes. After centrifugation, the cell pellets were resuspended in 0.2 ml of a freshly prepared miniprep solution (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 1mM EDTA, pH 8.0). A total of 0.2 ml of phenol-chloroform-isoamyl alcohol (25:24:1) and 0.3 g acid-washed glass beads were added and the tubes were vortexed continuously for 3 min. Afterwards, 0.2 ml TE buffer was added, mixed briefly and the samples were centrifuged for 5 min at full speed. The aqueous layers were transferred to new tubes and 1 ml ethanol (100%) was added. The tubes were mixed by inversion and centrifuged for 2 min. The precipitated DNA was then washed with ice-cold 70% ethanol, dried briefly, resuspended in an adequate volume of TE (25-100 µl) and subsequently used as template for PCR reactions or Southern blots.

Yeast transformations

- **By Li^+-ion method**

S. pombe cells were transformed using a protocol based on the method of Keeney and Boeke (1994). Cells were grown at 27°C to 1×10^7 cells/ml. Cells (20 ml per transformation) were washed once with an equal volume of water, resuspended in 1ml of water, transferred to an Eppendorf tube, and washed once with 1 ml of 1 x LiAc/TE made from a 10 × filter-sterilized stock (10 × LiAc: 1 M Lithium acetate, pH 7.5, adjusted with diluted acetic acid; 10 × TE: 0.1 M Tris-HCl, 0.01 M EDTA, pH 7.5). The cells were then resuspended in 1 x LiAc/TE to a final concentration of 2×10^9 cells/ml. Cells (100µl) were mixed with 2 µl sheared herring testes DNA (10 mg/ml Yeastmaker carrier DNA; Clontech Laboratories) and 10 µl of the transforming DNA. After 10 min incubation at room temperature, 260 µl of 40% PEG/LiAC/TE (for 20 ml solution: 8 g PEG 4000 in 2 ml 10 × LiAc, 2 ml 10 × TE, and 9.75 ml water) were added. The cell suspension was gently mixed and incubated for 30 to 60 min at 30°C. An amount of 43 µl of DMSO was added, and the cells were heat shocked for 5 min at 42°C. Cells transformed with the *kanMX2* marker were then washed once with 1 ml water, resuspended in 0.5 ml water, plated onto two YES plates and incubated at 30°C for 18 to 24 h, resulting in a lawn of cells. The cells were then replica plated onto YES plates containing 100 mg/l G418 (Geneticin, Life Technologies). The replica plates were incubated for two to three days at 30°C, and large colonies were restreaked onto fresh YES plates containing G418. Cells transformed with the *ura4⁺* marker were washed with water as described above, plated onto SD plates without uracil, and grown at 30°C for three days. Colonies were restreaked onto fresh SD

plates without uracil. Cells transformed with plasmids carrying specific genes were treated as described above and plated on appropriate SD plates.

S. cerevisiae cells were transformed using a modification of the Li^+-ion method as described by Gietz and coworkers (1992). Selections of transformants were performed on appropriate SGal/Raff plates.

- **By electroporation**

S. pombe cells were transformed using a protocol based on the method of Prentice (1992). *S. pombe* cells were grown at 27°C either on YES or SD media to a density of approximately 1×10^7 cells/ml and collected by centrifugation. Cells were then washed three times with an equal volume of ice-cold filter sterilized 1.2 M sorbitol. The cells were then resuspended in 1.2 M sorbitol to a concentration of 1×10^9 cells/ml. A total of 0.2 ml of the cell suspension was mixed with the DNA, and transferred to an ice-cold cuvette with a 0.2 cm electrode gap (Bio-Rad). Cells were then pulsed at 2.25 kV (11.25kV/cm), 200Ω, and 25μF. After the pulse, 0.5 ml of ice-cold 1.2 M sorbitol was added to the cuvette. Electroporated cells were diluted and plated on selective plates as described above.

PCR amplification of fragments for transformation

In order to delete the entire ORFs of *tps2*$^+$, *tps3*$^+$ and *tps4*$^+$, special PCR primers of 100 to 123 bp length were designed (Table 2). The deletion strategy is depicted in Figure 3.

- **Deletion of *tps2*$^+$ and *tps3*$^+$**

S. pombe tps2$^-$ strains were obtained by deleting the complete *tps2*$^+$ coding region by the PCR method described earlier (Wach *et al.*, 1994) using plasmid pFA6a (*kanMX2*) as template. The deletion primers 24770 and 21215 contained 99 nucleotides immediately upstream and downstream, respectively, of the *tps2*$^+$ coding region, and 19 and 22 nucleotides upstream and downstream, respectively, of the *kanMX2* module (Wach *et al.*, 1994). *tps3*$^-$ strains were obtained with the same strategy using primers 25317 and 25323. PCR reactions were performed in a Programmable Thermal Controller (PTC-100) machine (MJ Research, Inc.). The PCR mixture contained (in a total volume of 50 μl) 2.5 U of *Taq* DNA polymerase (Pharmacia), 0.2 mM of each dATP, dCTP, dTTP, and dGTP, 0.5 μM of each primer, and 25 ng of template DNA. On top of each mixture 2 drops of mineral oil (Sigma) were added. The reaction conditions were: 2 min at 92°C to denature the template DNA, followed by 29 cycles of 15 s at 92°C, 1 min at 55°C, and 1 min and 30 s at 72°C. The program ended with 4 min at 72°C, after that, the reaction was cooled to 4°C. In order to check whether the amplified PCR

fragment had the correct size (1.7 kb), 2 μl of the PCR mixture were analyzed by agarose gel electrophoresis. Four to ten PCR reactions were pooled, extracted with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1), ethanol-precipitated, and dissolved in 10 μl TE (pH 8.0) buffer. The concentrated DNA of the *tps2*$^+$and *tps3*$^+$ deletion cassettes were transformed into strain PB004 and PB003 to construct strains MRP3 (*tps2*$^-$) and MRP11 (*tps3*$^-$), respectively (Table 1). Geneticin-resistant transformants were further analyzed and correct integration of the *kanMX2* module was confirmed by PCR and Southern blot analysis (see below).

- **Deletion of *tps4*$^+$**

S. pombe tps4$^-$ strains were obtained by deleting the complete *tps4*$^+$ coding region by a PCR method using plasmid KS-*ura4*$^+$, which contains the entire *S. pombe ura4*$^+$ gene including flanking sequences (Grimm *et al.*, 1988), as template. Each of the primers used (23812 and 23814) had a total length of 100 nucleotides. Their 5' ends corresponded to the 76 nucleotides immediately upstream and downstream of the *tps4*$^+$ coding region, while their 3' ends corresponded to the 24 nucleotides of the M13 forward and reverse sequencing primers, respectively. The resulting PCR product had a stretch of 76 nucleotides of homology at the 5' and 3' ends to the regions immediately up- and downstream, respectively, of the *tps4*$^+$ coding region which was separated by the *ura4*$^+$ gene. The PCR conditions used were the same as described above. An aliquot (2 μl) of the PCR product was analyzed by agarose gel electrophoresis and a band of 2.2 kb was visible. Four to ten PCR reactions were pooled, and treated as described above. The concentrated DNA of the *tps4*$^+$ deletion cassette was used to transform strains PB004 and MRP3 to construct strains MRP4 and MRP5, respectively (Table 1). Transformants that had *tps4*$^+$ replaced with the *ura4*$^+$ marker were identified by their growth on SD plates without uracil and were subsequently confirmed by PCR and Southern blot analysis (see below).

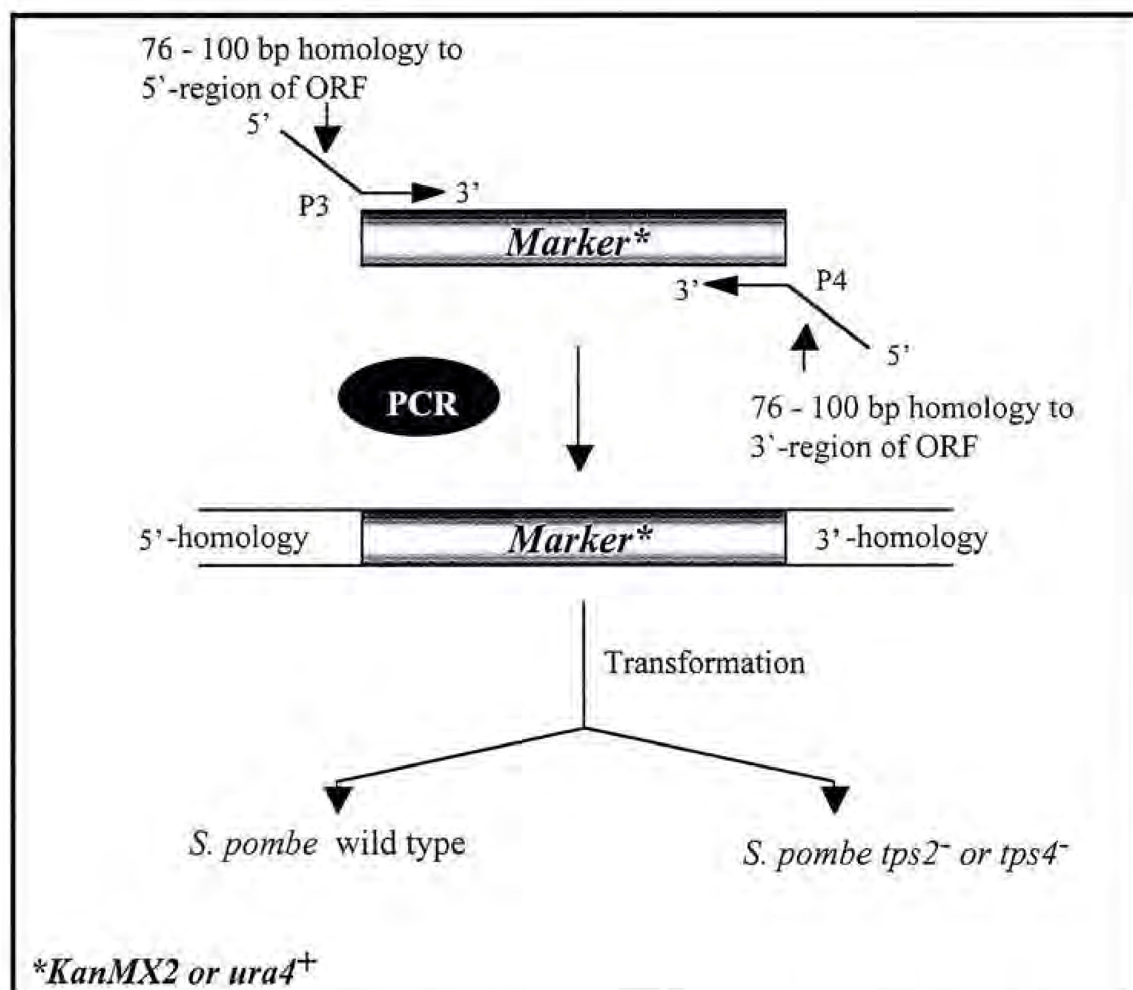

Figure 3. Construction of *tps2⁻*, *tps3⁻*, and *tps4⁻* strains. PCRs were performed with either plasmid pFAG-*kanMX2* or KS-*ura4⁺* as template and in each case two primers with homology to the marker as well as to sequences immediately up- and downstream of the *tps2⁺*, *tps3⁺*, or *tps4⁺* coding regions. The PCR products were transformed into wild-type strain PB003 to obtain homologous recombination and integration at the *tps2⁺*, *tps3⁺*, or *tps4⁺* loci resulting in the strains MRP3 (*tps2::kanMX2*), MRP11 (*tps3::kanMX2*), and MRP4 (*tps4::ura4⁺*), respectively. The double deletion mutant *tps2⁻ tps4⁻* was obtained by transforming the *tps2::kanMX2* deletion cassette into strain MRP4 resulting in the strain MRP5 (*tps2::kanMX2, tps4::ura4⁺*).

PCR-screening of transformants for correct integration of the deletion cassettes

The G418-resistant or URA⁺ transformants were checked by PCR for the correct integration of the DNA fragment. To this end, genomic DNA of transformants was prepared using a procedure based on that of Hoffman and Winston (1987) as described before. An aliquot of 0.5 µl of genomic DNA preparation was used as template in a PCR reaction with 0.5 mM of each of the dNTPs, 1.25 mM $MgCl_2$, 0.5 µM of each primer, 1 X PCR buffer, and 2.5 U of *Taq* polymerase (Pharmacia). The following PCR conditions were used: 2 min at 92°C

followed by 29 cycles of 1 min at 92°C, 30 s at 56°C, and 2 min at 72°C. Afterwards, an extension for 4 min at 72°C and subsequent cooling at 4°C were applied. To test the correct integration of the *ura4*$^{+}$ marker or the *kanMX2* module, one primer corresponding to a sequence upstream of the coding region to be deleted (primer 22541 for *tps2*$^{+}$, 25323 for *tps3*$^{+}$, and 24330 or 23815 for *tps4*$^{+}$) was used together with a second, reverse primer corresponding to a sequence in either the *ura4*$^{+}$ gene (25321) or in the *kanMX2* module (16421), as well as with a third, reverse primer corresponding to a sequence in the target gene (primer 21249 for *tps2*$^{+}$, 25317 for *tps3*$^{+}$, and 24362 for *tps4*$^{+}$). The PCR products were analyzed on agarose gels. The expected size of the bands in case of correct integration of the *kanMX2* marker at the *tps2*$^{+}$ and *tps3*$^{+}$ loci is 900 and 1923 bp, respectively, while incorrect integration should yield bands of 1.8 and 1.3 kb, respectively. The correct integration of the *ura4*$^{+}$ marker at the *tps4*$^{+}$ locus is expected to yielded a 1.6 kb PCR-fragment, while incorrect integration should yield a 968 bp fragment. All primer sequences are listed in Table 2. To check whether the DNA fragment had integrated at a single site in the genome, the positive clones were further subjected to Southern blot analysis.

Southern blot analysis

Southern blot analysis was performed in order to check the correct integration of the *kanMX2* and *ura4*$^{+}$ markers in the genome of *S. pombe* cells. Genomic DNA (10 µg) of each strain was digested overnight at 37°C with *Cla*I and *Sal*I (*tps2*$^{+}$ deletion), or with *Acc*I and *Pst*I (*tps4*$^{+}$ deletion). After digestion, 1 µl of RNase (10 mg/ml) was added and the mixture was further incubated at 37°C for 1 h. The DNA was precipitated with ethanol (2.5 volumes) and sodium acetate (0.1 volume). The pellets were washed with ice-cold 70% ethanol and resuspended in 10 µl TE (pH 8.0). Samples were mixed with loading buffer containing ethidium bromide (1µg/ml) and run on an 0.8% (w/v) agarose gel at 40 V for 4 h and 30 min. The bands were visualized under UV light. Subsequently, the DNA was denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl for 40 min with gentle agitation. The gel was then soaked for 15 min in 0.25 M HCl, rinsed several times with water, and neutralized by soaking in 0.5 M Tris, 1.5 M NaCl solution for 40 min with gently agitation. The DNA was then blotted to a nitrocellulose membrane (BA83, Schleicher & Schüll) by overnight capillary transfer using 10 x SSC (1.5 M NaCl, 170 mM sodium citrate, pH 7.0) as transfer buffer. The membrane was washed in 2 x SSC, dried with 3MM paper (Whatman), and baken in a vacuum oven at 80°C for 2 h to fix the DNA to the nitrocellulose. Prehybridization was carried out in 10 ml hybridization solution II (1% crystalline BSA, 1mM EDTA, pH 8.0, 0.5 M $NaHPO_4$ buffer, pH 7.2, and 7% SDS) at 65°C for at least 2 h. In the meantime, the [α-^{32}P]dATP-labeled DNA probe fragments were prepared using the Prime-It II Random Primer Labeling Kit (Stratagene) and purified using the NucTrap Probe Purification Columns (Stratagene). For analysis of the *tps2*$^{+}$ deletion, an internal 0.79 kb PCR-generated fragment (primers 22538 and 22534) of *tps2*$^{+}$

was used as probe. For analysis of the *tps4*$^+$ deletion, an internal 2.07 kb fragment of *tps4*$^+$, obtained by digestion of a PCR-generated fragment (primers 23815 and 25634) with *Acc*I and *Pst*I, was used as a probe. Hybridization was performed in 10-ml hybridization solution at 65°C overnight. The membranes were washed three or more times with high-stringency wash buffer II (1mM EDTA, pH 8.0, 40 mM $NaHPO_4$ buffer, pH 7.2, and 1% SDS) until low radioactivity background was detected. The membranes were wrapped in plastic wrap and exposed to Kodak X-OMAT AR film. Successful integrations of the markers at a single site were expected to yield 810 bp and 727 bp bands for the *tps2*$^+$ or *tps4*$^+$ deletions, respectively. In case of incorrect integrations, the expected size of the bands were 1859 bp and 2068 bp for the *tps2*$^+$ and *tps4*$^+$ strains, respectively (see results section of Chapter 4).

RNA isolation from yeast

RNA was prepared according to Piper (1994). Aliquots of 50 ml for log-phase and heat-shocked cells, or 25 to 35 ml for stationary-phase cells were harvested, chilled rapidly by adding ice-cold DEPC-treated sterile water, pelleted, and washed once more with ice-cold DEPC-treated sterile water. The cell pellets were either kept at –20°C for later extraction or immediately used for RNA extraction. To this end, the pellets were resuspended in 2 ml RNA extraction buffer (20 mM Tris-HCl, pH 8.5, 10 mM EDTA, pH 8.0, and 1% SDS) and 2 g acid-washed glass beads and 2 ml phenol (pH 8.0) were added. The suspensions were vortexed continuously for 6 min at room temperature and centrifuged for 5 min at 3500 rpm (IEC Centra GP8R). The upper aqueous layers were transferred to new tubes containing an equal volume of phenol-chloroform. The suspensions were vortexed for 1min, centrifuged for 5 min at 3500 rpm and the upper phases were transferred to new tubes containing an equal volume of chloroform. The suspensions were once more vortexed for 1 min, centrifuged for 2 min at 3500 rpm and the upper phases were transferred to Corex tubes. Ammonium acetate (6 M) was added to a final concentration of 1 M. Subsequently, 2 volumes of ice-cold ethanol were added and the tubes were placed at –20°C for at least 20 min. The RNA was pelleted by centrifugation at 8000 rpm for 20 min at 4°C, poured off, and the tubes were drained on paper tissue. The pellets were then resuspended in 1 ml TE and the RNA was precipitated again by adding 3 M sodium acetate to a final concentration of 0.2 M and 2.5 volumes of ice-cold ethanol. The tubes were placed at –20°C for 20 min and then centrifuged at 8000 rpm for 20 min at 4°C. Following centrifugation, the supernatants were poured off and the tubes were drained on paper tissue. The pellets were then washed with 70% ice-cold ethanol and dried under vacuum in a Speed Vac (UNIVAPO 150 H). The extracted RNAs were finally resuspended in an appropriate amount of TE (150 to 300 µl) and the concentration was determined by reading the Optical Density (OD) at 260/280 nm in a Shimadzu UV-160A

spectrophotometer. PolyA-mRNA was extracted from the total RNA using the Oligotex poly A mRNA Kit from Qiagen.

Northern blot analysis

The RNA extracted as described above was analyzed as follows: 10 μg of total RNA were resuspended in a final volume of 10 μl sample buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA, pH 8.0, 2.2 M formaldehyde, and 50% formamide) and incubated at 55°C for 10 min. Samples were mixed with loading buffer and 1 μl of ethidium bromide solution (1μg/ml) and subjected to electrophoresis on 1.1% agarose gels containing MOPS buffer (20 mM MOPS, pH 7.0, 5 mM Sodium acetate, and 1 mM EDTA, pH 8.0). and 0.65 M formaldehyde. Gels were run for 3 to 4 h at 80 V with MOPS buffer as running buffer. The blotting procedure was done as follows: first, the gels were washed twice in 10 X SSC for 20 min, then transferred overnight (by capillary transfer) onto nitrocellulose membrane (BA83, Schleicher & Schüll) using 20 X SSC as transfer buffer. Afterwards, the membranes were soaked in 6 X SSC for 5 min and then placed on a 3MM filter paper to dry for 30 min at room temperature. The membranes were then placed between two 3MM filter paper (Whatman), and baken in a vacuum oven for 1 h at 80°C to fix the RNA to the nitrocellulose. Prehybridization was performed in 10 ml of RNA hybridization solution II (1% crystalline BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, and 7% SDS) for 2 h at 60°C in a hybridization oven (Hybaid). Meanwhile, probes were labeled using the Prime-It II Random Primer Labeling Kit (Stratagene) and purified using the NucTrap Probe Purification Columns (Stratagene). Hybridization with the DNA probes was done overnight at 60°C in 10 ml of RNA hybridization solution II. Probes used for hybridization corresponded to internal fragments of *tps2*$^+$ (0.79 kb; primers 34 and 22538) and *tps4*$^+$ (0.94 kb; primers 24331 and 24362) and were generated by PCR. Northern blot analyses revealed that in both cases a 4.6 kb RNA species hybridized with the *tps2*$^+$ and *tps4*$^+$ probe (see results section Chapter 4).

Generation times of growth on SD or YES media

In order to assess the influence of *tps2*$^+$, *tps3*$^+$, and *tps4*$^+$ deletions on the generation times, strains PB003, MRP3, MRP4, MRP5, and MRP11 were grown overnight on either SD or YES media at 27°C (140 rpm). Cell densities were initially measured at 600 nm with a Shimadzu UV-160A spectrophotometer. Each strain was diluted with the appropriate medium to an OD of 0.05 and transferred to a microtiter plate which was incubated at 27°C in a Microtiter Shaker-Incubator (Dynatech). The OD at 620 nm was measured with an Anthos Labtec Instruments every hour.

Heat shock conditions

Log-phase cells (6×10^6 to 1×10^7 cells/ml) growing in glucose-containing liquid medium were submitted to a heat shock at 40°C in a water bath for the times indicated in the figure legends. Aliquots for trehalose, Tre6P, and enzyme determinations were taken before and after heat shock at the times indicated in the figure legends.

Osmotic shock conditions

Strains PB003, MRP3, MRP4, MRP5, and MRP11 were streaked on YES plates and incubated at 27°C for 24 hours. Subsequently, the plates were replica-plated on YES plates containing 0.6 or 1 M KCl and incubated at 30°C for 3 days.

Thermotolerance

Thermotolerance was determined following transfer of cells (1 ml of culture) to prewarmed glass tubes and submission to a challenging heat shock at 50°C for the times indicated in the figure legends. The challenging heat shock was either preceded, or not, by a preconditioning heat shock for 1 h at the temperature indicated in the figure legend (generally 40°C). Afterwards, samples were cooled on ice, diluted appropriately with sterile water, plated onto YES and incubated at 27°C for three days. Controls were left at 27°C for the same period of time as the samples submitted to the challenging heat shock. Colonies were counted and the percentage of survival was compared with the controls not subjected to the challenging heat shock (100%).

Enzyme assays

- **Preparation of permeabilized cells**

Aliquots of 20 to 40 ml of log-phase cells, or 5 ml of stationary-phase cells were filtered (Whatman GF/C) and washed 3 times with distilled water. For determination of the Tre6P synthase activity, samples were resuspended in 1 ml of 0.2 M Tricine (Na^+), pH 7.0, containing 0.05% Triton X-100, and immediately frozen in liquid nitrogen (Miozzari *et al.*, 1978). For determination of the Tre6P phosphatase activity, samples were resuspended in 1 ml 50 mM (K^+) phosphate buffer, pH 6.5, with 0.05% Triton X-100, and immediately frozen in liquid nitrogen. For determination of trehalase activity, samples were resuspended in 1 ml of 0.2 M Mes (K^+), pH 6.0, containing 0.05% Triton X-100 and immediately frozen in liquid nitrogen.

Before each assay, the samples were thawed (3min at 30°C), centrifuged for 10 min at 15000 rpm and 4°C, washed twice with the appropriate buffer without Triton X-100, and resuspended in 1 ml of the same buffer.

- **Preparation of crude extracts**

Aliquots of 40 to 60 ml of log-phase or 20 to 25 ml of stationary-phase cells were harvested at 3500 rpm for 5 min at 4°C. The pellets were washed twice with ice-cold water and once with 0.2 M Tricine, pH 7.0, for determination of Tre6P synthase activity, or once with 50 mM phosphate buffer, pH 6.5, for determination of Tre6P phosphatase, or once with 0.2 M MES, pH 6.0, for trehalase activity measurements. Cells were resuspendend in 0.5 ml of the corresponding buffer and disrupted by shaking vigorously during four to five 1 min periods with 1 min intervals on ice in the presence of protease inhibitors (1 tablet of Complete™ Protease Inhibitor Cocktail, Boehringer Mannheim, per 50 ml buffer) and 1.5 g of acid-washed glass beads. Disruption was confirmed under the light microscope. The extracts were centrifuged for 10 min at 14000 rpm (4°C) and the supernatants were used for enzymatic assays. For FPLC analysis, the disruption was performed in 150 mM NH_4CO_3 containing 1 mM EDTA, 1mM DTT, 1 mM PMSF, and 8 µg/ml pepstatin A, or in 0.2 M Tricine (Na^+), pH 7.0, containing 1 mM EDTA, 1mM DTT, 1 mM PMSF, and 8 µg/ml pepstatin A.

- **Trehalose-6P synthase and Trehalase**

Tre6P synthase activity was measured at 50°C using a coupled assay as described by Hottiger and coworkers (1987). Trehalase activity was measured at 30°C by incubation of cell extracts with 0.1 M trehalose and quantification of the glucose released in the supernatant using the GOD/POD kit (Boehringer Mannheim) as described by De Virgilio and coworkers (1991a).

- **Trehalose-6P phosphatase**

Tre6P phosphatase activity was assayed in a mixture containing 1.6 mM Tre6P (Sigma), 10 mM $MgCl_2$, 25 mM (K^+-) phosphate buffer, pH 6.5, and 50 µl extract in a total volume of 0.1 ml. After 10 minutes incubation at 32.5°C, the reaction was stopped in a boiling water bath for 3 min. The assay mixtures were then centrifuged (10 min at 14000 rpm) and the amount of trehalose formed was subsequently measured by HPLC analysis. Alternatively, the amount of trehalose was determined by a trehalase-mediated assay. To this end 25 µl of the supernatant were digested for 4 h at 37°C with 25 µl trehalase (porcine kidney, 0.27 U/ml, Sigma) and 50 µl digestion buffer (0.2 M NaOAc, 0.03 M $CaCl_2$, pH 5.7) The reaction was stopped by boiling

the tubes for 5 min. The glucose released by hydrolysis of trehalose was quantified using the GOD/POD kit (Boehringer Mannheim) as described by De Virgilio *et al.* (1991a).

Trehalose and Tre6P quantification

For trehalose and Tre6P determinations, 10 to 20 ml of log-phase cells and 1 to 2 ml of stationary-phase cells were filtered (Whatman GF/C), washed four times with distilled water, resuspended in 1 ml of water, and transferred to a boiling water bath for 10 min. After centrifugation (10 min at 15000 rpm and 4°C), trehalose and Tre6P were determined in the supernatants by HPLC analysis as described previously (De Virgilio *et al.*, 1993).

Protein quantification

Proteins were either determined by using a modified version of the Lowry method (Peterson, 1951), or by using the Bio-Rad protein assay with Bradford reagent and BSA as a standard.

Effect of pH and temperature on the activity of Tre6P phosphatase

To measure the effect of pH on the activity of Tre6P phosphatase, strain PB003 was grown to stationary phase on YES medium. Several 5ml-aliquots were filtered (Whatman GF/C), washed with distilled water, resuspended in 1ml of 50 mM (K^+-) phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, or 8.0) containing 0.05% Triton X-100, and immediately frozen in liquid nitrogen. The samples were then thawed (3min at 30°C), centrifuged (10 min at 15000 and 4°C), and washed twice with ice-cold 50 mM (K^+) phosphate buffer of the corresponding pH lacking Triton X-100. Tre6P phosphatase activity was assayed in a mixture containing 1.6 mM Tre6P (Sigma), 10 mM $MgCl_2$, 25 mM (K^+) phosphate buffer (pH 6.0, 6.5, 7.0, 7.5, or 8.0), and 50 μl extract in a total volume of 0.1 ml (De Virgilio *et al.*, 1993). The reaction mixture was incubated for 10 or 20 min at 30°C. The reaction was stopped by boiling for 3 min. Following centrifugation at 13000 rpm for 10 min the amount of trehalose formed in the supernatant was directly measured by high performance liquid chromatography (HPLC) analysis on a CarboPac PA-10 anion-exchange column using a DIONEX DX-300 Gradient chromatography system coupled with pulsed amperometric detection as described previously (De Virgilio *et al.*, 1993).

The effect of temperature on the enzyme activity was determined by preparation of the permeabilized cells in 50 mM (K^+) phosphate, pH 6.5, containing 0.05% Triton X-100. Tre6P phosphatase activity was assayed as described above incubating the mixture at different

temperatures from 20°C to 60°C for 10 min. Trehalose in the supernatant was quantified by HPLC analysis as described above.

FPLC experiments

Crude extracts obtained as described above were first applied to a Superose 6 HR 10/30 column (Pharmacia) for estimation of the complex size. Subsequent FPLC analyses were carried out using a Superdex 200 HR 10/30 FPLC column (Pharmacia). The extracts applied to the Superose 6 column were eluted with buffer containing 150 mM NH_4CO_3, 1 mM EDTA, 1 mM PMSF and a cocktail of protease inhibitors (1 tablet of Complete™ protease inhibitor cocktail, Boehringer Mannheim, per 50 ml buffer). The flow rate used was 12 ml/h and the volume of the fractions collected was 0.75 ml. The extracts applied to the Superdex 200 column were eluted with buffer containing 50 mM Tricine (Na^+), pH 7.0, 0.15 M NaCl, 1 mM EDTA, 1 mM PMSF and a cocktail of protease inhibitors (1 tablet of Complete™ protease inhibitor cocktail, Boehringer Mannheim per 50 ml buffer). The flow rate used was 15 ml/h, and the volume of the fractions collected was 0.4 ml. Tre6P synthase activities in the fractions were measured as described above. Tre6P phosphatase activity assays were performed as described above, except that the incubation time at 32.5°C was increased to 1 h. Protein concentrations were determined using the Bradford assay (see above).

Electrophoresis

Proteins were separated on one-dimensional SDS-polyacrylamide gels using the general technique outlined by Laemmli (1970). Electrophoresis was performed on a 10.5 cm slab gel (Bio-Rad) model number MV1-SC2. The stacking gel acrylamide concentration was 5% (0.33 ml of 30% acrylamide mix, 20 µl 10% w/v APS, 1.4 ml water, 20 µl 10% w/v SDS, 2 µl TEMED, and 0.5 ml 0.5 M Tris-HCl, pH 6.8) and the separating gel concentration was 12% (2.0 ml of 30% acrylamide mix, 50 µl 10% w/v APS, 1.6 ml water, 50 µl 10% w/v SDS, 2 µl TEMED, and 1.3 ml 1.5 M Tris-HCl, pH 8.8). Proteins were extracted from 1×10^8 cells by adding NaOH (0.25 M final concentration) and subsequent precipitation with trichloroacetic acid (TCA; 12% final concentration). The centrifuged pellets were washed three times with ice-cold acetone, resuspended in 0.1 M NaOH and mixed with an equal amount of 2 × sample buffer (Laemmli, 1970). After boiling for 3 min, the probes were centrifuged and equal amounts of proteins (10 µg) were loaded onto the SDS gel which was run at 180 V for 45 min. Low range molecular weight standards (Bio-Rad) were run on the same gel.

Autoradiography

SDS-gels were fixed in 40% methanol and 10% v/v acetic acid for 30 min before drying. The dried gels containing radioactivity were exposed on Kodak X-OMAT film at –70°C for 1 day. The exposed films were then developed (Kodak liquid X-ray developer), rinsed, and fixed (Kodak liquid X-ray fixer).

CHAPTER 3

TREHALOSE SYNTHESIS IS IMPORTANT FOR THE ACQUISITION OF THERMOTOLERANCE IN *SCHIZOSACCHAROMYCES POMBE*

Maria J.S.Ribeiro, Anke Reinders, Thomas Boller, Andres Wiemken, and Claudio De Virgilio

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Trehalose synthesis is important for the acquisition of thermotolerance in *Schizosaccharomyces pombe*

Maria J. S. Ribeiro, Anke Reinders, Thomas Boller, Andres Wiemken and Claudio De Virgilio*
Botanisches Institut der Universität, Hebelstrasse 1, CH-4056 Basel, Switzerland.

Summary

Yeast cells show an adaptive response to a mild heat shock, resulting in thermotolerance acquisition. This is accompanied by induction of heat-shock protein (hsp) synthesis and rapid accumulation of trehalose. Genetic approaches to determine the specific role of trehalose in heat-induced thermotolerance in *Saccharomyces cerevisiae* have been hampered by the finding that deletion of *TPS1*, the gene encoding trehalose-6-phosphate synthase, causes a variety of pleiotropic effects, including inability to grow on glucose-containing media. Here, we have studied a *tps1* mutant of the yeast *Schizosaccharomyces pombe* that reportedly has no such growth defects. We show that *tps1* mutants have a serious defect in heat shock-induced acquisition of thermotolerance if conditioned at highly elevated temperatures (40–42.5°C), which, in wild-type cells, prevent hsp but not trehalose synthesis. In contrast, hsp synthesis appears to become particularly important under conditions in which trehalose synthesis is either absent (in *tps1* mutant strains) or not fully induced (conditioning at moderately elevated temperatures, i.e. 35°C). In addition, *pka1* mutants deficient in cAMP-dependent protein kinase were examined. Unconditioned *pka1* cells had low levels of trehalose but a high basal level of thermotolerance. It was found that *pka1* mutant cells, contrary to wild-type cells, accumulated large amounts of trehalose, even during a 50°C treatment. *pka1 tps1* double mutants lacked this ability and showed reduced intrinsic thermotolerance, indicating a particularly important role for trehalose synthesis, which takes place during the challenging heat shock.

Introduction

Acquired thermotolerance of cells or organisms has been defined as a transient increase in resistance to heat induced by exposure to elevated temperatures or by various other stress-related treatments (Lindquist, 1986). During a conditioning heat shock, synthesis of a small set of highly conserved proteins, the heat-shock proteins (hsps), is dramatically increased. Some hsps have been shown to play a vital role in unstressed cells by associating with newly synthesized proteins (Beckman *et al.*, 1990), assisting in their folding (Ang *et al.*, 1991; Gething and Sambrook, 1992) and allowing these proteins to cross membranes (Cheng *et al.*, 1989; Kang *et al.*, 1990). However, analysis of hsp function in *Saccharomyces cerevisiae* has revealed that only a limited number of hsps have appreciable effects on the induction of thermotolerance during a conditioning heat shock (for a review, see Piper, 1993). In particular, Hsp104, Hsp70, Ctt1 and Ubi4 have been found to contribute to the acquisition of thermotolerance by promoting the resolubilization of aggregated proteins (Parsell *et al.*, 1994), the sequestering and refolding of denatured proteins (for a review, see Mager and Ferreira, 1993), the prevention of oxidative damage (Wieser *et al.*, 1991) and the proteolytic elimination of toxic proteins (Finley *et al.*, 1987) respectively.

An additional element of the adaptive response of yeast to elevated temperature is the accumulation of the non-reducing disaccharide trehalose (Attfield, 1987; Hottiger *et al.*, 1987; Ribeiro *et al.*, 1994), which is thought to increase the cell's thermotolerance by enhancing the thermal stability of proteins and reducing the heat-induced formation of protein aggregates (Crowe *et al.*, 1987; Hottiger *et al.*, 1994; for a review, see Wiemken, 1990). Synthesis of trehalose is mediated by the trehalose-6-phosphate (Tre6P) synthase–phosphatase complex, which, in *S. cerevisiae*, consists of at least four subunits carrying either Tre6P synthase (Tps1), Tre6P phosphatase (Tps2) or regulatory activities (Tps3 and Tsl1; Bell *et al.*, 1992; De Virgilio *et al.*, 1993; Vuorio *et al.*, 1993; Reinders *et al.*, 1997). In accordance with the observation that heat-induced accumulation of trehalose is partially dependent on protein synthesis in *S. cerevisiae* (De Virgilio *et al.*, 1991), both catalytic subunits (Tps1 and Tps2) have been shown to be hsps, and expression of all four genes encoding the subunits of the Tre6P synthase complex (*TPS1*, *TPS2*, *TPS3* and *TSL1*) has been found to be dramatically enhanced under heat-shock conditions (De Virgilio *et al.*, 1993; Winderickx *et al.*, 1996).

Received 7 May, 1997; revised 14 June, 1997; accepted 16 June, 1997. *For correspondence. E-mail devirgilioc@ubaclu.unibas.ch; Tel. (61) 267 2311; Fax (61) 267 2330.

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Degradation of cytosolic trehalose is mainly mediated by the neutral trehalase (Londesborough and Varimo, 1984), which has attracted considerable attention because its potential activation by the cAMP-dependent protein kinase (cAPK) allows monitoring of the activation status of the Ras/cAMP pathway (for a review, see Thevelein, 1992). Interestingly, cAPK activity was found to exert a dual role in the regulation of trehalose levels in yeast, namely by post-translational activation of the neutral trehalase and by transcriptional repression of *TPS* genes via a novel *cis*-acting stress-responsive element (STRE), which is independent of the heat-shock factor (HSF; Jakobsen and Pelham, 1988; Sorger and Pelham, 1988). This element, which was shown to be negatively regulated by cAPK, mediates the heat-induced expression of several hsps, including *HSP12* (Varela *et al.*, 1995), *CTT1* (Marchler *et al.*, 1993; Schüller *et al.*, 1994), *SSA3* (Boorstein and Craig, 1990), *TPS2* (Gounalaki and Thireos, 1994) and possibly also *TPS1*, *TPS3* and *TSL1* (Varela *et al.*, 1995). Accordingly, studies using *S. cerevisiae* mutants of the Ras/cAMP pathway have shown that low cAPK activity correlates with high levels of *HSP* expression, trehalose synthesis and thermotolerance even in the absence of an inducing heat stress, whereas high cAPK activity reduces the levels of *HSP* expression, trehalose synthesis and thermotolerance even under heat-shock conditions (Hottiger *et al.*, 1989; Iida, 1988; Shin *et al.*, 1987).

Genetic approaches to determine the specific role of trehalose for the heat-induced thermotolerance in wild-type cells (De Virgilio *et al.*, 1994), as well as for the intrinsic thermotolerance in mutants with attenuated cAPK activity, have been hampered by the finding that deletion of *TPS1*, the gene encoding Tre6P synthase, causes a variety of pleiotropic effects in *S. cerevisiae*. These effects include the inability to grow on rapidly fermentable sugars, such as glucose and fructose, and a deficiency in many glucose-induced regulatory mechanisms, indicating that trehalose biosynthesis, in addition to its role in the stress response, may also be involved in the general control of glycolysis (for a review, see Thevelein and Hohmann, 1995). Surprisingly, a *TPS1* homologue in the distantly related fission yeast, *Schizosaccharomyces pombe*, while also being essential for trehalose synthesis, was reported to be completely dispensable for growth on glucose (Blázquez *et al.*, 1994), rendering this yeast species a much more attractive model system for studies of the role of trehalose in the acquisition of thermotolerance. Moreover, several elements of the cAPK pathway in *S. pombe*, including an adenylate cyclase (encoded by *cyr1*$^+$; Yamawaki-Kataoka *et al.*, 1989; Young *et al.*, 1989), a regulatory subunit of cAPK (encoded by *cgs1*$^+$; DeVoti *et al.*, 1991) and a cAPK (encoded by *pka1*$^+$; Maeda *et al.*, 1994), have recently been identified. The role of the cAMP signalling pathway in *S. pombe* is pleiotropic, as in *S. cerevisiae*, and involves the regulation of cell cycle events, sexual differentiation and gene expression (DeVoti *et al.*, 1991; Hoffman and Winston, 1991; Mochizuki and Yamamoto, 1992). Moreover, it has been suggested that cAMP-dependent phosphorylation may also be involved in the post-translational regulation of neutral trehalase activity and in the modulation of the heat-shock response (Soto *et al.*, 1995; Fernández *et al.*, 1997).

Here, we report that deletion of *tps1*$^+$ in *S. pombe* seriously reduces the heat shock-induced acquisition of thermotolerance, especially if the conditioning heat shock is performed at very high temperatures (40–42.5°C), which prevent hsp synthesis but not trehalose accumulation in wild-type cells. We have also shown that general synthesis of hsps becomes particularly important under conditions in which trehalose synthesis is either absent (in *tps1* mutant strains) or not fully induced (in cells conditioned at 35°C). Moreover, even though an unconditioned *pka1* mutant cell was found to have low levels of trehalose, it had a high intrinsic level of thermotolerance, which was strongly dependent on the presence of a functional *tps1*$^+$ gene, indicating a particularly important role for the trehalose synthesis taking place during the challenging heat shock.

Results

Temperature limit for protein synthesis in S. pombe *defines the upper limit for a role of hsps in acquired thermotolerance*

To assess the relative importance of general protein synthesis, and the role of hsps in particular, in the acquisition of thermotolerance, we first determined the upper limit for (heat-shock) protein synthesis in *S. pombe*. The wild-type strain, PB003, showed sustained protein synthesis, as detected by the incorporation of L-[^{35}S]-methionine into TCA-precipitable material and visualization of the radiolabelled proteins following gel electrophoresis and autoradiography at 27°C, 35°C and 37.5°C, with the typical pattern of hsps at 35°C and 37.5°C (Fig. 1). At 40°C, protein synthesis was already dramatically reduced (13.1% when compared with the 35°C treatment) and at temperatures above 42.5°C virtually no protein synthesis could be detected. These results clearly indicate that hsp synthesis may only contribute significantly to the acquisition of thermotolerance in wild-type cells subjected to a conditioning heat shock at temperatures below 40°C.

Conditioning at moderate temperatures reveals an important role for hsp synthesis, but not of trehalose synthesis, for acquired thermotolerance

To study the role of trehalose and hsp synthesis for acquired thermotolerance, wild-type cells (PB003) were

Fig. 1. Protein synthesis in *S. pombe* strain PB003 (wild type) during a conditioning heat shock at various temperatures. Cultures were labelled at the temperatures indicated by the addition of L-[^{35}S]-methionine for 1 h. Radiolabelled proteins were extracted and resolved by gel electrophoresis as described in *Experimental procedures*. The positions of molecular weight standards are indicated on the right (in kDa), and those of the hsps on the left.

exposed to 50°C for 8 min, either after growth at 27°C (unconditioned cells) or following a 1-h conditioning heat shock at various temperatures (35°C, 37.5°C, 40°C, 42.5°C, 45°C and 47.5°C; conditioned cells) in the presence or absence of cycloheximide (100 μg ml^{-1}). As shown in Fig. 2, unconditioned cells were extremely heat sensitive (less than 0.3% survivors after 8 min at 50°C; Fig. 2A) and did not contain appreciable amounts of trehalose (Fig. 2B), following a 1-h incubation at 27°C in the presence or absence of cycloheximide. However, conditioning of the cells for 1 h at 35°C, 37.5°C and 40°C did result in low (0.03 g g^{-1} protein), moderate (0.19 g g^{-1} protein) and high (0.42 g g^{-1} protein) accumulation of trehalose, respectively, while the levels of acquired thermotolerance achieved during conditioning at these three temperatures did not significantly differ from each other (about 55% survivors after 8 min at 50°C). These results suggest that trehalose accumulation may play a subordinate role in thermotolerance acquisition if the conditioning heat shock is carried out at 35°C, but may become increasingly important at higher temperatures (i.e. between 37.5°C and 42.5°C). In contrast, hsp synthesis may be particularly important during conditioning at a temperature of 35°C. In accordance with this suggestion, inhibition of protein synthesis (by the addition of cycloheximide before the conditioning heat shock) did significantly reduce the level of acquired thermotolerance (by about 58%) of cells conditioned at 35°C, but not of cells conditioned at 37.5°C or at higher temperatures (Fig. 2A). This is also in agreement with the above finding that wild-type cells are unable to synthesize significant amounts of proteins at temperatures higher than 40°C (Fig. 1), and that hsp synthesis may therefore not contribute to the acquisition of thermotolerance under these conditions. In accordance with previously published results (De Virgilio *et al.*, 1990), cycloheximide did not affect the cell's ability to accumulate trehalose at any temperature tested, indicating that trehalose synthesis is mainly regulated at a post-translational level under heat-shock conditions. Cycloheximide addition also did not strongly affect the survival rate of cells during the conditioning heat shock (Fig. 2C). In control experiments, cycloheximide inhibited the incorporation of L-[^{35}S]-methionine into TCA-precipitable material by more than 90% in wild-type cells (strain PB003; data not shown). Except for a very small fraction of the cells (< 1.0%), 23% of which were able to survive the challenging heat shock at 50°C, the majority of the cells were unable to survive a 1-h conditioning heat shock at temperatures at or above 45°C, irrespective of the presence or absence of cycloheximide (Fig. 2C). Thus, a temperature of around 45°C constitutes the absolute physiological temperature limit within which *S. pombe* cells can acquire thermotolerance when pregrown at 27°C.

Conditioning at high temperatures reveals an important role for trehalose synthesis, but not for hsp synthesis, in acquired thermotolerance

Since the results in Fig. 2 suggested a potentially important role for trehalose synthesis in the acquisition of thermotolerance during a 1-h conditioning heat shock at temperatures between 40°C and 42.5°C, we decided to study the temperature-dependent induction of thermotolerance in a mutant defective in the gene coding for the Tre6P synthase (*tps1*$^{+}$). Unconditioned *tps1* mutant cells were as similarly heat sensitive as wild-type cells (less than 0.3% survivors after 8 min at 50°C), after a 1-h incubation at 27°C in the presence or absence of cycloheximide (Fig. 3A). While completely deficient for trehalose synthesis at any temperature tested (Fig. 3B), *tps1* mutant cells were able to acquire relatively high levels of thermotolerance during a heat shock at moderate temperatures (35°C and 37.5°C; about 70% survivors after 8 min at 50°C), but their ability to acquire thermotolerance was reduced if conditioned at 40°C or 42.5°C (Fig. 3A), when compared with the isogenic wild-type cells (Fig. 2A). Consequently, trehalose synthesis is an important factor for the acquisition of thermotolerance during conditioning at high temperatures between 40°C and 42.5°C. As in wild-type cells, the addition of cycloheximide before

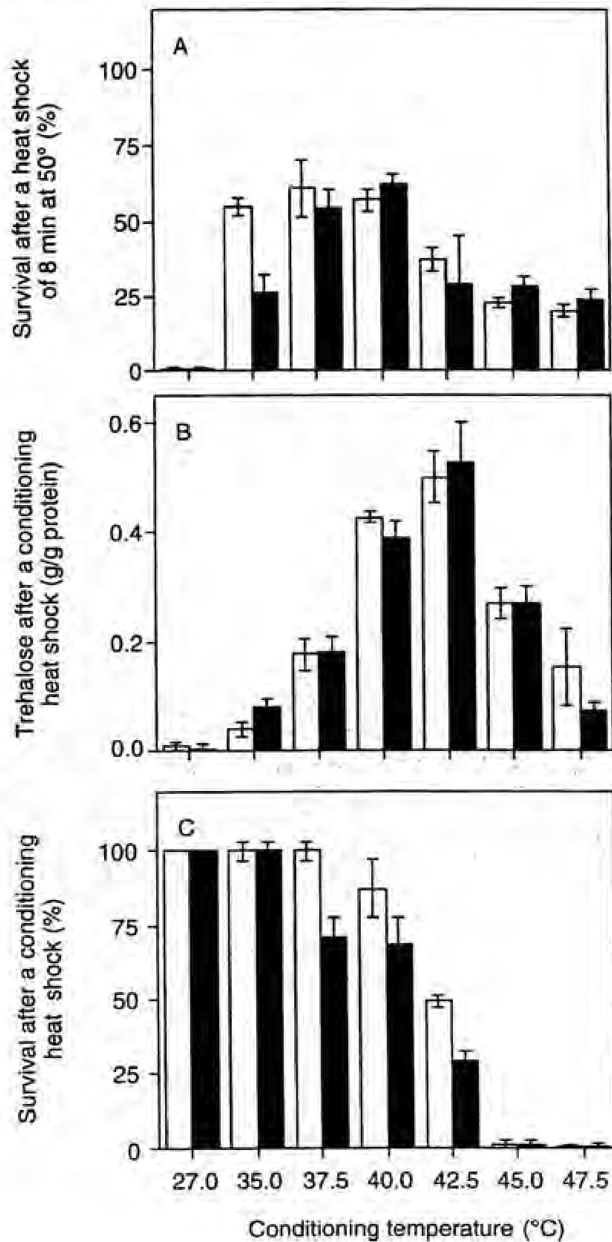

Fig. 2. Thermotolerance (A), trehalose levels (B) and survival (C) of *S. pombe* strain PB003 (wild type) after a conditioning heat shock at different temperatures in the absence or presence of cycloheximide. Cultures of wild-type cells were grown to early exponential phase ($<4\times10^6$ cells ml^{-1}) on YES medium and subjected to a conditioning heat shock for 1 h at the temperatures indicated in the absence (open bars) or presence (shaded bars) of cycloheximide (100 $\mu g\ ml^{-1}$) added 5 min before initiation of the heat shock. Thermotolerance (A) was measured as the survival following a subsequent incubation for 8 min at 50°C. Trehalose levels (B) were determined after a 1-h conditioning heat shock. Survival after the conditioning heat shock (C) was determined as a percentage of the 27°C control culture. The error bar for each sample represents the SEM of at least three independent measurements.

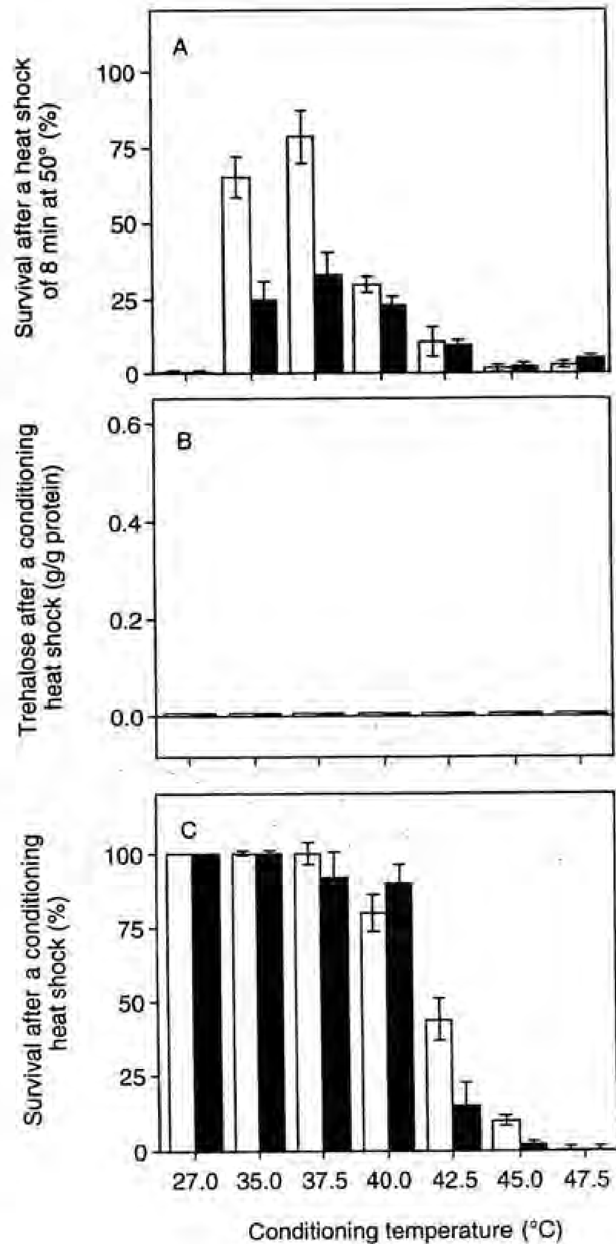

Fig. 3. Thermotolerance (A), trehalose levels (B) and survival (C) of *S. pombe* strain PBL-17 (*tps1::LEU2*) after a conditioning heat shock at different temperatures in the absence or presence of cycloheximide. Cultures of *tps1* mutant cells were grown to early exponential phase ($<4\times10^6$ cells ml^{-1}) on YES medium and subjected to a conditioning heat shock for 1 h at the temperatures indicated in the absence (open bars) or presence (shaded bars) of cycloheximide (100 $\mu g\ ml^{-1}$) added 5 min before initiation of the heat shock. Thermotolerance (A) was measured as the survival following a subsequent incubation for 8 min at 50°C. Trehalose levels (B) were determined after a 1-h conditioning heat shock. All trehalose values were found to be below the detection limit indicated by the bars ($<0.001\ g\ g^{-1}$ protein). Survival after the conditioning heat shock (C) was determined as a percentage of the 27°C control culture. The error bar for each sample represents the SEM of at least three independent measurements.

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Table 1. Trehalose content of *S. pombe* wild-type, *pka1*, *tps1* and *pka1 tps1* strains before and after a conditioning heat shock and after a challenging heat shock.

	Trehalose ($g\,g^{-1}$ protein)			
Relevant genotype	27°C	1 h at 40°C	27°C + 20 min at 50°C	1 h at 40°C + 20 min at 50°C
pka1⁺ tps1⁺	0.001	0.423	0.001	0.320
pka1::ura4⁺ tps1⁺	0.013	0.784	0.280	1.010
pka1⁺ tps1::LEU2	<0.001	<0.001	<0.001	<0.001
pka1::ura4⁺ tps1::LEU2	<0.001	<0.001	<0.001	<0.001

Cells were grown at 27°C to early exponential phase ($<4\times10^6$ cells ml^{-1}) on YES medium and assayed for their trehalose content either before or after a 1-h conditioning heat shock at 40°C. Unconditioned and conditioned cells were also tested for their ability to accumulate trehalose during the 20 min of the challenging heat shock at 50°C. Experiments were repeated at least three times, also using independent strains with the same relevant genotype (i.e. PB003, CHP421, MRP-7C and MRP-9C for wild-type strains; CHP453 and MRP-7A for *pka1::ura4⁺ tps1⁺* strains; PBL-17 and MRP-7B for *pka1⁺ tps1::LEU2* strains; and MRP-3A, MRP-7D and MRP-9D for *pka1::ura4⁺ tps1::LEU2* strains). Accordingly, values represent the means of at least three independent cultures and at least two independent strains. The SEMs were less than 10% of the corresponding means in each case.

a conditioning heat shock at 40°C or at higher temperatures did not significantly affect the thermotolerance levels of *tps1* mutant cells when compared with the cycloheximide-untreated cells (Fig. 3A). This is in agreement with our finding that general protein synthesis rates at 40°C were also dramatically reduced in *tps1* mutant cells (>86% reduction when compared with the 35°C treatment; data not shown) and that protein synthesis may therefore not be important for thermotolerance acquisition under these conditions. However, *tps1* mutant cells were impaired for their ability to acquire thermotolerance if cycloheximide was added during a conditioning heat shock at 35°C, and, unlike wild-type cells, also at 37.5°C. This indicates that protein synthesis (and especially hsp synthesis) is particularly important if trehalose synthesis is absent (i.e. conditioning at temperatures between 35°C and 37.5°C in a *tps1* mutant). In control experiments, cycloheximide inhibited the incorporation of L-[^{35}S]-methionine into TCA-precipitable material by more than 90% in *tps1* mutant cells (strain PBL-17; data not shown). As in wild-type cells, we detected a high sensitivity of *tps1* cells towards conditioning temperatures above 42.5°C, which was also barely altered by the presence of cycloheximide.

pka1 *mutant cells have a high level of basal thermotolerance that is partially dependent on the presence of* tps1⁺

Studies using *S. cerevisiae* mutants of the Ras/cAMP pathway have shown that mutants with low cAPK activities have high levels of thermotolerance, even without a conditioning heat shock (Shin *et al.*, 1987; Hottiger *et al.*, 1989). While the presence of at least one of the three *TPK* genes encoding the catalytic subunits of cAPK is essential in *S. cerevisiae*, the single *pka1⁺* gene in *S. pombe* was reported to be dispensable for growth (Maeda *et al.*, 1994). To study the role of the *pka1⁺* gene in basal and induced thermotolerance and in trehalose metabolism, *pka1* mutant and wild-type cells were exposed to a temperature of 50°C, either after growth at 27°C (unconditioned cells) or following a 1-h heat shock at 40°C (conditioned cells). Even though both unconditioned wild-type and *pka1* mutant cells did not contain appreciable amounts of trehalose (Table 1), the *pka1* mutant cells were extremely thermotolerant (about 10^4-fold more survivors after 20 min at 50°C) when compared with the wild-type cells (Fig. 4A). These results are consistent with data reported by Fernández *et al.* (1997) and may, at first glance, indicate that trehalose synthesis does not contribute to the high basal thermotolerance in *pka1* mutants. Surprisingly, however, we found that *pka1* mutant cells, in contrast to wild-type cells, were rapidly able to accumulate large amounts of trehalose during the 20 min of the challenging heat shock at 50°C (Table 1; 0.150 $g\,g^{-1}$ protein and 0.279 $g\,g^{-1}$ protein after 5 min and 10 min at 50°C respectively), suggesting that trehalose synthesis may in fact be important under these conditions. To test this assumption further, we constructed *pka1 tps1* double mutants and analysed their basal trehalose and thermotolerance levels. As expected, the *pka1 tps1* double mutants were completely defective for trehalose synthesis at 27°C or following an incubation for 20 min at 50°C (Table 1). In addition, thermotolerance levels of unconditioned *pka1 tps1* double mutants were found to be significantly lower than those of unconditioned *pka1* single mutants (about 40-fold fewer survivors after 20 min at 50°C; Fig. 4A), yet significantly higher than those of wild-type and *tps1* single mutant cells (about 200-fold more survivors after 20 min at 50°C), indicating that trehalose synthesis is an important, but not the only, element of the basal thermotolerance of *pka1* mutant cells.

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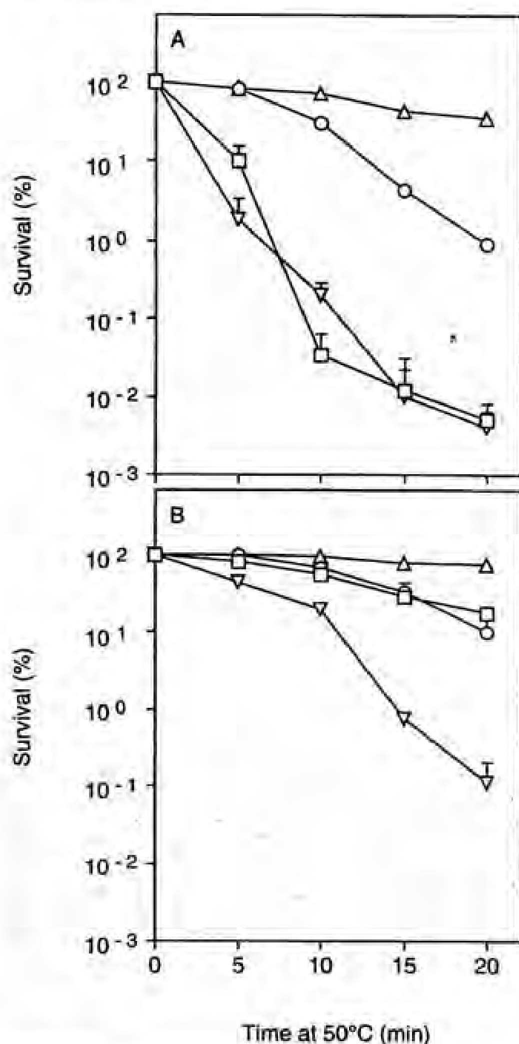

Fig. 4. Thermotolerance of *S. pombe* wild-type, *pka1*, *tps1* and *pka1 tps1* mutant strains before (A) and after (B) a conditioning heat shock. Cells were grown to early exponential phase ($<4\times10^6$ cells ml^{-1}) on YES medium and subjected to a conditioning heat shock for 1 h at 40°C. Thermotolerance of wild-type (□) and *pka1* (Δ), *tps1* (∇) and *pka1 tps1* (○) mutant strains before or after the conditioning heat treatment was measured as the survival following incubation at 50°C for the times indicated. Experiments were repeated at least three times with two (*pka1*$^+$ *tps1::LEU2*, PBL-17 and MRP-7B; *pka1::ura4*$^+$ *tps1*$^+$, CHP453 and MRP-7A), three (*pka1::ura4*$^+$ *tps1::LEU2*, MRP-3A, MRP-7D and MRP-9D) or four (wild-type: PB003, CHP421, MRP-7C and MRP-9C) independent strains with the same relevant genotype. Accordingly, the error bars, some of which are smaller than the symbols, represent the SEM of at least three independent measurements.

Heat-induced acquisition of thermotolerance in wild-type and pka1 *mutant cells is partially dependent on the presence of* tps1$^+$

A conditioning heat shock for 1 h at 40°C not only induced significant thermotolerance levels in wild-type cells, but also led to a further increase of the basal thermotolerance in *pka1* mutant cells. Accordingly, conditioned *pka1* mutant cells had almost fourfold higher survival rates (74%) than wild-type cells (18%) after a challenging heat shock (20 min at 50°C). Remarkably, compared with wild-type cells, *pka1* mutant cells also had a considerably (twofold to threefold) higher capacity to accumulate trehalose during the conditioning heat shock (including the following challenging heat shock; Table 1), which coincided with twofold to threefold higher Tre6P synthase activity in these cells (Table 2). Deletion of *tps1* in either a wild-type or a *pka1* mutant background not only caused inability to synthesize trehalose (Table 1), but also significantly reduced the capability of the cells to acquire thermotolerance during a conditioning heat shock (about 165-fold reduction in the wild-type and sevenfold reduction in the *pka1* background after 20 min at 50°C), demonstrating also the particularly important role of trehalose synthesis in the full acquisition of heat-induced thermotolerance. However, as in unconditioned cells, the conditioned *pka1 tps1* double mutant cells acquired much higher thermotolerance levels than the single *tps1* mutant cells (about 100-fold more survivors after 20 min at 50°C), indicating the existence of additional *tps1*-independent elements of thermotolerance that are under the negative control of cAPK.

Different effects of pka1 *and* tps1 *mutations on enzymes of trehalose metabolism*

To study a possible involvement of cAPK in the activation/inactivation of the enzymes of trehalose metabolism in *S. pombe*, we determined the activities of neutral trehalase and Tre6P synthase in wild-type and *pka1* mutant cells before and after a 1-h heat shock at 40°C (Table 2). In accordance with our own previously reported results (De Virgilio *et al.*, 1990), both neutral trehalase and Tre6P synthase activities in wild-type cells were found to increase during a heat shock at 40°C. Since these activity increases were previously shown to be unaffected by the addition of cycloheximide (De Virgilio *et al.*, 1990), and since general protein synthesis rates at 40°C are dramatically reduced in *S. pombe* wild-type cells (Fig. 1), these activity increases are most probably caused by post-translational activation mechanisms and not by *de novo* synthesis of the corresponding enzymes. Interestingly, the heat-induced activity increases of both neutral trehalase and Tre6P synthase were also found in *pka1* mutant cells. Since general protein synthesis rates at 40°C were also dramatically reduced in *pka1* strains (>85% reduction when compared with the 35°C treatment; data not shown), both enzymes are likely to be activated by cAPK-independent post-translational mechanisms. This conclusion is in accordance with a previous report showing that the heat shock-induced activation of neutral trehalase and synthesis of trehalose are not affected by the prior addition of cycloheximide in an *S. pombe pka1* mutant (Fernández *et al.*,

Table 2. Trehalose-6-phosphate synthase and trehalase activities in *S. pombe* wild-type, *pka1*, *tps1* and *pka1 tps1* strains before and after a conditioning heat shock.

	Tre6P synthase (μkat g^{-1} protein)		Trehalase (μkat g^{-1} protein)	
Relevant genotype	27°C	40°C	27°C	40°C
pka1$^+$ tps1$^+$	0.71 ± 0.07	1.41 ± 0.31	0.39 ± 0.09	1.11 ± 0.23
pka1::ura4$^+$ tps1$^+$	2.42 ± 0.17	3.37 ± 0.08	0.28 ± 0.06	0.97 ± 0.17
pka1$^+$ tps1::LEU2	0.03 ± 0.02	0.03 ± 0.02	0.38 ± 0.05	0.13 ± 0.03
pka1::ura4$^+$ tps1::LEU2	0.06 ± 0.03	0.02 ± 0.01	0.14 ± 0.03	0.15 ± 0.01

Cells were grown to early exponential phase (<4×10^6 cells ml^{-1}) on YES medium and assayed for their trehalose-6-phosphate synthase and trehalase activities either before or after a conditioning heat shock of 1 h at 40°C. Experiments were repeated at least three times, also using independent strains with the same relevant genotype (i.e. CHP421, MRP-7C and MRP-9C for wild-type strains; CHP453 for a *pka1::ura4$^+$ tps1$^+$* strain; PBL-17 and MRP-7B for *pka1$^+$ tps1::LEU2* strains; and MRP-3A, MRP-7D and MRP-9D for *pka1::ura4$^+$ tps1::LEU2* strains). Accordingly, values are means ± the SEM of at least three independent cultures and at least two independent strains.

1997). The same report, however, claims that the heat-induced activation of trehalase and the synthesis of trehalose are both significantly reduced in wild-type cells by the addition of cycloheximide before the conditioning heat shock, which is clearly at variance with our former results (De Virgilio *et al.*, 1990) and the conclusions of the present study (see above). At present, we do not know the reasons for these discrepancies. However, our finding that trehalase activation and trehalose accumulation took place in wild-type cells even under heat-shock conditions that allow only residual protein synthesis (i.e. 1 h at 40°C) strongly indicates that these processes are not dependent on *de novo* protein synthesis and are mainly regulated at a post-translational level.

In this context, it is interesting that the putative gene product of a recently identified multicopy suppressor of a *pka1* mutation, namely *sck1$^+$*, was reported to display a high degree of homology to the catalytic domain of cAPK (Jin *et al.*, 1995). Analysis of a *pka1 sck1* double mutant revealed that both gene products may share a redundant role during the exit from stationary phase and during spore germination, which may be associated with the post-translational activation of neutral trehalase. It will, therefore, be interesting to determine whether Sck1 may indeed be involved in trehalase activation under these conditions and whether it could also be involved in the alternative cAMP-independent pathway for heat shock-induced activation of neutral trehalase.

The observation that unconditioned *pka1* mutant cells had threefold to fourfold increased levels of Tre6P synthase activity indicates that cAPK may, as in *S. cerevisiae* (Winderickx *et al.*, 1996), have a role in the negative regulation of *tps1$^+$* transcription. As expected, *tps1$^+$* was found to be essential for Tre6P synthase activity in wild-type and *pka1* mutant cells. Surprisingly, however, *tps1* mutants, as well as *pka1 tps1* double mutants, were also found to be defective for the heat-induced activation of neutral trehalase, indicating that the absence of trehalose may activate feed-back mechanisms preventing the unnecessary activation of neutral trehalase (Table 2).

Discussion

Upon exposure to a mild heat shock, yeast cells acquire thermotolerance, i.e. the capacity to survive a subsequent severe heat stress that would be lethal in the absence of the conditioning heat shock. There is substantial evidence from studies in *S. cerevisiae* that both hsp and trehalose synthesis may be important elements in acquired thermotolerance (for a review, see Piper, 1993). One central goal of the present study was to determine the relative contribution of trehalose synthesis to the acquisition of thermotolerance in the fission yeast *S. pombe*. Two observations make this yeast a particularly interesting object for studies of trehalose metabolism. First, the *S. pombe TPS1* homologue has been reported to be essential for Tre6P synthesis, but, unlike in *S. cerevisiae*, to be dispensable for growth on glucose (Blázquez *et al.*, 1994). Analysis of a *tps1* mutation in *S. pombe* may therefore allow a more specific assessment of the role of trehalose for the acquisition of thermotolerance than in *S. cerevisiae* (De Virgilio *et al.*, 1994). Second, using the protein translation inhibitor, cycloheximide, and temperature-sensitive mutants for protein synthesis, we have previously shown that the heat-induced increase in trehalose accumulation is partly dependent on protein synthesis in *S. cerevisiae*, whereas it is completely independent of protein synthesis in *S. pombe* (De Virgilio *et al.*, 1990; 1991). Cycloheximide studies in *S. pombe* wild-type and *tps1* mutant cells may, therefore, allow a dissection of the specific roles of (heat-shock) protein and trehalose synthesis in the acquisition of thermotolerance.

One main conclusion of our studies is that the relative importance of hsp and trehalose synthesis in the acquisition of thermotolerance is strongly dependent on the temperature during the conditioning heat shock. The underlying

physiological reason for this is most probably the fact that the two adaptive responses have different temperature optima for maximal induction. Accordingly, in cells pregrown at 27°C, maximal induction of hsp synthesis is achieved by a heat shock at a temperature of around 35°C, and trehalose synthesis is maximally induced by a heat shock at a temperature of around 42.5°C. While conditioning heat shocks at temperatures between these maxima were found to result in partial activation of both responses, little overlap between the two responses was found at either 35°C or 42.5°C. It is therefore not surprising that cycloheximide treatment during a conditioning heat shock at 35°C revealed an important role for (heat-shock) protein synthesis, but not for trehalose synthesis, in the acquisition of thermotolerance. Similarly, it is not surprising that the studies of *tps1*$^+$ mutants revealed an important role for trehalose synthesis, but not for hsp synthesis, in the acquisition of thermotolerance if the conditioning heat shock was performed at 42.5°C (or 40°C). It is interesting to note, however, that, even in the absence of both trehalose synthesis and hsp synthesis (by the addition of cycloheximide), *tps1* mutant cells acquired significant levels of thermotolerance during a conditioning heat shock at 40°C, indicating the existence of additional post-translationally activated thermotolerance factors.

Previously, we have suggested that the function of trehalose and hsps may be complementary in that trehalose acts to prevent heat-induced inactivation and aggregation of proteins (protection), while hsps act in the resolubilization and refolding of aggregated and denatured proteins (repair) respectively (De Virgilio *et al.*, 1991; Hottiger *et al.*, 1994 and references therein). In accordance with such a model, it has recently been reported that trehalose and Hsp104 may have synergistic effects for thermotolerance in *S. cerevisiae* (Elliott *et al.*, 1996). It would therefore be sensible to assume that hsp synthesis becomes particularly important if trehalose synthesis is absent. Interestingly, and in accordance with this assumption, we have found in this study that the acquisition of thermotolerance in *tps1* mutant cells conditioned at 37.5°C was particularly sensitive to cycloheximide addition before the conditioning heat shock.

A second important conclusion of this study is that rapid adaptive responses during the challenging heat shock may contribute significantly to the levels of thermotolerance. This conclusion is based on our surprising finding that unconditioned *pka1* mutants, in contrast to wild-type cells, were able to react to the challenging heat shock at 50°C with rapid synthesis of large amounts of trehalose. Moreover, the analysis of unconditioned *pka1 tps1* double mutants revealed that this rapid adaptive response is important for the high basal thermotolerance levels of *pka1* cells. Thus, future studies of acquired thermotolerance should undoubtedly also take into account the fact that certain cells may have the ability for rapid post-translational activation of protective mechanisms during the challenging heat shock.

Studies in *S. cerevisiae* have revealed that the transcription of a set of genes, including *TPS1* and other heat shock genes, is under the negative control of cAPK and that mutants with attenuated cAPK activity have high constitutive levels of the corresponding transcripts and/or proteins (Boorstein and Craig, 1990; Marchler *et al.*, 1993; Schüller *et al.*, 1994; Varela *et al.*, 1995; Winderickx *et al.*, 1996). If cAPK has a similar role in *S. pombe*, *pka1* mutants would be expected to have high constitutive levels of hsps, including also Tps1 (Blázquez *et al.*, 1994; Degols *et al.*, 1996). In accordance with this assumption, it has recently been reported that the level of *tps1*$^+$ mRNA in an unconditioned *pka1* mutant is about threefold higher than in the corresponding control strain (Fernández *et al.*, 1997). This is also consistent with our finding that Tre6P synthase activity is enhanced about threefold in an unconditioned *pka1* mutant when compared with its wild-type parent. Interestingly, it has recently been suggested that *tps1*$^+$ transcription is positively regulated in response to heat shock through the Wis1–Spc1 mitogen-activated protein kinase (MAPK) pathway (Degols *et al.*, 1996). This raises the possibility that two different pathways, the Wis1–Spc1 MAPK pathway and the cAPK signalling pathway, may act antagonistically to control the transcription of *tps1*$^+$. This presumably dual regulation of *tps1*$^+$ transcription by two different pathways may provide a basis for further analyses addressing the question of how these pathways interact to control elements of the heat-shock response.

Unconditioned *pka1 tps1* double mutants, despite their reduced basal thermotolerance levels when compared with *pka1* single mutants, were still found to be quite thermotolerant when compared with wild-type cells. As discussed above, it is possible that unconditioned *pka1* mutants may have elevated levels of various hsps, which may confer partial resistance to the challenging heat shock even if trehalose synthesis is absent (such as in *pka1 tps1* double mutants). While future studies should undoubtedly address this question in more detail (using also specific antibodies to determine the exact levels of various hsps), these results allow us to propose an interesting working model. The high basal level of thermotolerance in *pka1* mutants may be caused by at least two different processes, namely the constitutive synthesis of a set of hsps before the challenging heat shock and the rapid accumulation of trehalose during the challenging heat shock. A particularly interesting aspect of this model is the possibility that elevated levels of molecular chaperones may directly enhance the stability of the Tre6P synthase and therefore be a prerequisite for trehalose synthesis during the challenging heat shock at 50°C.

Table 3. *S. pombe* strains used in this study.

Strain	Genotype	Source (reference)
PB003	h^+ *ade6-M216 leu1-32 ura4-D18*	Blázquez *et al.* (1994)
PBL-17	h^+ *ade6-M216 leu1-32 ura4-D18 tps1::LEU2*	Blázquez *et al.* (1994)
CHP421	h^- *his7-366 leu1-32 ura4-D18*	Jin *et al.* (1995)
CHP453	h^- *his7-366 leu1-32 ura4-D18 pka1::ura4*$^+$	Jin *et al.* (1995)
MRP-3A	h^+ *his7-366 leu1-32 ura4-D18 pka1::ura4*$^+$ *tps1::LEU2*	This study
MRP-7A	h^+ *leu1-32 ura4-D18 pka1::ura4*$^+$	This study
MRP-7B	h^+ *ade6-M216 leu1-32 ura4-D18 tps1::LEU2*	This study
MRP-7C	h^- *his7-366 leu1-32 ura4-D18*	This study
MRP-7D	h^- *ade6-M216 his7-366 leu1-32 ura4-D18 pka1::ura4*$^+$ *tps1::LEU2*	This study
MRP-9C	h^- *ade6-M216 his7-366 leu1-32 ura4-D18*	This study
MRP-9D	h^+ *leu1-32 ura4-D18 pka1::ura4*$^+$ *tps1::LEU2*	This study

Experimental procedures

Strains and growth conditions

The genotypes of all *S. pombe* strains used in this study are listed in Table 3. Strains MRP-3A, MRP-7A, MRP-7B, MRP-7C, MRP-7D, MRP-9C and MRP-9D are all haploid meiotic segregants of three independent diploid strains constructed by crossing strains PBL-17 and CHP453. Mating, sporulation and tetrad analysis of *S. pombe* were performed as described previously (Moreno and Nurse, 1991). Cells were grown in YES medium (0.5% yeast extract and 3% glucose), supplemented with the appropriate auxotrophic requirements and grown at 27°C on a rotary shaker (140 r.p.m.) to mid-exponential growth phase at a density of 4×10^6 cells ml^{-1}. For isotopic labelling studies, cells were transferred into minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose), supplemented with the appropriate auxotrophic requirements.

Heat-shock conditions and analysis of thermotolerance

Log-phase cells grown at 27°C in liquid medium were subjected to a conditioning treatment for 1 h at either 35°C, 37.5°C, 40°C, 42.5°C, 45°C or 47.5°C. Cycloheximide was used at a final concentration of 100 μg ml^{-1} and added 5 min before the conditioning heat shock where indicated (including the labelling experiments). For the determination of thermotolerance, aliquots of the cultures (1 ml) were transferred to prewarmed glass tubes, incubated at 50°C for the times indicated, rapidly cooled on ice, appropriately diluted with sterile water and plated on YES agar. Colonies were counted after 4 days at 27°C, and the percentage survival was assessed by comparison with controls not subjected to the 50°C treatment (100%).

Analytical procedures

Activities of both neutral trehalase and Tre6P synthase were measured in permeabilized cells. To this end, 20-ml aliquots of the log-phase cultures were filtered (Whatman GF/C), resuspended in 1 ml of 0.2 M Mes (K^+), pH 6.0, 0.05% Triton X-100, or in 1 ml of 0.2 M Tricine (Na^+), pH 7.0, 0.05% Triton X-100 and immediately frozen in liquid nitrogen. After thawing (3 min at 30°C), the cells were centrifuged, washed twice with ice-cold 0.2 M Mes (K^+), pH 6.0, or 0.2 M Tricine (Na^+), pH 7.0, and immediately used for the assays of trehalase and Tre6P synthase, respectively, as described previously (De Virgilio *et al.*, 1990). For trehalose determination, 10–20 ml of exponentially growing cells were filtered (Whatman GF/C), washed four times with 5 ml of distilled water, resuspended in 1 ml of water and transferred to a boiling water bath for 10 min. After centrifugation (5 min at $15\,000\times g$), trehalose was determined in the supernatant by high-performance liquid chromatography (HPLC) analysis as described previously (De Virgilio *et al.*, 1993). Protein was quantitated as described by Lowry *et al.* (1951) using BSA as standard.

For the analysis of protein synthesis at different temperatures (27–47.5°C), exponentially growing cultures were centrifuged for 5 min at $3000\times g$ and resuspended in minimal medium at a final concentration of 1×10^8 cells ml^{-1}. Cultures (1 ml) were labelled for 1 h at the temperatures indicated by the addition of 14 μCi of carrier-free L-[^{35}S]-methionine (1.1 μCi μl^{-1}). Radiolabelled proteins were extracted by adding NaOH (0.25 M final concentration) and subsequent precipitation with trichloroacetic acid (TCA; 12% final concentration); the pellets were washed three times with ice-cold acetone, resuspended in 0.1 M NaOH and mixed with an equal amount of twice-concentrated sample buffer (Laemmli, 1970). Radiolabelled proteins were resolved by one-dimensional SDS–PAGE; the gels were then stained, destained and dried before autoradiography at −70°C, using Kodak X-OMAT AR film.

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CHAPTER 4

DELETION OF THE *SCHIZOSACCHAROMYCES POMBE* *tps2*$^{+}$, *tps3*$^{+}$, AND *tps4*$^{+}$ GENES AND THEIR ROLES IN TREHALOSE METABOLISM UNDER HEAT-SHOCK CONDITIONS

INTRODUCTION

In *S. cerevisiae*, several lines of evidence suggest that trehalose may serve primarily as a protectant against adverse environmental conditions such as dehydration, freezing, osmotic and heat stress, rather than as a carbon and energy source (for a review see General Introduction; chapter 1). In the yeast *S. pombe*, trehalose was initially found to accumulate in spores just before they reach maturity and enter into the dormant state (Inoue and Shimoda, 1981a,b). As in *S. cerevisiae*, trehalose accumulation could also be induced when *S. pombe* log-phase cells were submitted to a mild heat shock (a shift from 27°C to 40°C) (De Virgilio *et al.*, 1990). In these studies two key enzymes of trehalose metabolism were described: a trehalase with neutral pH optimum and a Tre6P synthase.

There is no doubt that trehalose has a prominent role in protecting *S. pombe* cells during stress. However, while some aspects of trehalose metabolism and function in *S. cerevisiae* and *S. pombe* are very similar, others differ significantly. As in *S. cerevisiae*, *S. pombe* cells possess two different trehalases; a neutral, cytosolic trehalase and an acidic trehalase. In contrast to *S. cerevisiae*, where the acidic trehalase is localized to the vacuoles, the *S. pombe* acidic trehalase is only found in the cell walls of mature asci (Inoue and Shimoda, 1981b). The constitutive neutral trehalase is present in vegetative cells, during the log and stationary growth phase, as well as in asci (De Virgilio *et al.*, 1991a). The confirmation that the neutral trehalase is encoded by *ntp1*$^+$ was recently provided by Cansado and coworkers (1998). Cloning of *ntp1*$^+$ and analysis of the deduced Ntp1 protein sequence revealed 45 to 55% identity with neutral trehalases from other yeasts. However, in contrast to *S. cerevisiae* Nth1, it seems to have only one consensus site for cAMP-dependent protein phosphorylation (Soto *et al.*, 1998). Neutral trehalase from *S. pombe* shares some properties with that of *S. cerevisiae*: it is almost completely inhibited by 3 mM Zn^{2+} and it is heat-shock inducible even in the presence of protein translation inhibitor cycloheximide, suggesting a post-translational regulation of its activity (De Virgilio *et al.*, 1991a; Thevelein, 1984; Carrilo *et al.*, 1994). Notably, *in vitro* activation of the neutral trehalase by cAPK was confirmed by Soto and coworkers (1995b). This finding was quite surprising because in *S. pombe*, in contrast to *S. cerevisiae*, the Ras1 protein is not involved in the activation of adenylate cyclase (see General Introduction; chapter 1). Thus, despite the fact that in both *S. pombe* and *S. cerevisiae* neutral trehalase is activated by cAPK, this activation appears to be regulated by different signalling pathways. Phosphorylation of *S. pombe* trehalase was also demonstrated to be partially independent of cAPK (Soto *et al.*, 1995b). In agreement with this, a second protein kinase, Sck1, was proposed to activate neutral trehalase upon heat shock or exposure to a nitrogen source (Jin *et al.*, 1995). Unlike *S. cerevisiae* cells, *S. pombe* cells seem to use an osmosensing signalling pathway (via Wis1/Spc1 MAPK) to regulate the osmotically-induced neutral trehalase activity (Zähringer *et al.*, 1997; Cansado *et al.*, 1998; Soto *et al.*, 1998). The increase in trehalase activity upon osmotic stress is probably due to transcriptional regulation, since the trehalase activation under

the same conditions is completely abolished in the presence of cycloheximide (Fernández *et al.*, 1997; Cansado *et al.*, 1998a).

The gene encoding Tre6P synthase, *tps1*$^{+}$ has been isolated and characterized by Blázquez and coworkers (1994). It was shown to be necessary for spore germination, but its deletion had no effect on the ability of cells to conjugate or to sporulate (Blázquez *et al.*, 1994). *S. pombe tps1*$^{+}$ is essential for Tre6P synthesis, but unlike in *S. cerevisiae*, is dispensable for growth on glucose and deletion of *tps1*$^{+}$ does not cause the pleiotropic phenotype observed in *S. cerevisiae*. This unexpected finding provided the basis for the experiments presented in chapter 3. Heat-shock conditions also led to an increase in *tps1*$^{+}$ mRNA transcripts, as previously described for *S. cerevisiae*, suggesting that trehalose accumulation may be achieved by an increase in the synthesis of Tre6P synthase during heat shock. However, in contrast to the situation in *S. cerevisiae*, the addition of cycloheximide to *S. pombe* cells prior to the heat-shock treatment neither blocks trehalose synthesis, nor the activation of Tre6P synthase (De Virgilio *et al.*, 1990, Ribeiro *et al.*, 1997; chapter 3). This suggests that *tps1*$^{+}$ in *S. pombe* is likely to be regulated via a post-translational mechanism. In *tps1*$^{-}$ cells, acquisition of thermotolerance is severely impaired, even when the cells are first submitted to a prior preconditioning heat treatment (Ribeiro *et al.*, 1997; chapter 3). However, deletion of the *pka1*$^{+}$ gene in this mutant can substantially increase the thermotolerance under the same conditions (Ribeiro *et al.*, 1997; chapter 3). The levels of *tps1*$^{+}$ mRNA are induced approximately threefold in a *pka1*$^{-}$ strain compared to a wild-type strain, suggesting that Pka1 is a negative regulator of *tps1*$^{+}$ expression (Fernándes *et al.*, 1997). In contrast, transcription of the *S. pombe tps1*$^{+}$ is positively regulated by the Wis1/Spc1 MAPK cascade in response to heat shock (Degols *et al.*, 1996).

In *S. cerevisiae*, trehalose biosynthesis is known to occur in a two-step process catalyzed by Tps1 and Tps2 enzymes. It is also known that both proteins reside, together with two additional proteins, Tps3 and Tsl1, in a multimeric complex. One explanation for such structural composition is based on the model that Tre6P regulates the early steps of glycolysis by inhibiting hexokinase (see introduction of chapter 5 for further explanation, and chapter 6). The inhibitory effects of free Tre6P would not occur if both enzymatic functions of Tre6P synthase and Tre6P phosphatase are combined in a multimeric complex. Importantly, in *S. pombe*, hexokinase is not inhibited by Tre6P, suggesting that Tre6P may possess a role in *S. cerevisiae* which it does not have in *S. pombe*. This finding also suggests that the enzymes participating in trehalose formation in *S. pombe* may be differently organized and regulated. In order to address this question, it was challenging to first shed light on the mechanisms involved in trehalose formation in *S. pombe* cells by isolating and characterizing the still unknown *S. cerevisiae TPS2* homologue in *S. pombe*.

Taking advantage of the fact that the *tps2* mutant from *S. cerevisiae* is unable to grow at elevated temperatures, a complementation strategy with a cDNA library from *S. pombe* was used to isolate an *S. pombe TPS2* homologue. In this chapter the isolation of the *S. pombe TPS2*

homologue (*tps2*$^+$), and the effects of its deletion in *S. pombe* cells are reported. The results presented indicate that, as in *S. cerevisiae*, deletion of *tps2*$^+$ in *S. pombe* cells causes loss of Tre6P phosphatase activity *in vitro*, and an *in vivo* accumulation of Tre6P during heat shock. In addition, loss of Tps2 also causes a growth defect at elevated temperatures. Recently, two other genes have been identified by the European *Schizosaccharomyces* Genome Sequencing Project as potential genes involved in trehalose formation in *S. pombe*. One of them was identified as a second gene encoding a potential Tre6P phosphatase, here named *tps4*$^+$, and the other is a homologue of the *S. cerevisiae TPS3* gene, here named *tps3*$^+$. The effects of their deletions on trehalose metabolism under heat-shock conditions were analyzed and will be presented in this chapter.

RESULTS

Effect of pH and temperature on Tre6P phosphatase activity

To study the effects of pH and temperature on the activity of *S. pombe* Tre6P phosphatase, permeabilized extracts from strain PB003 (wild type; Table 1, see page 38 in chapter 2) grown to stationary phase were obtained (see Materials and Methods; chapter 2). Tre6P phosphatase activity was first assayed under standard conditions described previously by De Virgilio and coworkers for *S. cerevisiae* Tre6P phosphatase (1993) using 25 mM (K^+) phosphate buffer prepared in different pHs (from 6.0 to 8.0). The effect of pH on the activity of Tre6P phosphatase activity is shown in Figure 1A. The optimum pH for *S. pombe* Tre6P phosphatase under the standard conditions (incubation at 30°C) was in phosphate buffer at pH 6.5. Interestingly, this value is close to those found for Tre6P phosphatases from other organisms (Friedman, 1960, cited in Vandercamen *et al.*, 1989; Matula *et al.*, 1971, cited in Vandercamen *et al.*, 1989). Tre6P phosphatase activity was then assayed at pH 6.5 at different temperatures (20°C to 60°C), showing the highest activity at 32.5°C (Figure 1B).

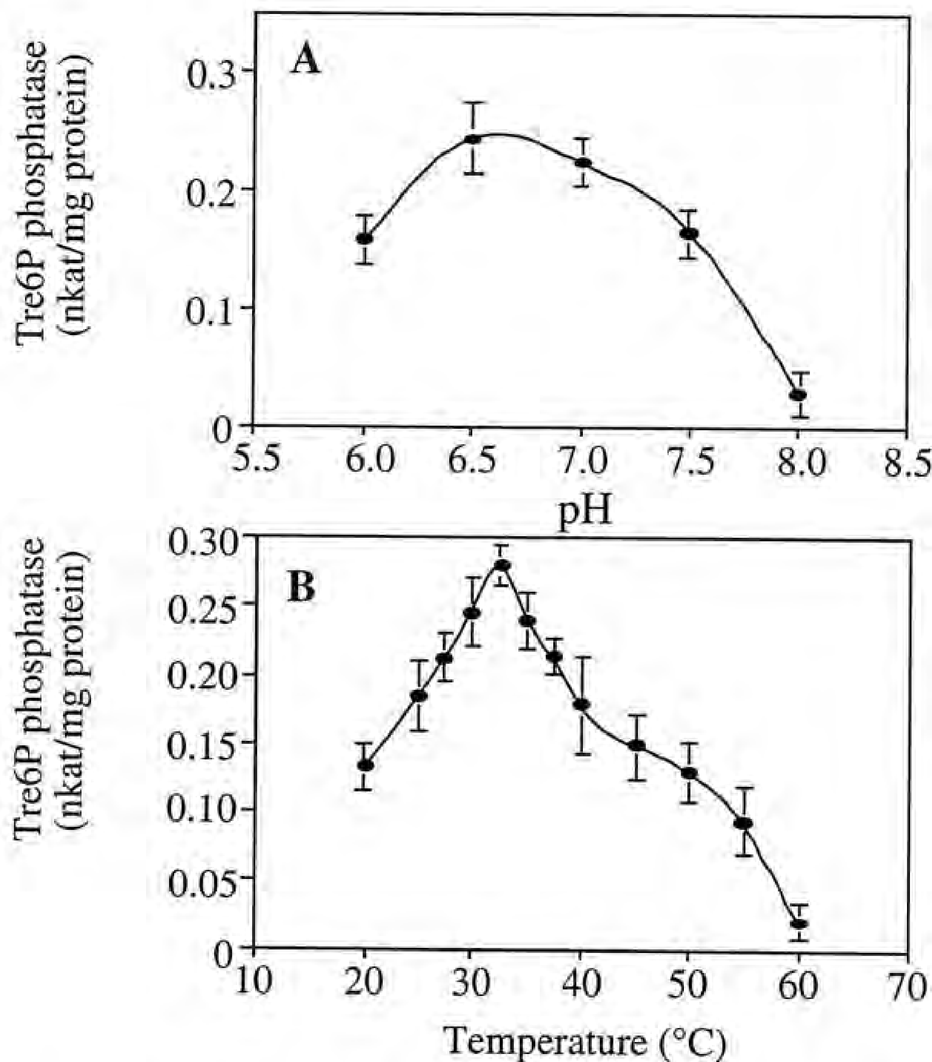


Figure 1. The pH and temperature optima for Tre6P phosphatase activity. Effects of pH (**A**) and temperature (**B**) on Tre6P phosphatase activity in wild-type cells. Strain PB003 was grown at 27°C to stationary phase on YES medium containing 1% glucose and fivefold excess of auxotrophic requirements to ensure that the carbon source was the limiting factor. Activities were assayed in permeabilized extracts as described in Materials and Methods (chapter 2). Tre6P phosphatase activity was assayed under standard conditions, varying the pH of the phosphate buffer or at constant pH 6.5, varying the incubation temperatures of the assay. The results shown are the mean ± standard deviation of at least three independent experiments.

Isolation and sequence analysis of the *S. pombe tps2⁺* gene

In order to isolate from *S. pombe* the *TPS2* homologue, a *tps2* mutant of *S. cerevisiae* was transformed with an *S. pombe* cDNA library which is expressed under the control of the *S. cerevisiae ADH1* promoter (see Materials and Methods; chapter 2). The positive clones

contained short inserts of around 1.5 kb and were assumed to encode only part of a putative full-length *S. pombe TPS2* homologue, since the *TPS2* gene in *S. cerevisiae* has an ORF of 2685 nucleotides. In order to get the full-length *S. pombe tps2$^+$* gene, 5' RACE-PCR experiments were performed as described in Materials and Methods (chapter 2). At the same time, a partial sequence of a putative Tre6P phosphatase in *S. pombe*, sequenced by the European *Schizosaccharomyces* Genome Sequencing Project was entered into the database. Later, the complete sequence of the *S. pombe tps2$^+$* gene became available at the GenBank database (accession number Z97209). Nevertheless, the entire *S. pombe tps2$^+$* gene was sequenced in our lab and found to differ in two nucleotides from the sequence in the database. The differences are in nucleotide numbers 378 and 935 resulting in the following amino acid changes: the asparagine (N) at position 126 is a lysine (K) in the deduced sequence of the isolated gene and the valine (V) at position 312 is an alanine (A) (Figure 2A). The Tre6P phosphatase sequence obtained included 130 nucleotides upstream of the start codon and 80 nucleotides downstream of the stop codon of the *tps2$^+$* gene. Figure 2 depicts the *S. pombe tps2$^+$* DNA sequence and the deduced Tps2 amino acid sequence. Additional 880 bp of the 5' untranslated sequence (-1011 to 131) and 391 bp of the 3' untranslated sequence (2598 to 2989) were taken over from the databank sequence. A possible binding site for the Atf1 transcription factor was found at position –218 to –211 and is depicted in Figure 2A in bold, underlined letters.

The *S. pombe TPS2* homologue was termed *tps2$^+$*, and encodes a protein of 817 amino acids with 42% identity over the entire stretch to Tps2 of *S. cerevisiae*. The amino acid sequence also shows similarity to Tre6P phosphatases from *Aspergillus nidulans*, *E. coli*, and to a possible second Tre6P phosphatase from *S. pombe*, named here Tps4 (Figure 2B). In *S. cerevisiae*, Tps2 has 896 amino acid residues and has been divided into three major domains. The N-terminal domain (residues 1-552), the central domain (residues 573-784), and the C-terminal domain. The N-terminal domain of Tps2 is homologous to the other subunits of the Tre6P synthase/phosphatase complex, including Tps1, Tsl1 and Tps3, and it is also quite homologous to Tps1 from *S. pombe*. The central part could be further split into two other domains according to Thaller and coworkers (1998): domain A (residues 575-587) and B (residues 777-784), which presumably encode the Tre6P phosphatase catalytic sites (Figure 2B).

A

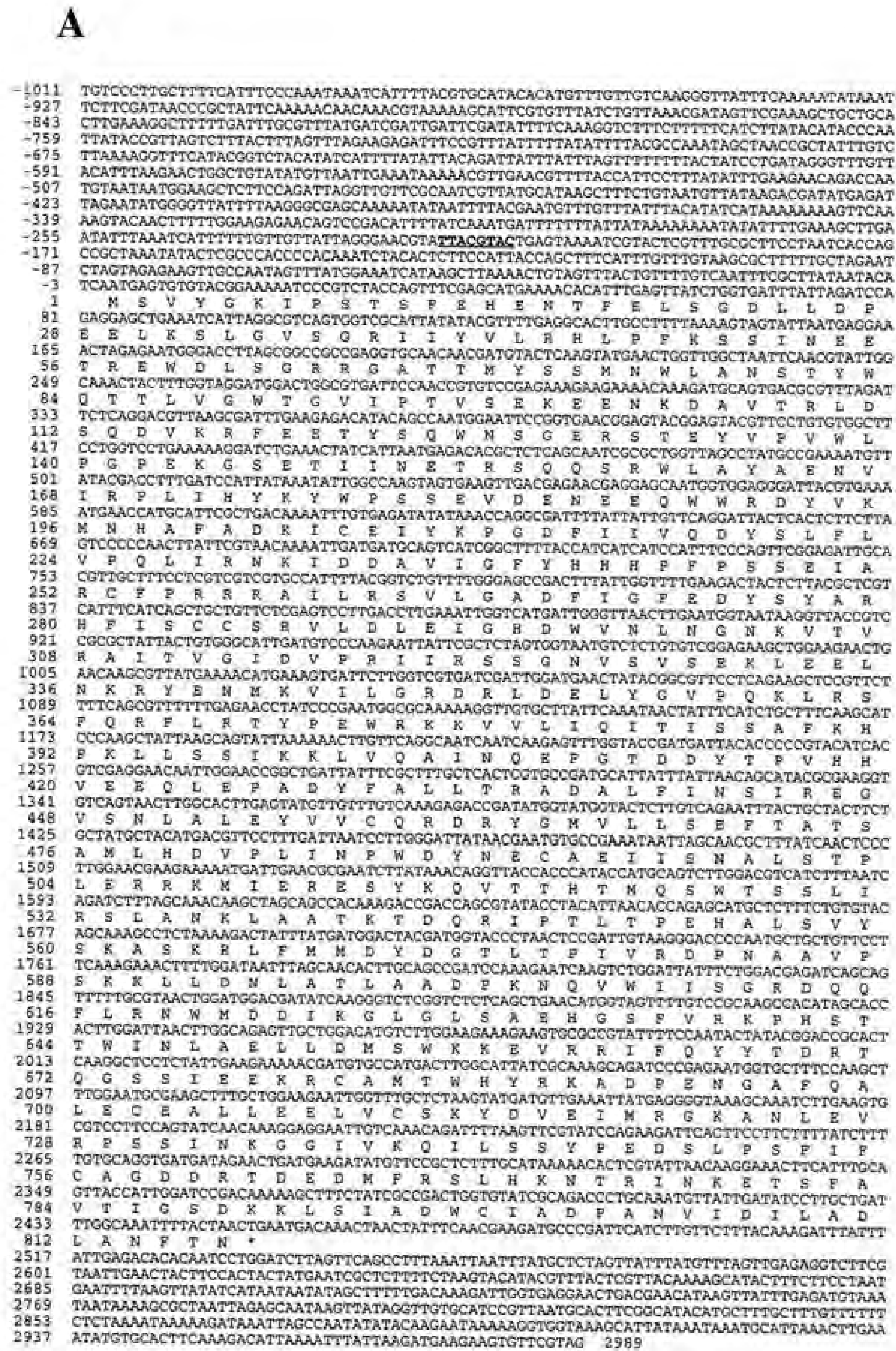


Figure 2A. Sequence analysis of *tps2*$^+$ and its deduced Tps2 sequence. Nucleotide sequence and deduced amino acid sequence of *S. pombe tps2*$^+$. A stretch of 1011 nucleotides up- and 473 nucleotides downstream to the ORF are also shown. A possible site for Atf1 binding is shown in bold underlined letters. The stop codon is indicated by an asterisk.

Figure 2B. Comparison of the amino acid sequences of Tre6P phosphatase of *S. pombe* and other organisms. Alignment of Tre6P phosphatase-related amino acid sequences from various organisms. The bold underlined letters represent the two possible phosphatase domains. The sequences are: *Sptps2* (*Schizosaccharomyces pombe*, Z97209), *AnorlA* (*Aspergillus nidulans*, U35731), *ScTPS2* (*Saccharomyces cerevisiae*, P31688), *Sptps4* (*S. pombe*, Z99167), *EcotsB* (*Escherichia coli*, P31678), *RzotsB* (*Rhizobium* sp., P55611), *AtTPPA* (*Arabidopsis thaliana*, AF007778), *AtTPPB* (*A. Thaliana*, AF007779), and *MlotsP* (*Mycobacterium leprae*, S72829).

Deletion of the *tps2*⁺ gene in *S. pombe* leads to a complete loss of Tre6P phosphatase activity

To show that *S. pombe tps2*⁺ encodes a genuine Tre6P phosphatase, and to analyze its function in this yeast, the complete coding region of *tps2*⁺ was deleted by a PCR-based gene deletion method, as described in Wach and coworkers (1994), using the *kanMX2* module (see Materials and Methods; chapter 2). Southern-blot analysis, with an internal 0.79 kb PCR-generated fragment of *tps2*⁺ as a probe (see Materials and Methods; chapter 2), confirmed the integration of the *kanMX2* module at the *tps2*⁺ locus (Figure 3) of strain MRP3 (Table 1, page 38 in chapter 2). Successful single integration of the marker at the *tps2*⁺ locus yielded a 0.81 kb band. A band of 1.86 kb was observed in the wild-type strain PB003 (Figure 3).

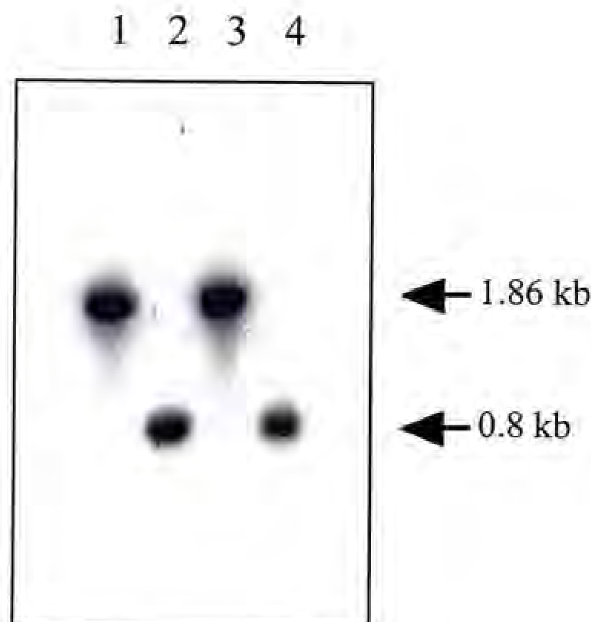


Figure 3. Southern-blot analysis of yeast genomic DNA. The DNAs of PB003 (wild-type, lane 1), MRP3 (*tps2*$^-$, lane 2), MRP4 (*tps4*$^-$, lane 3), and MRP5 (*tps2*$^-$ *tps4*$^-$, lane 4) strains were digested with *ClaI-SalI* and probed with an internal 0.79-kb PCR fragment of *tps2*$^+$. Sizes of the bands are indicated on the right.

In the following, Tre6P levels (Figure 4A), Tre6P phosphatase activity (Figure 4B), Tre6P synthase activity (Figure 4C), and trehalose levels (Figure 4D) were analyzed in extracts from *S. pombe* wild-type (PB003) cells and in the *tps2*$^-$ (MRP3) mutant cells harvested during exponential phase or after a heat shock at 40°C for 1 h. In parallel, the same parameters were evaluated in extracts of *S. cerevisiae* wild-type cells (YSH6.106.-3A) and of the corresponding isogenic *tps2* mutant cells (YSH6.106.-8C). When the *S. cerevisiae tps2* mutant strain was heat-shocked, an excessive accumulation of Tre6P was detected (Figure 4A). Tre6P phosphatase activity was completely absent under the same conditions (Figure 4B). Interestingly, strain YSH6.106.-8C, carrying a *tps2* deletion, but a functional *TPS1* gene, was not only defective for Tre6P phosphatase activity, but also had significantly reduced levels of Tre6P synthase (Figure 4B and 4C). Deletion of *tps2* caused a fourfold decrease in Tre6P synthase activity in log-phase and a fivefold decrease in heat-shocked cells when compared to the activities in the wild-type strain YSH6.106.-3A (Figure 4C). Reduced Tre6P synthase activity in the *S. cerevisiae tps2* mutant has also been described by Reinders and coworkers (1997) and Bell and coworkers (1998). Despite the low levels of *in vitro* detectable Tre6P

synthase activity, the *tps2* deletion mutant strains accumulate high amounts of Tre6P during heat shock, indicating that their *in vivo* Tre6P synthase activities were sufficient to sustain this accumulation and were unaffected by the loss of Tps2.

When the *S. pombe tps2* mutant strain MRP3 was grown to log-phase or heat shocked on glucose-containing media, Tre6P synthase activity values were comparable to those of the wild-type strain (Figure 4C). However, Tre6P phosphatase activity was totally absent under the same conditions (Figure 4B). In contrast, wild-type cells showed basal levels of Tre6P phosphatase activity in log-phase cells and an approximately twofold increase after heat shock (Figure 4B). A similar increase in the activity of Tre6P phosphatase was observed in the *S. cerevisiae* wild-type strain YSH6.106.-3A after heat shock (Figure 4B).

In agreement with the Tre6P phosphatase activity profile observed in the *S. pombe tps2*$^-$ strain, high amounts of Tre6P were found (0.249 g/g protein), but only a significantly reduced amount of trehalose (0.157 g/g protein) was accumulated as compared to wild-type cells (0.312 g/g protein) under heat-shock conditions (Figure 4A and 4D). No Tre6P could be detected in wild-type cells under any of the conditions tested. Together, these results indicate that the *S. pombe tps2*$^+$ gene encodes a Tre6P phosphatase.

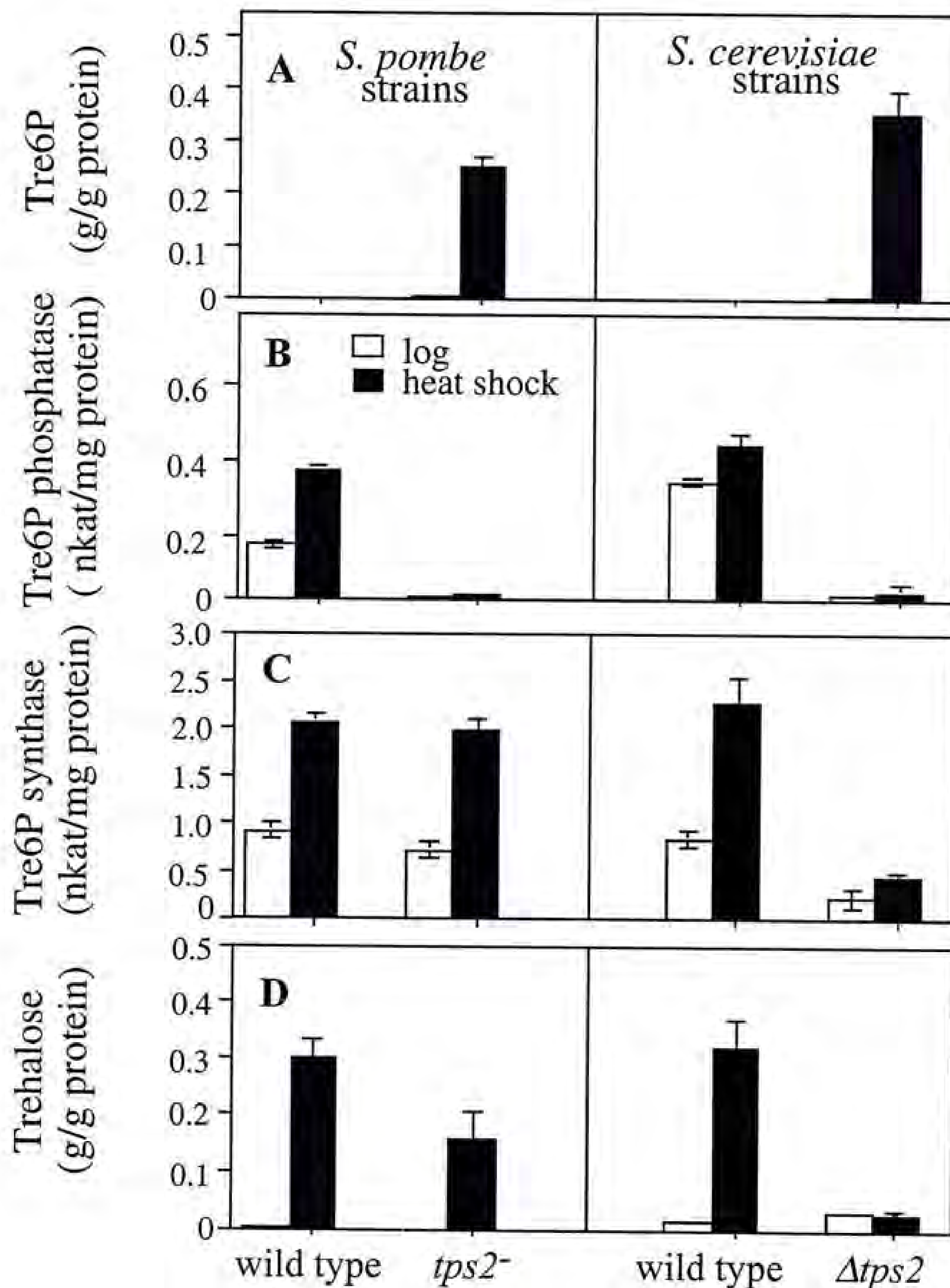


Figure 4. Tre6P levels, Tre6P phosphatase and Tre6P synthase activities, and trehalose levels before and after a heat shock. Tre6P levels **(A)**, Tre6P phosphatase **(B)** and Tre6P synthase activities **(C)**, and trehalose levels **(D)** in log-phase and heat-shocked cells. Exponentially growing cultures were heat shocked for 1 h at 40°C. Activities were assayed in permeabilized extracts as described in Materials and Methods (chapter 2). Strains PB003 (wild type) and MRP3 (*tps2⁻*) were grown on minimal medium containing 2% glucose. Strains YSH6.106.-3A (wild type) and YSH6.106.-8C (*Δtps2*) were grown on minimal medium containing 2% galactose and 1% raffinose. The results shown are the mean ± standard deviation of at least three independent experiments.

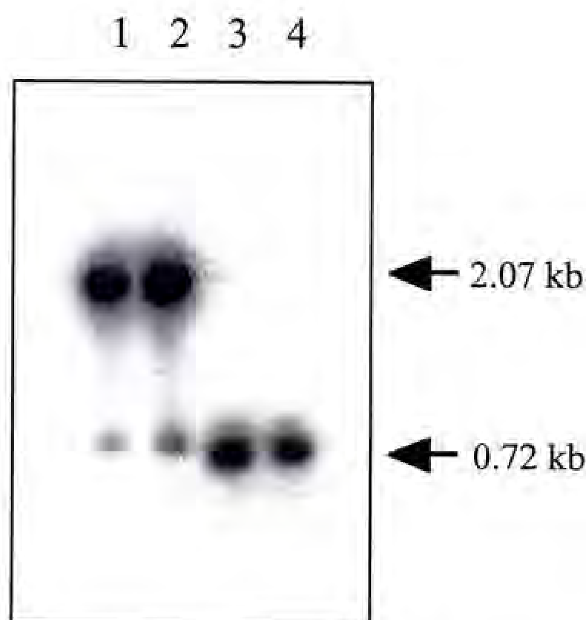


Figure 5. Southern-blot analysis of yeast genomic DNA.
Genomic DNA of PB003 (wild-type, lane 1), MRP3 (*tps2*⁻, lane 2), MRP4 (*tps4*⁻, lane 3), and MRP5 (*tps2*⁻ *tps4*⁻, lane 4) strains was digested with *AccI*-*PstI* and probed with the internal 2.07-kb fragment of *tps4*⁺. Sizes of the bands are indicated on the right.

Deletion of *tps4*⁺ gene in *S. pombe* does not cause accumulation of Tre6P, but decreases trehalose accumulation

Recently, a second gene coding for a potential Tre6P phosphatase protein of 849 amino acids with 43% identity to the *S. pombe* Tps2 sequence has been described in the GenBank database (accession number Z99167). Henceforth it is termed *tps4*⁺. In order to analyze its function, its coding region was entirely deleted by applying the PCR-based deletion method using a *ura4*⁺ marker from *S. pombe* (see Materials and Methods; chapter 2). In this way, it was possible to construct a single *tps4*⁻ mutant (MRP4) and a *tps2*⁻ *tps4*⁻ double mutant strain (MRP5) (see Table 1; page 38 in chapter 2). Homologous integration in both strains was confirmed by Southern-blot analysis with an internal 2.07 kb PCR-fragment of *tps4*⁺ as a probe (Figure 5). Successful integration of the *ura4*⁺ marker at a single site at the *tps4*⁺ locus yielded a 2.07 kb band. A band of 0.72 kb was observed in the wild type strain PB003 for *tps4*⁺ (Figure 5).

In contrast to the *tps2⁻* and *tps2⁻tps4⁻* cells, single *tps4⁻* cells grown to log-phase or heat-shocked, did not accumulate Tre6P (Figure 6A). The *tps4⁻* strain showed a 43% increase in the Tre6P phosphatase activity after the heat shock. In contrast, Tre6P phosphatase activity was absent in the *tps2⁻* and *tps2⁻tps4⁻* strains in log-phase or following heat-shock (Figure 6B). Deletion of the *tps2⁺* gene also caused a slight decrease in the Tre6P synthase activity. Deletion of *tps4⁺* in the wild type background or in the *tps2⁻* background caused 42% or 31% decrease, respectively, in Tre6P synthase activity in log-phase or following heat-shock (Figure 6C).

Concerning trehalose levels, as expected from the Tre6P synthase activity profile, *tps4⁻* or *tps2⁻ tps4⁻* cells did not accumulate normal levels of trehalose during heat shock, suggesting a possible role of *tps4⁺* in the formation of trehalose under these conditions (Figure 6D).

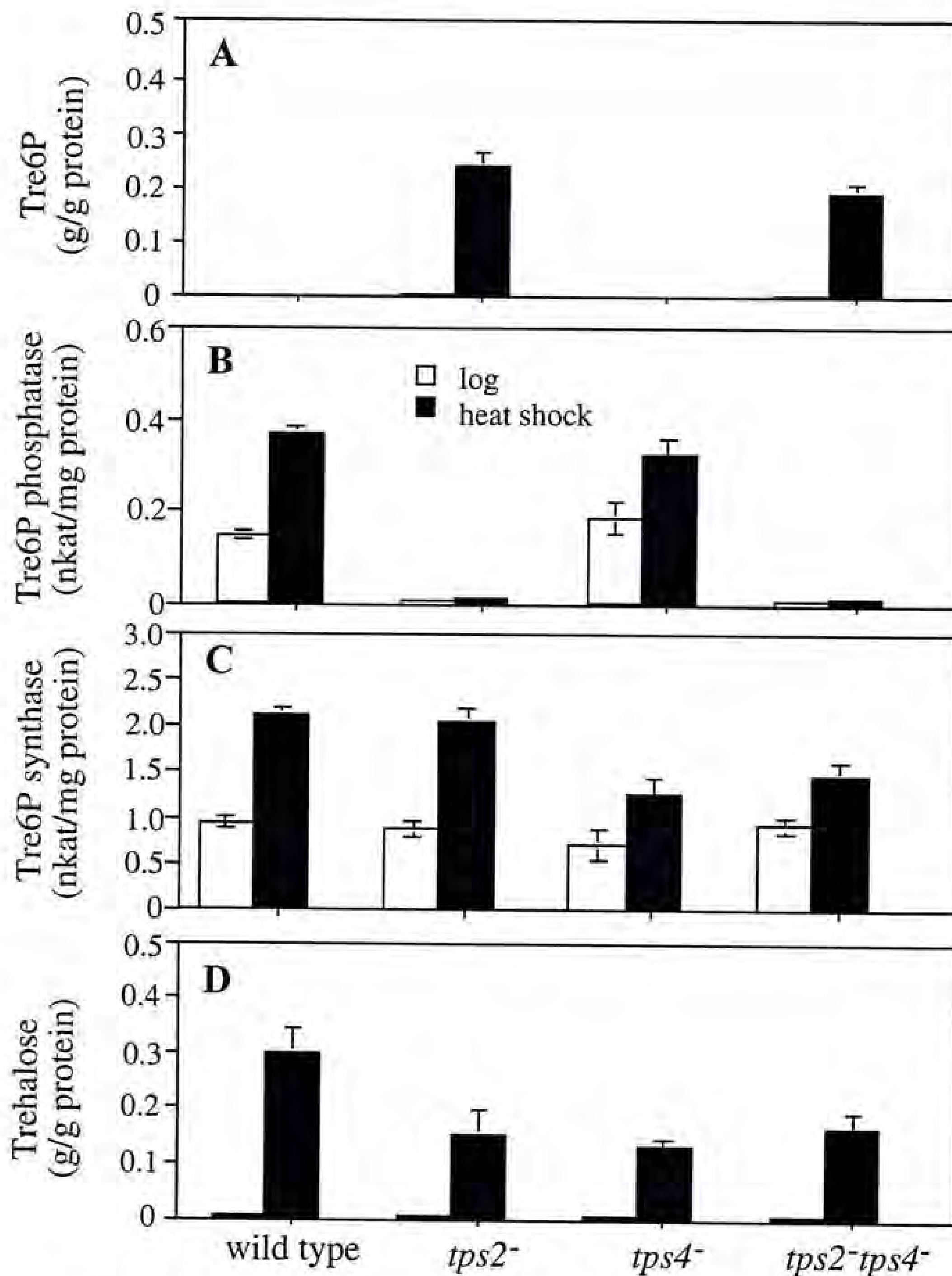


Figure 6. Tre6P levels, Tre6P phosphatase and Tre6P synthase activities, and trehalose levels before and after a heat shock. Tre6P levels **(A)**, Tre6P phosphatase **(B)** and Tre6P synthase activities **(C)**, and trehalose levels **(D)** in log-phase and heat-shocked cells. Exponentially growing cultures were heat shocked for 1 h at 40°C. Activities were assayed in permeabilized extracts as described in Materials and Methods (chapter2). Strains PB003 (wild type), MRP3 (*tps2-*), MRP4 (*tps4-*), and MRP5 (*tps2- tps4-*) were grown on minimal medium containing 2% glucose. The results shown are the mean ± standard deviation of at least three independent experiments.

The *S. pombe tps2*$^+$, but not *tps4*$^+$, complements an *S. cerevisiae tps2* mutant

An *S. cerevisiae tps2* mutant strain, YSH6.106.-8C, was transformed with an empty plasmid (YCpIF5) or with plasmids YCpIF5-*tps2*$^+$ and YCpIF5-*tps4*$^+$, as described in Materials and Methods (chapter 2).

As shown previously, strain YSH6.106.-8C containing the empty plasmid accumulated Tre6P instead of trehalose under heat-shock conditions (Figure 7A). Further, Tre6P phosphatase activity was completely abolished in this strain under these conditions (Figure 7B). Plasmid YCpIF5 contains the *S. cerevisiae* galactose-inducible *GAL1* promoter, which is turned on when cells are grown in minimal medium containing galactose as carbon source. Introduction of plasmid YCpIF5-*tps2*$^+$ into *S. cerevisiae tps2* mutant cells restored Tre6P phosphatase activity during heat shock when the cells were pre-grown on galactose-containing media (Figure 7B). Tre6P synthase activity was strongly reduced in the *S. cerevisiae tps2* mutant containing an *S. pombe tps2*$^+$ gene (Figure 7C). Despite being able to complement Tre6P phosphatase activity during heat shock, the *S. pombe tps2*$^+$ gene could not restore the full Tre6P synthase activity to wild-type level (2.0 nkat/mg protein see also Figure 4C). YCpIF5-*tps2*$^+$ into *S. cerevisiae tps2* mutant cells restored trehalose accumulation during heat shock when the cells were pre grown on galactose-containing media (Figure 7D).

In contrast, introduction of plasmid YCpIF5-*tps4*$^+$ into the *S. cerevisiae tps2* mutant could neither restore Tre6P phosphatase activity nor trehalose accumulation under any of the conditions tested (Figure 7B and 7D). Together with the finding that deletion of *tps4*$^+$ caused no reduction in Tre6P phosphatase activity, this suggests that *tps4*$^+$ does possibly not encode a functional Tre6P phosphatase, or at least does not encode a Tre6P phosphatase that is functional under the tested conditions.

Deletion of *tps2*$^+$, but not *tps4*$^+$, leads to a growth defect at elevated temperature

A comparison of growth ability at 27°C and 38.6°C of wild type (PB003), *tps2*$^-$ (MRP3), *tps4*$^-$ (MRP4), and *tps2*$^-$*tps4*$^-$ (MRP5) cells is shown in Figure 8. Wild-type cells, as well as *tps4*$^-$ mutant cells, were able to grow at both temperatures (Figure 8). However, deletion of the *tps2*$^+$ gene in a wild-type or *tps4*$^-$ mutant background led to a growth defect at 38.6°C (Figure 8). This result could be explained by the fact that both *tps2*$^-$ and *tps2*$^-$*tps4*$^-$ strains accumulated high amounts of Tre6P upon a heat shock, which has been proposed to inhibit growth upon accumulation due to its toxicity (De Virgilio *et al.*, 1993).

The growth defect of *S. cerevisiae tps2* mutant cells could be complemented by transformation of these cells with plasmid YCpIF5-*tps2*$^+$ (Figure 9), but not with plasmid

YCpIF5-*tps4*$^+$ (Figure 9). Moreover, *S. pombe tps2*$^-$ cells could grow at 38.6°C, if transformed with plasmid pUR19-*TPS2* or pUR19-*tps2*$^+$ (Figure 10).

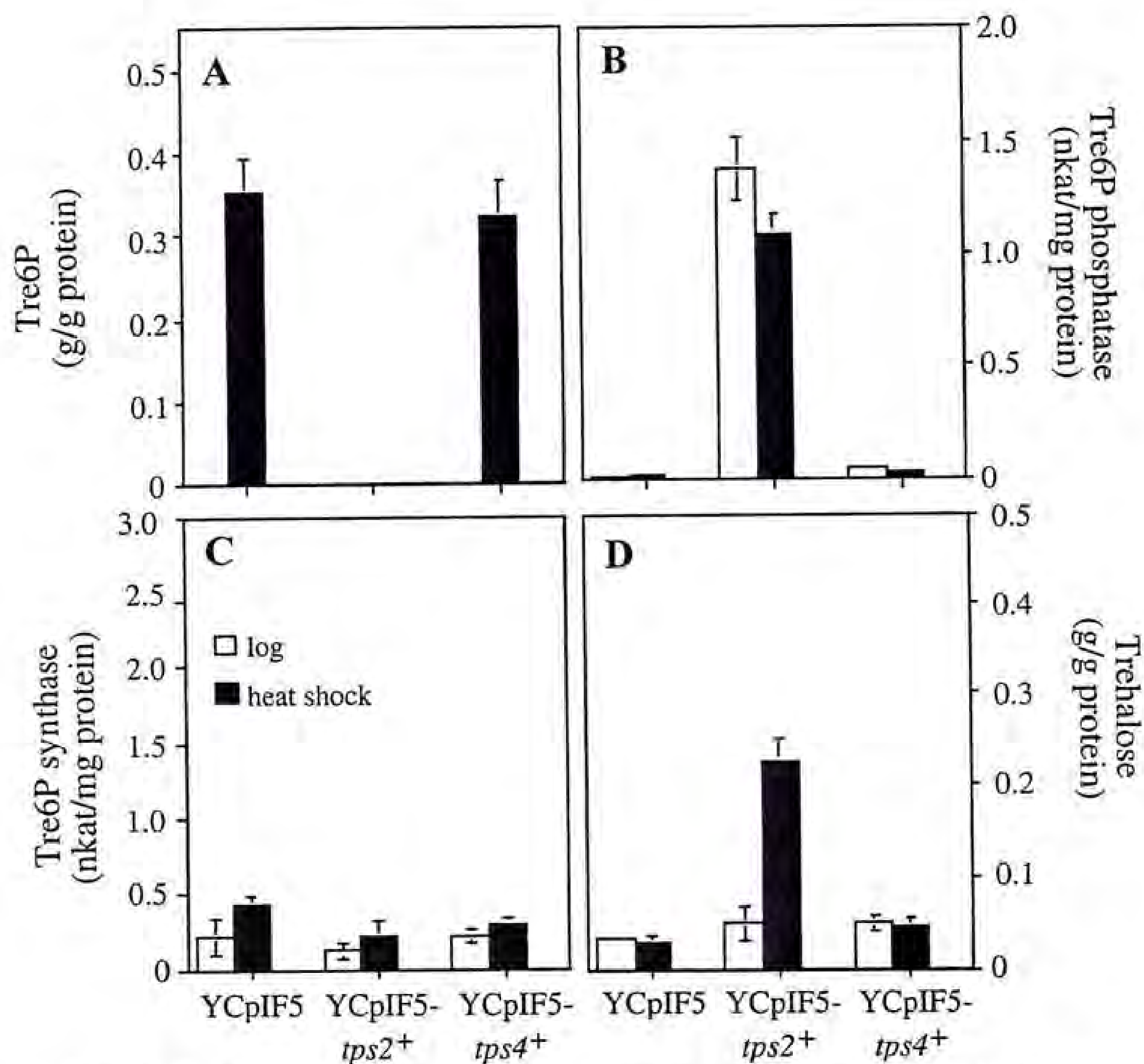


Figure 7. Complementation of an *S. cerevisiae tps2* mutant by *S. pombe tps2*$^+$ or *tps4*$^+$. *S. pombe* genes were cloned into plasmid YCpIF5, which allows galactose-inducible expression from the *S. cerevisiae GAL1* promoter. *S. cerevisiae* strain YSH6.106.-8C (*Δtps2*) was transformed with either YCpIF5 (control), YCpIF5-*tps2*$^+$, or YCpIF5-*tps4*$^+$. The strains were grown on medium containing galactose to induce the *GAL1* promoter. Exponentially growing cultures were heat shocked for 1 h at 40°C. Activities were assayed in permeabilized extracts as described in Materials and Methods (chapter 2). The results shown are the mean ± standard deviation of at least three independent experiments.

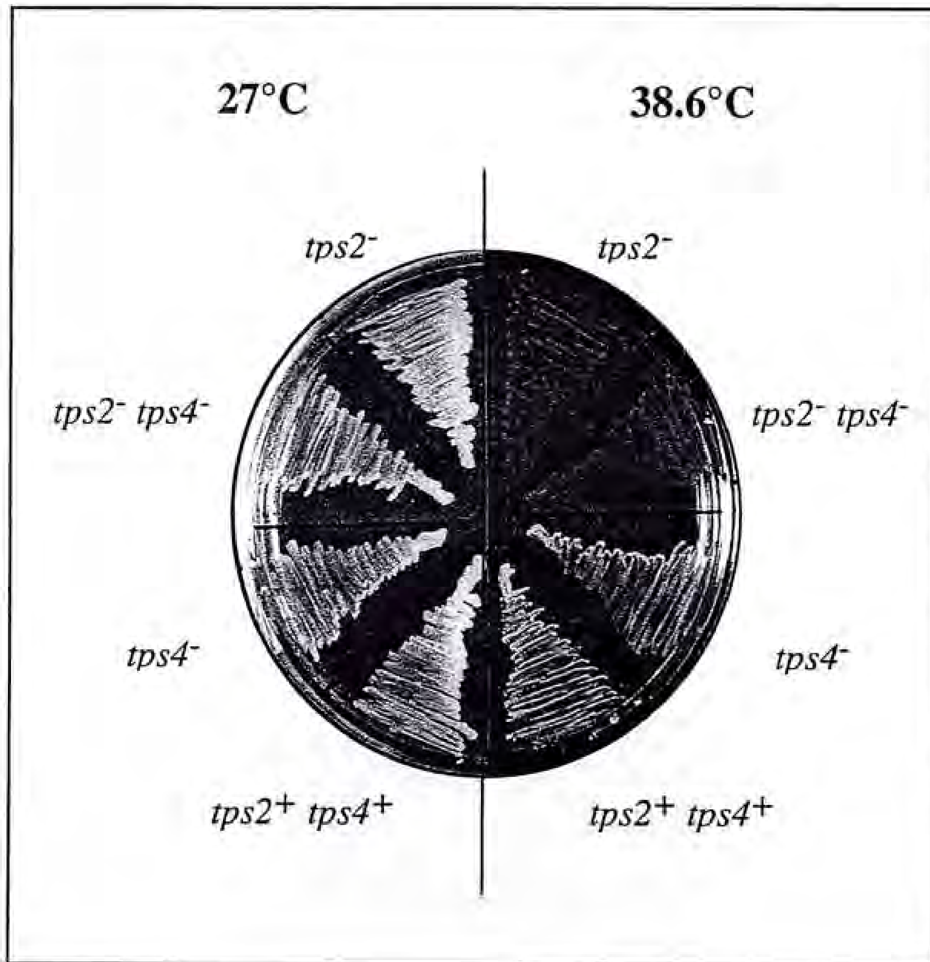


Figure 8. Growth defect at elevated temperatures caused by deletion of the *S. pombe tps2*$^+$ gene. Strains PB003 (wild type), MRP3 (*tps2*$^-$), MRP4 (*tps4*$^-$), and MRP5 (*tps2*$^-$*tps4*$^-$) were streaked out onto YES plates and incubated foi 3 days at 27°C, or 38.6°C .

Northern blots analysis reveales that *tps2*$^+$ and *tps4*$^+$ are differently expressed

Since several genes that respond to stress, such as heat-shock genes, are transcriptionally activated at higher temperatures, the heat shock induction profiles of *tps2*$^+$ and *tps4*$^+$ mRNA were examined and compared to their expression in log phase and stationary phase on glucose-containing medium (Figure 11A and 11B). Total RNA was extracted from strains PB003, MRP3, MRP4, and MRP5 either growing exponentially on glucose (L), heat shocked for 15 min (HS1) or 30 min (HS2) at 40°C, or growing to early (S1) and late (S2) stationary phase. A comparison of the transcription level profiles under these conditions is shown in Figure 11A and Figure 11B. As can be seen on the autoradiograms in Figure 11A,

wild-type cells express *tps2⁺* constitutively under all conditions tested. However, a strong induction of its transcript was observed upon heat shock. The same transcriptional profile of *tps2⁺* was observed in a *tps4⁻* mutant (Figure 11A). These results are in agreement with the observed induction of Tre6P phosphatase activity upon heat shock (Figure 4B). Analysis of *tps4⁺* gene expression in wild-type cells or in *tps2⁻* mutant cells revealed an increase in transcription mainly upon entry into stationary phase (Figure 11B). However, this induction could be more clearly observed in the *tps2⁻* strains, suggesting that *tps4⁺* expression may be influenced by the presence of the *tps2⁺* gene (Figure 11B).

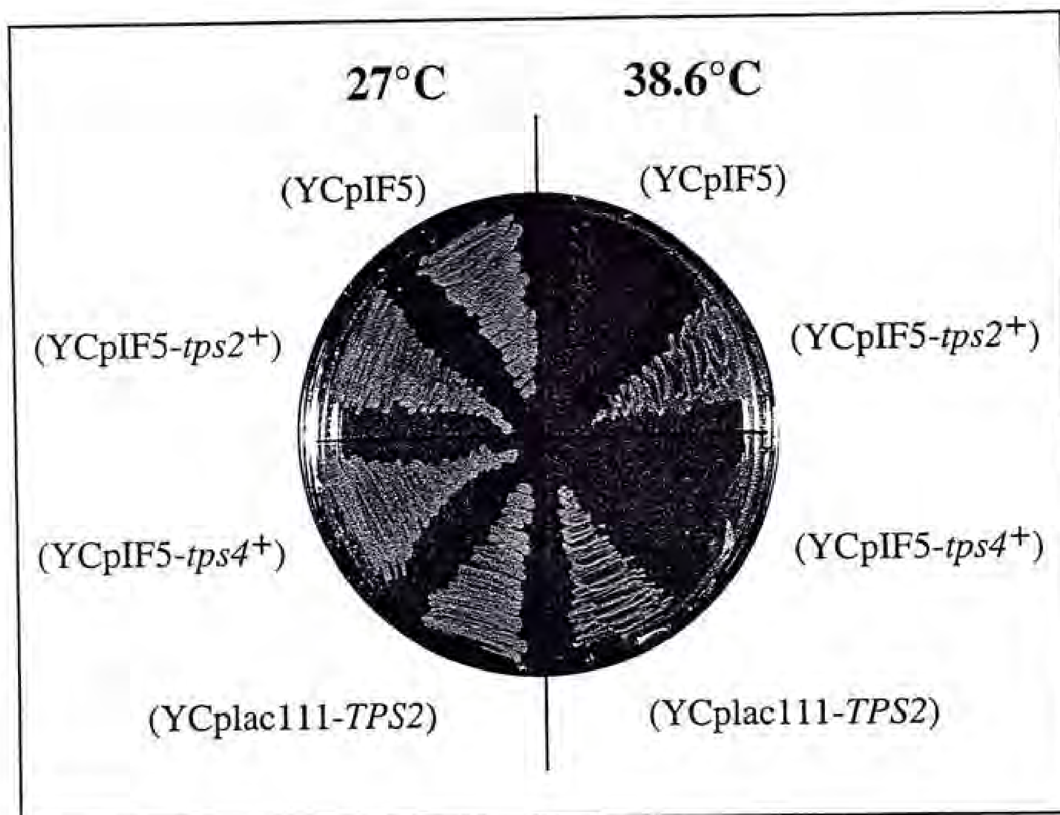


Figure 9. Complementation assay of the growth defect at 38.6°C of an *S. cerevisiae Δtps2* mutant by *S. pombe tps2⁺* or *tps4⁺*. The *S. pombe tps2⁺* and *tps4⁺* genes were cloned into plasmid YCpIF5, which allows galactose-inducible expression from the *S. cerevisiae GAL1* promoter, and transformed into an *S. cerevisiae Δtps2* mutant strain (YSH6.106.-8C). Controls were obtained by transforming an *S. cerevisiae tps2* deletion mutant with either YCpIF5 (negative control) or YCplac111-*TPS2* (positive control). The cells were streaked on minimal media containing galactose as carbon source, and incubated for 4 days at 27°C, or 38.6°C.

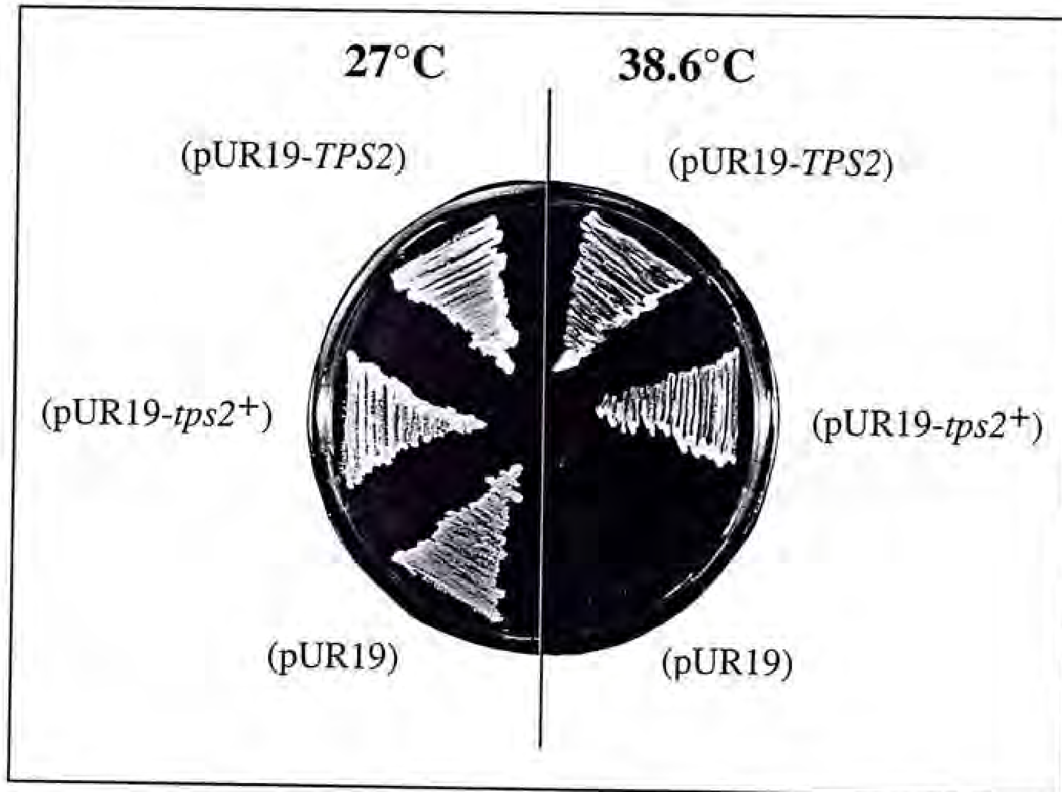


Figure 10. Complementation of the growth defect at 38.6°C of an *S. pombe tps2⁻* mutant. The *S. cerevisiae TPS2* gene under its own promoter was cloned into pUR19 and introduced in an *S. pombe tps2⁻* strain (MRP3). Controls were obtained by transformation of an *S. pombe tps2⁻* mutant strain carrying either pUR19 (negative control) or pUR19-*tps2*+ (positive control).

Deletion of *tps2*+, but not *tps4*+, leads to a loss of cell viability in stationary phase

Fission yeast enters stationary phase from either G_2 or G_1. Glucose and sulphate starvation are known to induce stationary phase entry mainly from G_2, while nitrogen and phosphate starvation induce cells to enter stationary phase from G_1. When growing to saturation on YES medium, cells lacking *tps2*+ rapidly lost viability, and most cells died between 48 and 72 hours, while more than 50% wild-type cells retained viable under the same conditions (Figure 12). The *tps2⁻* mutant cells that reached saturation had a morphology similar to wild-type cells, typical for carbon starved stationary-phase cells (*i.e.*, small rounded cells). In contrast, cells lacking only *tps4*+ were as viable as wild-type cells. Deletion of *tps2*+ in a wild type background or in a *tps4⁻* background strains did not cause any significant alterations, with respect to growth on glucose media (Table 1), or with respect to the total amount of protein content in stationary phase. However, their mating and sporulation efficiencies were severely

reduced (Table 2; see below). Deletion of *tps4⁺* did not cause any significant alterations, with respect to growth on glucose media (Table 1), nor to the total amount of protein content in stationary phase, nor to the sporulation efficiency (Table 2).

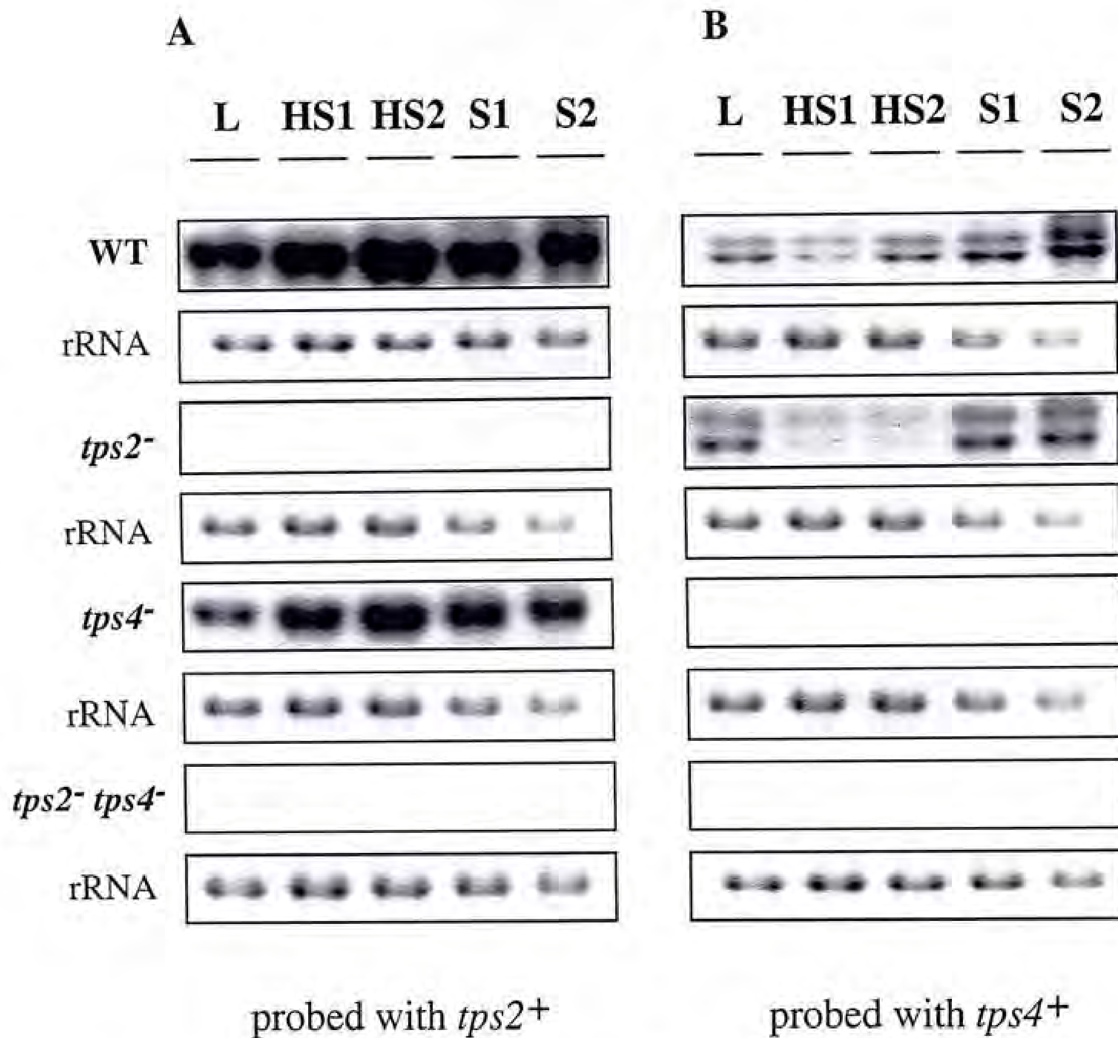


Figure 11. Northern blot analysis of gene expression under different growth conditions. (A) Total RNA of strains PB003 (wild type), MRP3 (*tps2⁻*), MRP4 (*tps4⁻*), and MRP5 (*tps2⁻ tps4⁻*) were probed with an internal fragment from the *tps2⁺* gene . (B) RNA from the same strains were probed with an internal *tps4⁺* fragment. Total RNA was extracted from log phase (L), 15 min (HS1) and 30 min (HS2) after transfer to 40°C, and in early (S1; 2 days of growth) and late (S2; 4 days of growth) stationary phase. Strains were grown on YES medium. For further details see Materials and Methods in chapter 2.

Table 1. Generation Times

SD media	Relevant genotype	YES media
3 h 00 min	Wild-type	2 h 20 min
3 h 10 min	*tps2⁻*	2 h 30 min
2 h 55 min	*tps4⁻*	2 h 15 min
3 h 15 min	*tps2⁻ tps4⁻*	2 h 35 min

The influence of *tps2⁺* and *tps4⁺* deletions on generation time was monitored following dilution of overnight cultures with the appropriate medium to an OD of 0.05 at 620 nm in a microtiter plate. At every hour the OD at 620 nm was measured as described previously in Materials and Methods (chapter 2).

Deletion of *tps2⁺* results in sporulation deficiency

Meiosis, and consequently spore formation, in *S. pombe* is triggered by transferring vegetatively growing cells into medium with low nitrogen or medium lacking a nitrogen source (Schweingruber and Edenharter, 1990). In order to check whether a *tps2⁺* or a *tps4⁺* deletion affect sporulation efficiency, haploid strains from opposite mating types (h^+/h^-), lacking *tps2⁺* or *tps4⁺* were first grown for 24 h separately on minimum medium (EMMA) containing low amounts of nitrogen. This procedure was repeated two times. Afterwards, heterothallic haploids from opposite mating types were mixed in minimum medium (EMMA) and further incubated for 24 hours. Sporulation efficiency was assessed by counting 500 cells under the microscope, including vegetative cells, zygotes and asci (see Materials and Methods; chapter 2). Three diploid strains were analyzed: wild type (PB003/PB004), a homozygous *tps2⁻* mutant (MRP3/MRP9) and a homozygous *tps4⁻* mutant (MRP4/MRP10). When strain PB003 (h^+) was mixed with strain PB004 (h^-) the haploid cells were able to mate and form zygotes, which then underwent meiosis and generated haploid spores. Sporulation efficiency was in the range of 43%, which was not significantly different from the sporulation efficiency of the *tps4⁻* mutant strain (38%; Table 2). However, a decrease of 50% in the sporulation efficiency was observed in the *tps2⁻* (strains MRP3 and MRP9; Table 2). These observations lead to the conclusion that *tps2⁺* in *S. pombe*, as in *S. cerevisiae*, is required for proper sporulation.

Table 2. Effect of *tps2*$^+$ and *tps4*$^+$ deletions on sporulation efficiency

Relevant genotype	Sporulation Efficiency (%)
wild-type	43
tps2$^-$/*tps2*$^-$	20
tps4$^-$/*tps4*$^-$	38

Strains from opposite mating types, PB003 (h^+, wild type) and PB004 (h^-, wild type), MRP3 (h^-, *tps2*$^-$) and MRP9 (h^+, *tps2*$^-$), and MRP4 (h^-, *tps4*$^-$) and MRP10 (h^+, *tps4*$^-$) were first separately precultured in EMMA medium and then mixed in the same medium as described in Materials and Methods (chapter 2). About 500 cells including vegetative cells, zygotes and asci were counted under the microscope to assess the sporulation efficiency (see Materials and Methods for details; chapter 2).

Growth on high osmolarity medium

To test whether *tps2*$^+$ and/or *tps4*$^+$ have a role in osmotic stress response, strains PB003 (wild type), MRP3 (*tps2*$^-$), MRP4 (*tps4*$^-$), and MRP5 (*tps2*$^-$*tps4*$^-$) were streaked out on YES plates and incubated for 24 h at 30°C. Afterwards, these strains were streaked out onto YES plates containing 0.6 or 1.0 M KCl, and incubated at 30°C for 3 days. As depicted in Figure 13A, all strains tested were able to grow normally on YES plates containing 0.6 M KCl. However, a different profile was observed when the same strains were grown on YES plates containing 1.0 M KCl (Figure 13B). The increase in osmolarity caused the *tps2*$^-$ strain (MRP3), to grow poorly on such conditions. Importantly, the osmosensitive growth caused by *tps2*$^-$ was not enhanced in *tps2*$^-$ *tps4*$^-$. The single *tps4*$^-$ mutation also did not affect the high osmolarity sensitivity (Figure 13B), indicating that *tps4*$^+$ does not play an important role under these conditions.

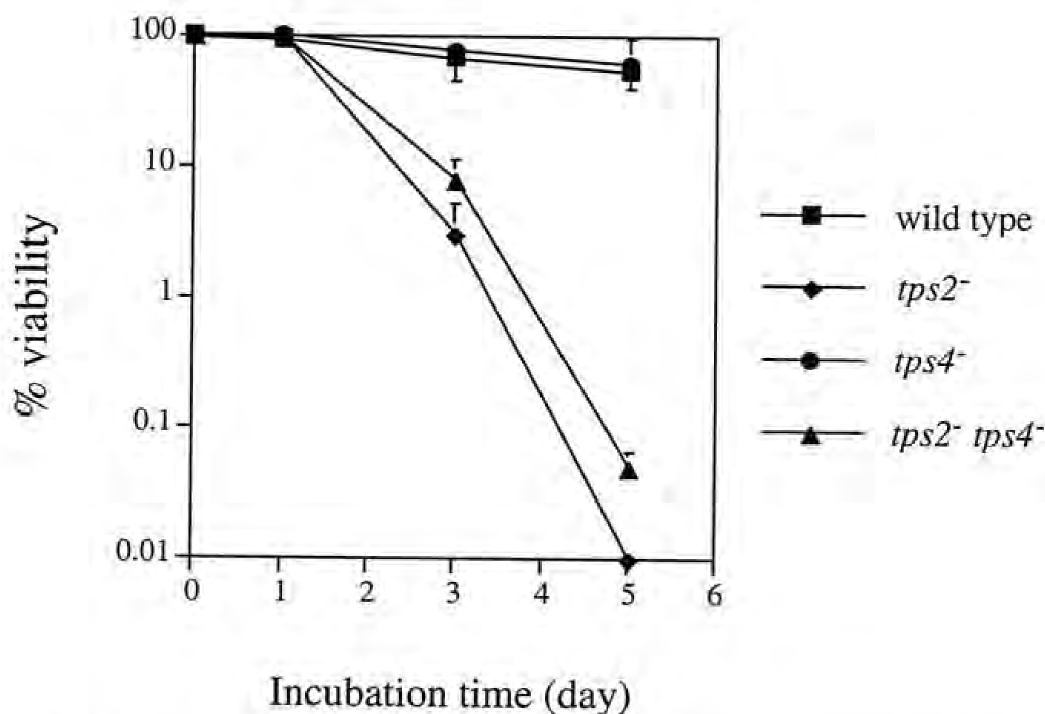


Figure 12. Comparison of long term stationary phase survival of *S. pombe* cells in YES medium. Wild type (PB003; ■), *tps2⁻* (MRP3; ◆), *tps4⁻* (MRP4; ●), and *tps2⁻ tps4⁻* (MRP5; ▲) strains were grown on YES medium containing 1% glucose. Percentage of survival was calculated with respect to the number of cells at early stationary phase (two days after inoculation) (100%). The values are the mean ± standard deviation of three independent experiments.

Deletion of *tps3⁺* gene in *S. pombe* strongly decreases Tre6P synthase activity but not trehalose levels following heat shock

The *S. pombe TPS3* homologue was entered at EMBL GenBank (under accession number Z98850) and termed *tps3⁺*. It encodes a predicted protein of 944 amino acids with an estimated molecular weight of 108.2 kDa, showing 41.3% identity over the entire stretch to Tps3 of *S. cerevisiae*. To assess its function in trehalose formation, the coding region was entirely deleted as described above for *tps2⁺* (see Materials and Methods; chapter 2). The correct integration of *kanMX2* at the *tps3⁺* locus in strain PB003 (Table 1, page 38 in chapter 2) was confirmed by PCR (see Materials and Methods; chapter 2).

Tre6P levels (Figure 14A), Tre6P phosphatase activity (Figure 14B), Tre6P synthase activity (Figure 14C), and trehalose (Figure 14D) levels were analyzed in the *S. pombe tps3⁻* (MRP11; Table 1, page 38 in chapter 2) strain and in its isogenic wild type strain PB003, during log phase and after 1-h heat shock at 40°C. When the *S. pombe tps3* mutant strain

MRP11 was grown to log phase or heat shocked in glucose-containing media, no Tre6P was detected (Figure 14A). In *tps3⁻* cells only a limited effect on Tre6P phosphatase activity was observed in log phase (Figure 14B). Like wild-type cells, the *tps3⁻* mutant cells showed an approximate twofold increase in Tre6P phosphatase activity under heat-shock conditions (Figure 14B). A strong decrease in Tre6P synthase activity was observed in *tps3⁻* cells. Deletion of the *tps3⁺* gene caused an approximate twofold decrease in Tre6P synthase activity in log-phase, and a fourfold decrease following heat shock when compared to the wild-type strain PB003 (Figure 14C). Differently from the Tre6P synthase activity profile, deletion of *tps3⁺* had no effect on trehalose levels under any of the conditions tested (Figure 14D). Although, *tps3⁻* strains showed a substantial decrease in the Tre6P synthase activity measured *in vitro*, their *in vivo* Tre6P synthase activities seem to be sufficient to support normal trehalose accumulation (Figure 14D).

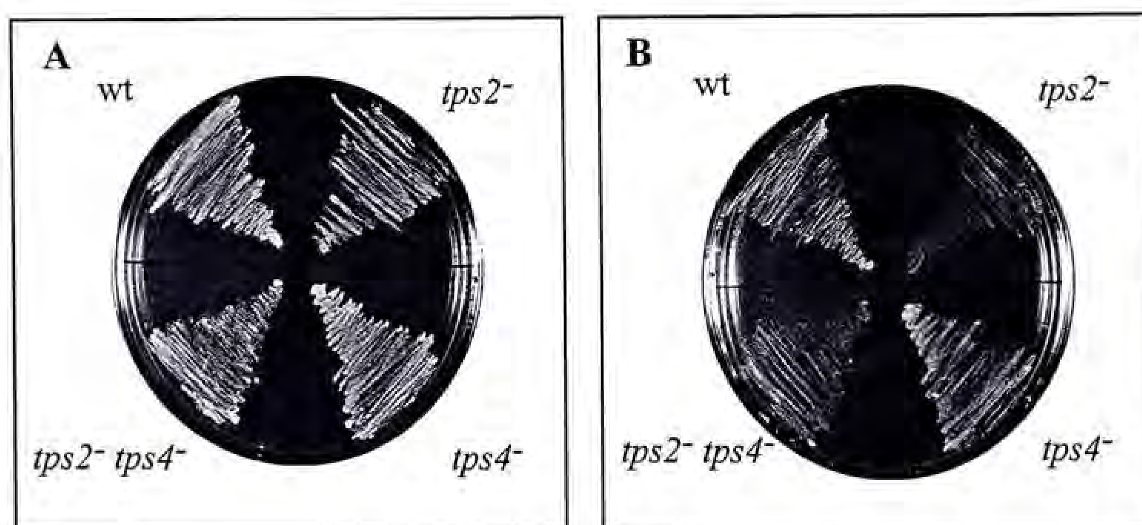


Figure 13. Growth on high osmolarity medium. Growth on YES plates containing 0.6 M KCl (**A**) and 1.0 M KCl (**B**). Strains PB003 (wild type), MRP3 (*tps2⁻*), MRP4 (*tps4⁻*), and MRP5 (*tps2⁻tps4⁻*) were first streaked out onto YES plates and incubated at 30°C for 24 h. Subsequently, the same strains were streaked onto YES plates containing 0.6 or 1.0 M KCl and further incubated at 30°C for three days.

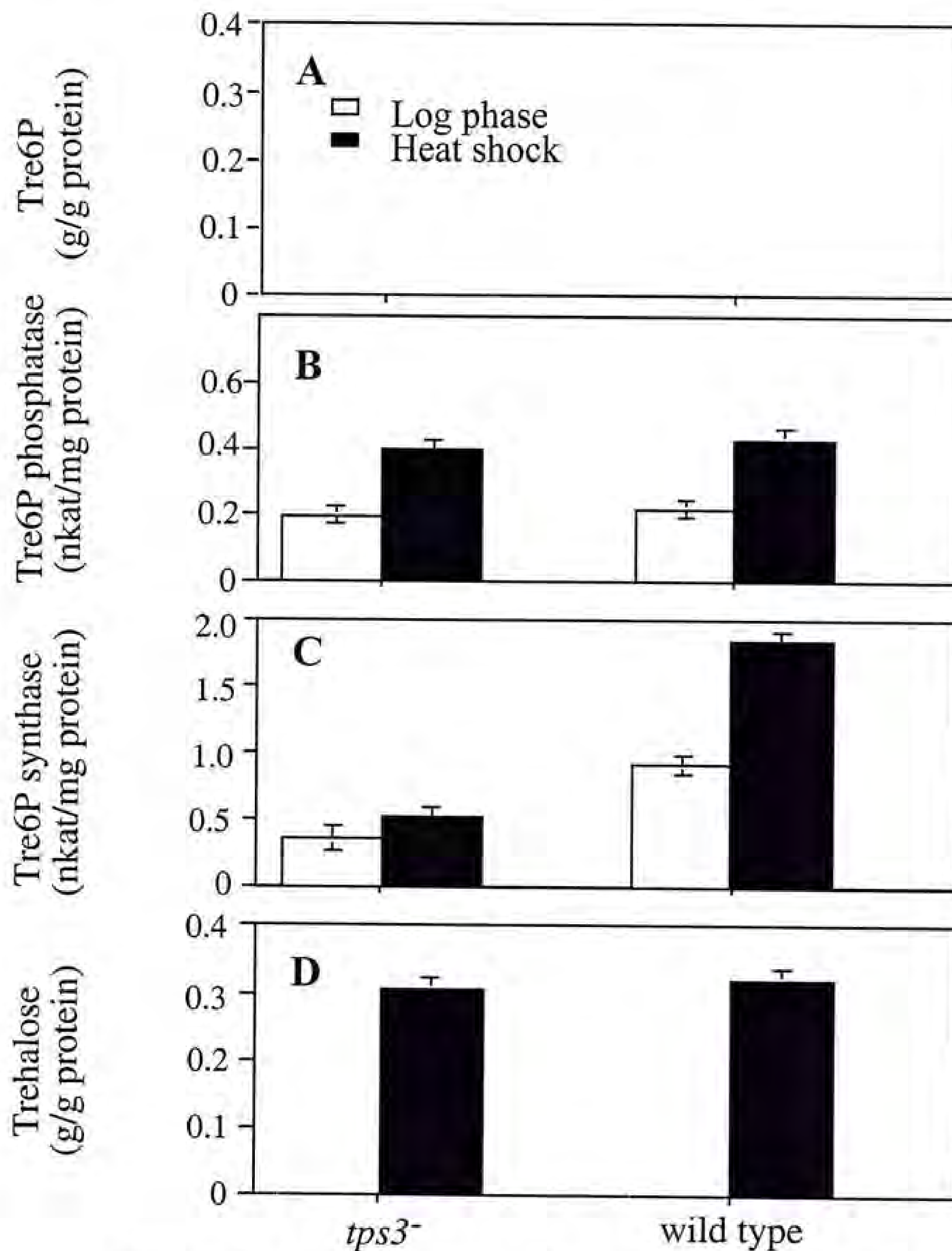


Figure 14. Tre6P levels, Tre6P phosphatase and Tre6P synthase activities, and trehalose levels before and after a heat shock. Tre6P levels **(A)**, Tre6P phosphatase **(B)** and Tre6P synthase activities **(C)**, and trehalose **(D)** levels of PB003 (wild type) and MRP11 (*tps3⁻*) strains before and after a heat shock for 1h at 40 °C. Exponentially growing cultures were heat shocked for 1 h at 40°C. Activities were assayed in permeabilized extracts. The results shown are the mean ± standard deviation of at least three independent experiments.

DISCUSSION

In this chapter the isolation of the *tps2*$^{+}$ gene was reported. It was identified by a screening protocol designed to identify clones that could complement the growth defect of an *S. cerevisiae tps2* mutant at elevated temperatures. It was assumed that the *S. pombe tps2*$^{+}$ gene encoding Tre6P phosphatase was isolated and characterized, because (i) the deduced Tps2 amino acid sequence has a high similarity to Tre6P phosphatases from various organisms, (ii) *S. pombe tps2*$^{-}$ cells have no detectable Tre6P phosphatase activity and accumulate Tre6P instead of trehalose under heat-shock conditions, and (iii) *tps2*$^{+}$ expressed from the galactose-inducible promoter restored the Tre6P phosphatase activity and complemented the temperature sensitive growth defect in an *S. cerevisiae tps2* mutant strain. As in *S. cerevisiae*, the *S. pombe tps2*$^{-}$ showed a temperature-sensitive growth defect, which might be due to the excessive accumulation of Tre6P at elevated temperatures or due to a shortage of intracellular P_i and ATP, since a great part of the cell's P_i is fixed in Tre6P. Moreover, *S. cerevisiae tps2* mutant cells are less thermotolerant than wild-type cells possibly because of the accumulation of Tre6P instead of trehalose (De Virgilio *et al.*, 1993). Tre6P, despite its structural similarity to trehalose, may not be able to protect proteins against thermal denaturation (Hottiger *et al.*, 1994). These findings demonstrate the important role of the *TPS2* gene from *S. cerevisiae* in thermotolerance induction (De Virgilio *et al.*, 1993; De Virgilio *et al.*, 1994; Hottiger *et al.*, 1994). The temperature-sensitive growth of *S. pombe tps2* mutants could be restored by introduction of the *S. cerevisiae TPS2* gene into these cells.

It was also observed that heat shock caused an increase in the activity of Tre6P phosphatase in wild-type cells. This increase was preceded by an accumulation in *tps2*$^{+}$ mRNA. A similar expression pattern has been described for *S. pombe tps1*$^{+}$ gene (Blázquez *et al.*, 1994). Thus, expression of genes coding for the catalytic activities of both Tre6P synthase and Tre6P phosphatase are strongly induced under heat-shock conditions. Nevertheless, *de novo* synthesis of proteins has been shown to be very low at 40°C in *S. pombe* (Ribeiro *et al.*, 1997; chapter 3). These high levels of *tps1*$^{+}$ and *tps2*$^{+}$ mRNA may therefore not be translated into corresponding Tps1 and Tps2 proteins. This observation is also in accordance with the previous finding that heat-induced synthesis of trehalose is not affected by the presence of the translation inhibitor cycloheximide (De Virgilio *et al.*, 1990; 1991a; Ribeiro *et al.*, 1997; see also chapter 3). Examination of the *tps2*$^{+}$ promoter region revealed the presence of a potential Atf1 binding site at position – 218 to –211 (TTACGTAC). In addition, the induction of *tps2*$^{+}$ transcript by heat shock, the requirement of Tps2 for proper sporulation and for growth in high osmolarity medium, and the rapid loss of viability of *tps2*$^{-}$ cells in stationary phase suggest that this gene may, like *tps1*$^{+}$, be regulated by the Wis1/Spc1 MAPK cascade and possibly also through Atf1 dependent activation.

Deletion of *tps4*$^{+}$ in an *S. pombe* wild-type background neither abolished Tre6P phosphatase activity, nor lead to Tre6P accumulation under any of the conditions tested.

Accordingly, *tps4⁺* expressed from the galactose-inducible promoter did not restore the Tre6P phosphatase activity nor complement the temperature sensitive growth defect of an *S. cerevisiae tps2* mutant strain. These findings suggest that Tps4 possesses no functional catalytic site for cleavage of the phosphate group from Tre6P. Comparison of Tps4 sequence with other Tre6P phosphatase sequences, showed that the *tps4⁺* amino acid sequence has the consensus motif of the B domain, but some amino acids are at variance with the consensus motif of the A domain (Figure 2B). Vogel and coworkers (1998) examined the role of the A and B domains by testing the ability of truncated Tps2 proteins to complement Tre6P phosphatase activity in an *S. cerevisiae tps2* mutant. The first two truncated *TPS2* constructs included the A and B domains, while the third construct only included the B domain. Activity of Tre6P phosphatase could only be detected in the truncated *TPS2* constructs containing both A and B domains. Tre6P phosphatase activity in the truncated *TPS2* construction containing only the B domain was virtually absent, indicating that motif A may have a crucial role for catalytic Tre6P phosphatase activity. Northern blot analysis showed that *tps4⁺* expression was mainly induced upon entry into stationary phase.

Surprisingly, a substantial decrease in trehalose accumulation under heat-shock conditions was observed in the mutants lacking *tps4⁺*. In addition, deletion of the *tps3⁺* gene caused a strong reduction in the Tre6P synthase activity *in vitro* but did not affected the *in vivo* accumulation of trehalose under the same conditions. Interestingly, deletion of the homologue *TPS3* gene in *S. cerevisiae* caused a similar phenotype. *S. cerevisiae* Tps3 has been shown to function, together with Tsl1, as potential stabilizer/and or regulator of the Tre6P synthase/phosphatase complex in *S. cerevisiae* (Reinders *et al.*, 1997; Bell *et al.*, 1998). If *S. pombe* cells also form trehalose by a multimeric protein complex, Tps4 and Tps3 may also be important candidates involved in its stabilization or regulation.

The general picture emerging from the molecular and genetic studies in this and in the previous chapter, depicts an important role of trehalose synthesis under several environmental conditions such as heat shock, high osmotic stress, and nutrient starvation. Cloning and characterization of the *tps2⁺*, *tps3⁺*, and *tps4⁺* genes has provided the basis for further studies of interesting questions which have now arisen in this context: is trehalose synthesis in *S. pombe* also performed by a multifunctional protein complex and, if so, what are the precise roles of the various subunits? These questions will be addressed in the next chapter.

CHAPTER 5

STRUCTURAL COMPOSITION OF THE TRE6P SYNTHASE COMPLEX IN *S. POMBE*

INTRODUCTION

Based on the knowledge gained during this thesis it is obvious that the key enzymes of trehalose metabolism and the induction of thermotolerance are regulated differently in *S. pombe* and *S. cerevisiae*. However, both *S. pombe* and *S. cerevisiae* cells promote trehalose formation mainly in a two-step process. In the first step, Tre6P synthase catalyzes Tre6P formation using UDPG and G6P as substrates. The second step is catalyzed by a specific Tre6P phosphatase, which cleaves off the phosphate group from Tre6P, thus liberating trehalose. The same pathway for trehalose biosynthesis has also been demonstrated in several other organisms, including several bacteria, filamentous fungi, insects and yeasts (see General Introduction; chapter 1). However, in contrast to *Escherichia coli*, where the two enzymatic activities reside in separate enzymes encoded by *otsA* (Tre6P synthase activity) and *otsB* (Tre6P phosphatase activity), *S. cerevisiae* cells synthesize trehalose by a large multimeric complex containing both Tps1 and Tps2 activities. Besides Tps1 and Tps2, two further proteins have been identified to participate in the Tre6P synthase/phosphatase complex in *S. cerevisiae*, Tsl1 and Tps3. The evidence that Tps3 as well as Tsl1 are part of the Tre6P synthase/phosphatase complex is based on two-hybrid analyses, as well as on genetic analyses (Reinders *et al.*, 1997; Bell *et al.*, 1998). Accordingly, both Tsl1 and Tps3 are able to interact *in vivo* with Tps1 and Tps2. Furthermore, single deletions of *TPS3* or *TSL1* have only limited effects on the heat-induced accumulation of trehalose, while a double mutant is severely impaired in heat-induced trehalose accumulation (Reinders *et al.*, 1997; Bell *et al*, 1998). The functions of Tsl1 and Tps3 are less clear, but these proteins seem to contribute to regulation and/or stabilization of the Tre6P synthase/phosphatase complex.

Surprisingly, the *S. cerevisiae TPS1* has been shown to be allelic to *FDP1* (Van de Poll and Schamhart, 1977), *CIF1* (Navon *et al.*, 1979), *BYP1* (Breitenbach-Schmitt *et al.*, 1984), *GGS1* (Thevelein 1992; Van Aelst *et al.*, 1993), and *GLC6* (Cannon *et al.*, 1994). With the exception of *glc6*, which specifically affects glycogen accumulation, all mutant alleles displayed the same pleiotropic effects including a deficiency in growth on fructose and glucose, lack of glucose induced signalling events and poor sporulation. Moreover, these mutants have characteristics that resemble those of strains with a constitutively high activity of cAPK, such as a low trehalose and glycogen content under starvation conditions. Addition of glucose to an *S. cerevisiae tps1* mutant strain causes hyperaccumulation of sugar phosphates, a drop in the levels of ATP and free P_i, and an arrest of glycolysis (Thevelein and Hohmann, 1995). How mutations in an enzyme involved in trehalose biosynthesis can lead to such diverse phenotypic effects, in particular the inability to grow on glucose, was puzzling. It is worth recalling at this point that disruption of the *TPS1* homologue in the distantly related yeast *Kluyveromyces lactis* causes the same phenotype as in *S. cerevisiae* (Luyten *et al.*, 1993). Notably, however, disruption of the *TPS1* homologue in *S. pombe*, in the dimorphic yeast *Candida albicans*, and in the thermophilic yeast *Hansenula polymorpha* does not lead to the inability of growth on glucose.

The first attempt to explain the pleiotropic phenotype of the *TPS1* deletion was made by Blázquez and coworkers (1993), who discovered that Tre6P competitively inhibits hexokinase II (*HXK2*) of *S. cerevisiae*, as well as hexokinases of some other yeasts including *Yarrowia lipolytica* and *K. lactis*, but not the hexokinase of *S. pombe*. In accordance with these results, deletion of the *HXK2* in an *S. cerevisiae tps1* deletion mutant was found to suppress both the growth and signalling defects of the *tps1* deletion mutant on glucose. Thus, Tps1 could be involved in regulation of sugar phosphorylation, via Tre6P-mediated inhibition of hexokinase (Hohmann *et al.*, 1993). Alternative models addressing the mechanism by which Tps1 could restrict glycolysis have been proposed (Thevelein and Hohmann, 1995). The first model suggests the existence of a "General Glucose Sensor" (GGS), a complex comprising a glucose carrier, a sugar kinase, and Tps1. Tps1 would possess an additional function besides that in trehalose formation: as a direct regulator of sugar uptake and phosphorylation (Thevelein, 1992). The second model is the so-called phosphate recovery model. In this model, trehalose synthesis could serve as a metabolic buffer system by recycling P_i when sugar phosphates accumulate and, thereby, ensure downstream glycolytic flux through glyceraldehyde-3-phosphate dehydrogenase reaction (Hohmann *et al.*, 1993). The last model is based on the *in vitro* inhibition of *S. cerevisiae* hexokinase activity by Tre6P (Blázquez *et al.*, 1993; for a review of these models, see Thevelein and Hohmann, 1995).

In *S. cerevisiae* it can be expected that a great part of Tre6P has to be channelled through a complex in order to avoid high cytosolic Tre6P concentrations. Since *S. pombe* hexokinase is not inhibited by Tre6P, it may be unnecessary to control metabolic channelling of Tre6P so tightly. Therefore, it was investigated in this chapter whether Tre6P synthase is, as in *S. cerevisiae*, part of a multimeric complex, or whether it functions rather as an isolated monomeric enzyme.

RESULTS

Tre6P synthase and Tre6P phosphatase activities, Trehalose and Tre6P levels in different deletion strains

To assess the relative importance of the known genes possibly involved in trehalose formation in *S. pombe*, a set of four isogenic deletion strains was constructed in the PB003/PB004 background (Table 1; see page 38 in chapter 2). The entire open reading frames of all genes were deleted using a PCR-based method as described in Materials and Methods (chapter 2). To first check whether $tps1^+$, $tps2^+$, $tps3^+$, or $tps4^+$ deletion affects Tre6P synthase and Tre6P phosphatase activities and the levels of trehalose and Tre6P, PB003 (wild type), PBL-17 ($tps1^-$), MRP3 ($tps2^-$), MRP4 ($tps4^-$), MRP5 ($tps2^- tps4^-$), and MRP11 ($tps3^-$) cells were grown to stationary phase. Tre6P synthase and Tre6P phosphatase activities were measured in

permeabilized extracts as described in Materials and Methods (chapter 2). Trehalose and Tre6P were extracted and quantified as described in Materials and Methods (chapter 2).

The *tps1⁺* gene is essential for Tre6P synthase activity, since no activity could be measured in all strains lacking this gene (Figure 1A). However, in strains with a functional *tps1⁺* gene, deletion of the other genes also affected Tre6P synthase activity (Figure 1A). Deletion of *tps2⁺* caused a decrease of 30% in the Tre6P synthase activity. Deletion of *tps4⁺* in wild type background or in a *tps2⁻* background caused a 40% decrease in Tre6P synthase activity (Figure 1A). More drastic was the deletion of the *tps3⁺* gene, which caused a 50% decrease in Tre6P synthase in stationary-phase cells when compared to the activities observed in the wild-type strain PB003 under the same conditions (Figure 1A).

Tre6P phosphatase activity was completely absent in all strains lacking *tps2⁺* confirming that the presence of *tps2⁺* is essential for Tre6P phosphatase (Figure 1B; see also chapter 4). Different from the situation in *S. cerevisiae*, where *TPS1* deletion led to almost complete loss of Tre6P phosphatase activity (Reinders *et al.*, 1997; Bell *et al.*, 1998), deletion of the *S. pombe tps1⁺* gene caused a decrease of only 38% in Tre6P phosphatase activity in stationary-phase. Deletion of *tps4⁺* caused a 35% decrease in Tre6P phosphatase activity under the same condition. Furthermore, less drastic was the deletion of the *tps3⁺* gene, which caused a 20% decrease in Tre6P phosphatase activity (Figure 1B).

In strains lacking the *tps1⁺* gene no trehalose accumulation could be detected (Figure 1C; see also chapter 3). Although *tps3⁻* strains showed a strong decrease in the Tre6P synthase activity measured *in vitro* their *in vivo* Tre6P synthase activities seemed to be sufficient to support normal trehalose accumulation (Figure 1C). However, *tps4⁺* deletion caused both a decrease in Tre6P synthase activity and a 70% decrease in trehalose levels (Figure 1C). Deletion of *tps2⁺* caused a complete loss of Tre6P phosphatase activity and, therefore, high amounts of Tre6P accumulated in such strains (Figure 1D). Nevertheless, trehalose accumulation was still observed in *tps2⁻* strains, probably because of the presence of unspecific phosphatases in the permeabilized extracts (Figure 1C and 1D). Strains lacking *tps1⁺*, *tps4⁺*, or *tps3⁺*, as expected from their enzyme profiles (Figure 1A and 1B), did not accumulate Tre6P (Figure 1D). Taken together, the data collected in stationary phase *tps1⁻*, *tps2⁻*, *tps3⁻*, *tps4⁻*, and *tps2⁻ tps4⁻* cells reflect a similar picture as emerged in the previous chapter when the same mutants were analyzed under heat shock conditions.

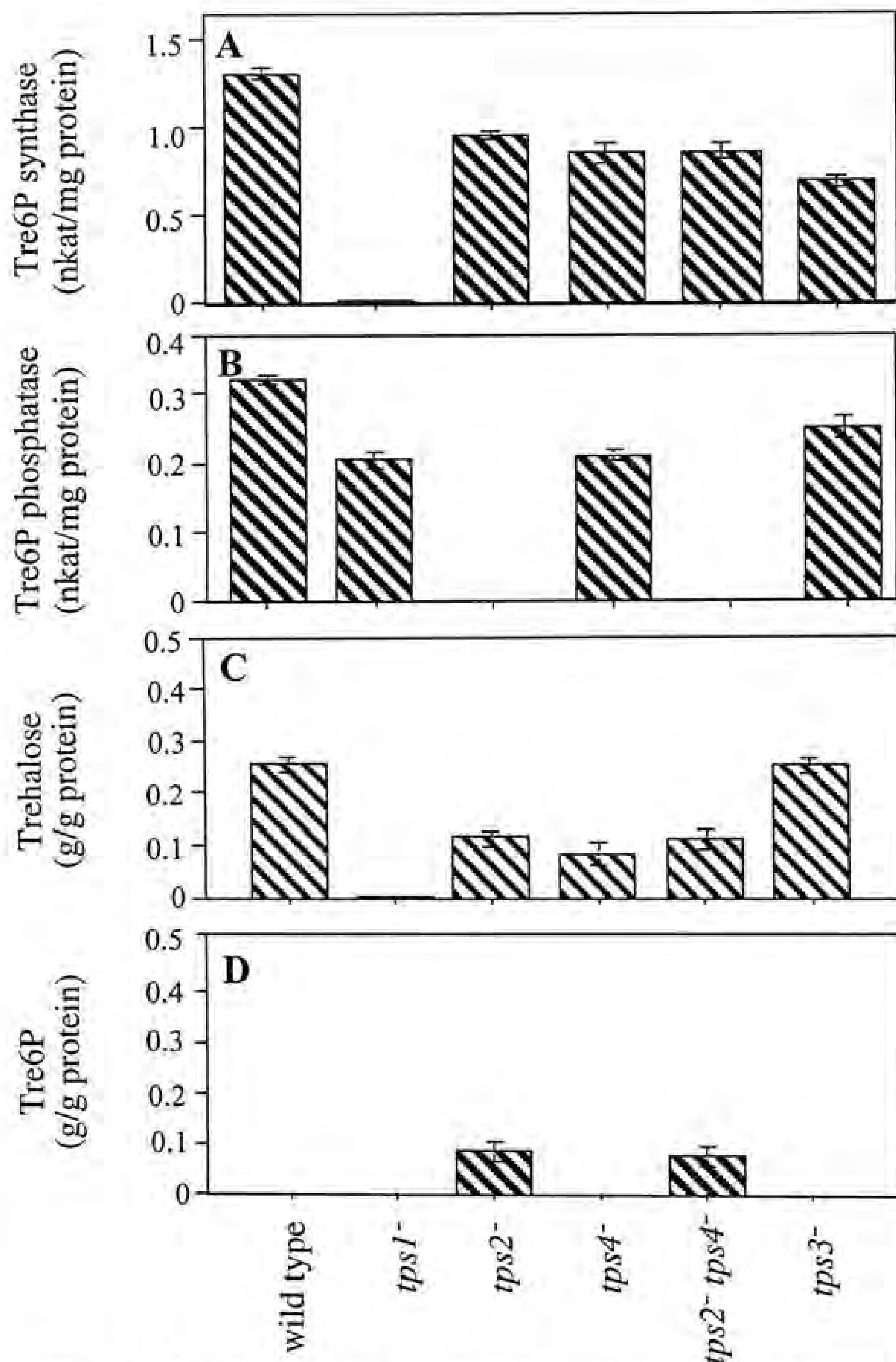


Figure 1. Tre6P synthase and Tre6P phosphatase activities, trehalose, and Tre6P contents in stationary phase. Strains PB003 (wild type), PBL-17 (*tps1⁻*), MRP3 (*tps2⁻*), MRP4 (*tps4⁻*), MRP5 (*tps2⁻ tps4⁻*), and MRP11 (*tps3⁻*) were grown for 3 days at 27°C in SD medium containing 1% glucose and fivefold excess of auxotrophic requirements to ensure that the carbon source was the limiting factor. Cell extracts were obtained as described in Materials and Methods (chapter 2) and Tre6P synthase activities **(A)**, Tre6P phosphatase activities **(B)**, Trehalose **(C)**, and Tre6P levels **(D)** were determined. Activities were assayed in permeabilized extracts as described in Materials and Methods (chapter 2). The results shown are the mean ± standard deviation of at least three independent experiments.

Molecular weight determination of Tps1 and Tps2 in *S. pombe*

FPLC on Superose 6

In order to evaluate whether trehalose formation in *S. pombe* is also performed by a multifunctional protein complex, yeast cell extracts from an *S. pombe* wild-type strain (PB003) grown to stationary phase were fractionated according to the molecular mass of the proteins using a Superose 6 HR 10/30 FPLC column (Pharmacia). An *S. cerevisiae* wild-type (YSH6.106.-3A; Table 1; page 38; chapter 2) was used for comparison. The Superose 6 FPLC was chosen because the molecular size of the Tre6P synthase/phosphatase complex in *S. cerevisiae* is between 600 and 800 kDa (Bell *et al.*, 1992; Vuorio *et al.*, 1993; De Virgilio *et al.*, 1993; Reinders *et al.*, 1997; Bell *et al.*, 1998) and because the column separates proteins of 5 kDa to 5000 kDa (see chapter 2 for further details). After elution from the Superose 6 column the fractions were kept on ice and immediately used for determination of Tre6P synthase and Tre6P phosphatase activity as described in Materials and Methods (chapter 2).

As expected, when the *S. cerevisiae* wild-type extract was fractionated on the Superose 6 column, the elution position of Tre6P synthase activity coincided with the elution position of Tre6P phosphatase activity. This elution position corresponded to a molecular weight of 600 to 800 kDa (Figure 2A) which is in agreement with previous reports (Bell *et al.*, 1992; Vuorio *et al.*, 1993; De Virgilio *et al.*, 1993; Bell *et al.*, 1998). Interestingly, a similar profile was observed with *S. pombe*. Both Tre6P synthase and Tre6P phospatase activities eluted in fractions 20 to 22 (Figure 2B). Thus, *S. pombe* Tps1 and Tps2 proteins may also be in a multimeric complex. The possible complex is smaller than that of *S. cerevisiae* and has a molecular mass of around 200 to 400 kDa.

FPLC on Superdex 200

In order to achieve a more precise estimate of the trehalose synthesis complex of *S. pombe*, a Superdex 200 HR 10/30 FPLC column (Pharmacia) was used. This column separates proteins with an optimal separation range from 10 kDa to 600 kDa.

Initially, a molecular weight calibration curve was obtained using a protein calibration kit (Pharmacia). It is depicted in Figure 3. The kit included the following standard proteins: ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (BSA; 67 kDa), and β-lactoglobulin (37 kDa). K_{av} was the chosen parameter for the calibration curve mainly because it is less sensitive to errors which may be introduced as a result of variation in column preparation and column dimensions.

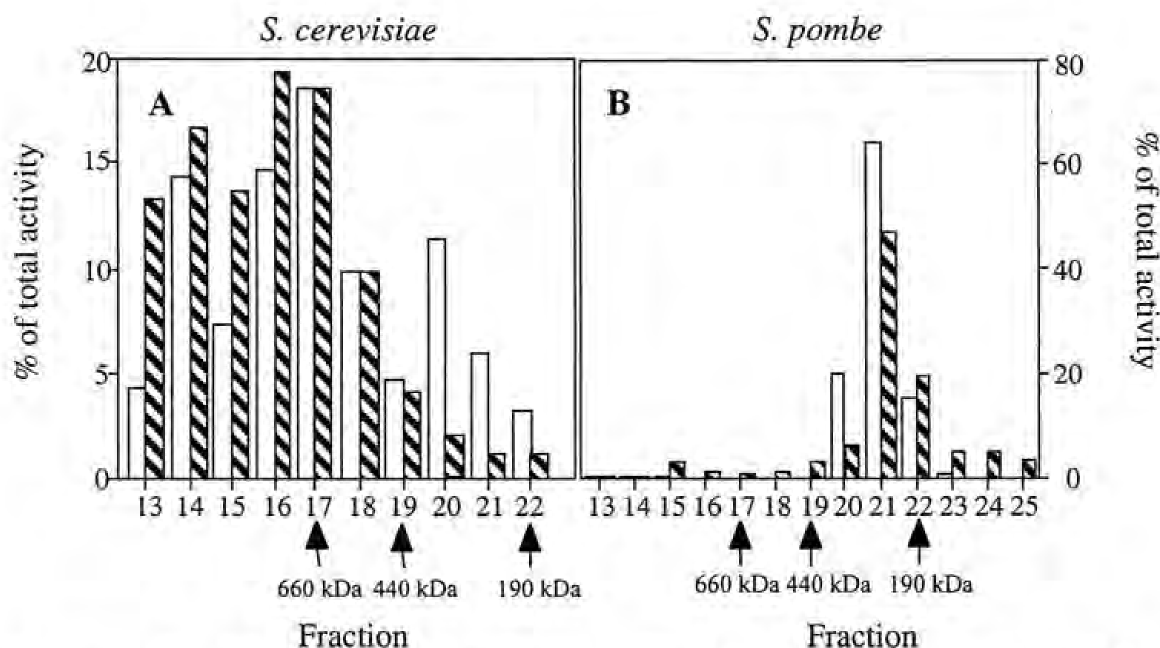


Figure 2. Molecular weights of Tre6P synthase and Tre6P phosphatase determined on a Superose 6 FPLC. Tre6P synthase (□) and Tre6P phosphatase (▧) activities in cell fractions of wild type strains from *S. cerevisiae* **(A)** and *S. pombe* **(B)** separated by Superose 6 FPLC. Crude extracts of stationary-phase cells grown for 2 days on YPD or YES medium were applied to the Superose 6 HR 10/30 FPLC column (Pharmacia) and eluted as described in Materials and Methods (chapter 2). Activities were assayed as described in Materials and Methods (chapter 2). Fraction 17 corresponds to a molecular mass around 660 kDa. Fraction 19 corresponds to a molecular mass around 440 kDa and fraction 22 corresponds to a molecular weight around 190 kDa.

Subsequently, *S. pombe* cells were grown to stationary phase on YES media. The cells were grown for 48 to 52 hours because, as previously shown in chapter 4, deletion of *tps2⁺* gene led to a loss of cell viability when cells spent longer time periods in stationary phase (see Figure 12; chapter 4). Crude extracts from the different deletion strains were obtained as described in Materials and Methods (chapter 2) and fractionated on the FPLC Superdex 200 column. Figure 4 shows the percentage per fraction of the total activity of Tre6P synthase and Tre6P phosphatase activities in a wild-type strain (PB003; Figure 4A), in a *tps1⁻* strain (PBL-17; Figure 4B), in a *tps2⁻* strain (MRP3; Figure 4C), in a *tps4⁻* strain (MRP4; Figure 4D), in a *tps2⁻tps4⁻* strain (MRP4; Figure 5E), and in a *tps3⁻* strain (MRP11; Figure 4F). Surprisingly, when smaller fractions were collected, Tps1 and Tps2 did not elute at the same position as observed before (Figure 2B) and two distinct pools were clearly observed (Figure 4A to Figure 4F). In the wild-type extract, Tps1 eluted in fractions 27 to 30 corresponding to molecular weights in the range of 454 to 247 kDa (Figure 4A). Tps2 eluted from the column later and its maximum of activity was in fractions 30 to 33, corresponding to molecular weights in the range of 247 to 134 kDa (Figure 4A). As expected, in *tps1⁻* cells no Tre6P synthase activity could be

measured, but, interestingly, the absence of Tps1 did not change the elution position of Tps2 (Figure 4B). Similarly, Tre6P synthase was still present in a higher molecular weight complex, when Tps2 was not present (Figure 4C). Since the *tps2⁺* gene was shown to be required for Tre6P phosphatase activity, its activity was absent in strains lacking this gene (Figure 4C). The high molecular weight Tre6P synthase complex (250 to 450 kDa) was still present and eluted at the same position as showed before in cell extracts of the wild-type strain even in the absence of Tps4 (Figure 4D), of both Tps2 and Tps4 (Figure 4E), and of Tps3 (Figure 4F). Deletion of *tps4⁺* did not affect the Tre6P phosphatase elution profile when compared to the wild-type profile (Figure 4D). However, Tre6P phosphatase activity was also absent in the double mutant because of the lack of *tps2⁺* gene (Figure 4E). In a cell extract of a *tps3⁻* strain the Tre6P phosphate was also present at the normal elution position (*i.e.*, in fractions 30 to 33; Figure 4F). Taken together, *S. pombe* Tre6P synthase activity could only be measured in fractions with a high molecular mass of 250-450 kDa and could not be found in fractions which corresponded to lower molecular weights. Thus, in contrast to the situation in *S. cerevisiae*, the *S. pombe* Tps1 protein seems not to occur as a monomeric subunit and seems to be present in a complex which does not comprise Tre6P phosphatase activity.

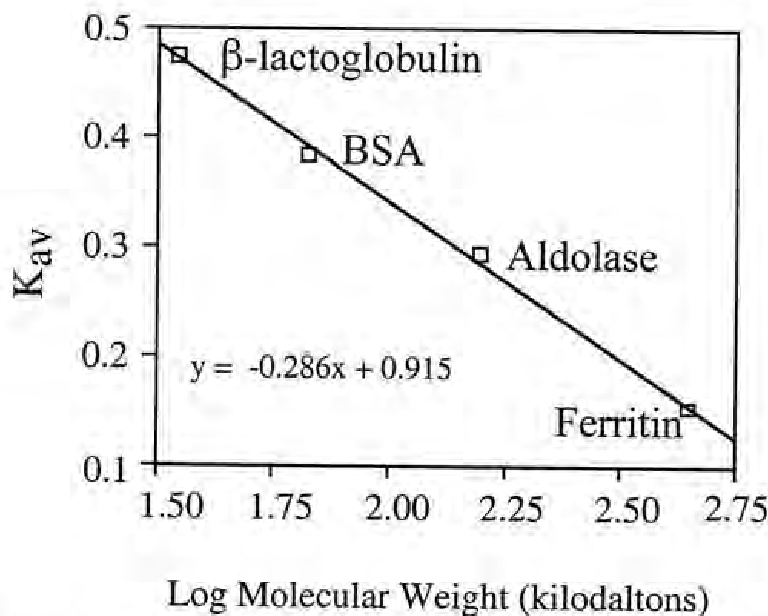


Figure 3. Calibration curve for the Superdex 200 HR 10/30 column (Pharmacia). A calibration kit of proteins (Pharmacia) containing ferritin (440 kDa), aldolase (158 kDa), BSA (67 kDa), and β-lactoglobulin (36 kDa) was applied to the column and eluted as described in Materials and Methods (chapter 2). The half-height of the leading edge of the eluted protein was taken as its elution volume (Ve); the leading edge half-height of blue dextran was taken as the void volume (Vo= 8.4 ml) and the total bed volume (Vt) for the column was taken as 24 ml. The Kav for each protein was then calculated as (Ve-Vo)/(Vt-Vo).

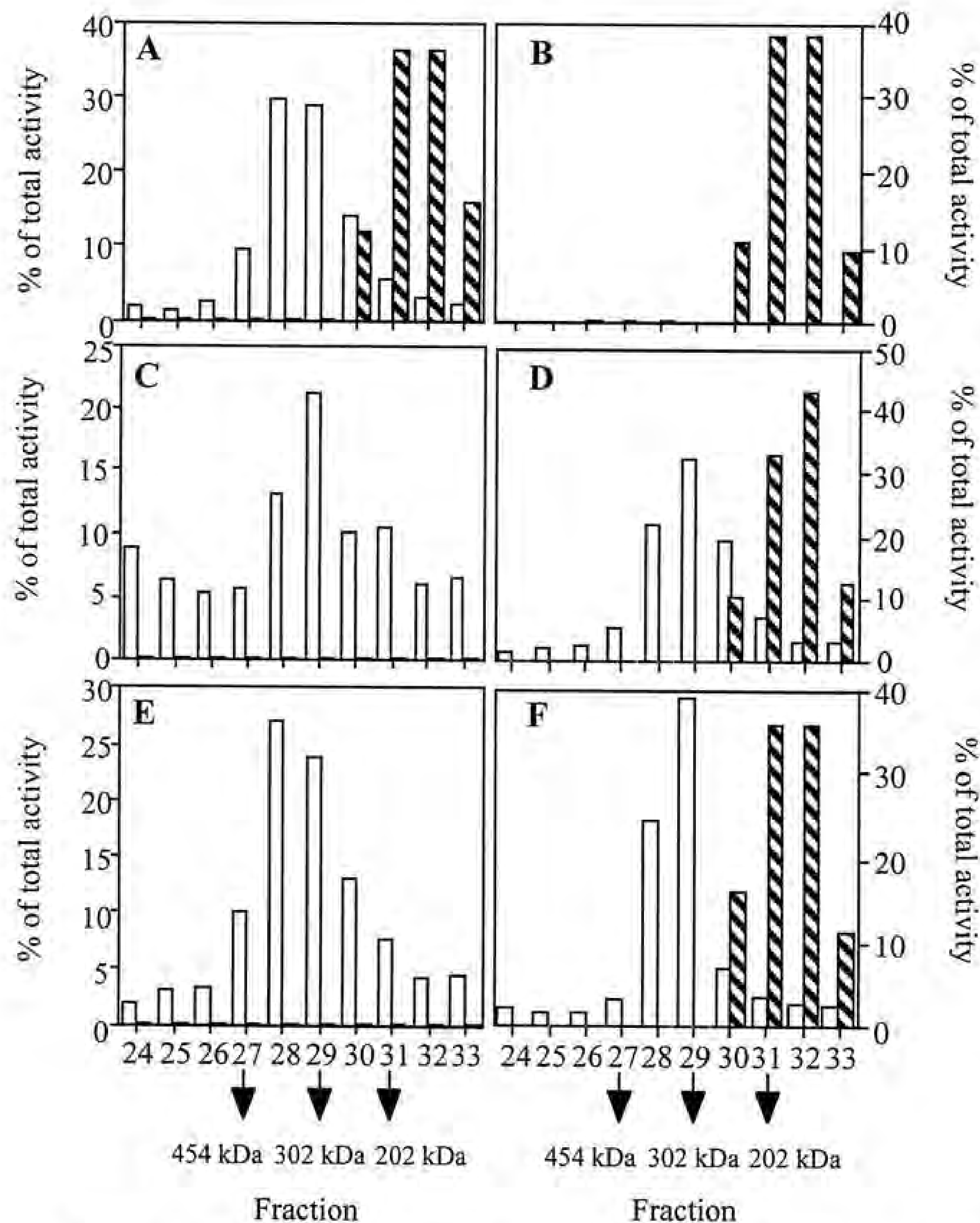


Figure 4. Determination of the molecular weight of Tre6P synthase and Tre6P phosphatase enzymes in *S. pombe*. Crude extracts of early stationary-phase cells from PB003 (wild type; **A**), PBL-17 (*tps1*$^-$; **B**), MRP3 (*tps2*$^-$; **C**), MRP4 (*tps4*$^-$; **D**), MRP5 (*tps2*$^-$ *tps4*$^-$; **E**) and MRP11 (*tps3*$^-$; **F**) were applied to a Superdex 200 HR 10/30 FPLC column (Pharmacia) and eluted as described in Materials and Methods (chapter 2). The strains were grown for two days on YES medium containing 1% glucose and fivefold excess of auxotrophic requirements to ensure that the carbon source was the limiting factor. Tre6P synthase (o) and Tre6P phosphatase (▨) activities were assayed in the fractions as described in Materials and Methods (chapter 2). Fractions 27 and 28 correspond to 454 to 370 kDa and fractions 29 and 30 correspond to 302 to 247 kDa. Fractions 31 and 32 correspond to 202 to 165 kDa.

DISCUSSION

S. cerevisiae cells perform trehalose formation by a large multimeric complex comprising both Tre6P synthase and Tre6P phosphatase activities. The reason why *S. cerevisiae* cells employ a multimeric complex to synthesize trehalose is still puzzling, but there is some evidence that it might have to do with the striking role of Tps1 in regulating glycolysis (Thevelein and Hohmann, 1995; Reinders *et al.*, 1997, Bell *et al.*, 1998).

In this chapter it was shown for the first time that in the distantly related yeast *S. pombe*, trehalose formation might also be catalyzed by a multimeric complex comprising Tps1 activity. Fractionation of *S. pombe* wild-type extracts on a size-exclusion column showed that Tps1 activity was present in fractions which correspond to the size of 250 to 450 kDa. No monomeric activity could be detected even in the fractionated extracts of the different deletion strains.

In contrast to the situation in *S. cerevisiae*, deletion of the *tps1*$^+$ gene had only a limited effect on the Tps2 activity. Moreover, deletion of the *tps2*$^+$ gene did not cause a substantial decrease in Tps1 activity in stationary phase. These findings suggest that if Tps1 and Tps2 activities are present in the same complex they must only be weakly associated. However, the most remarkable observation was that deletion of the *tps2*$^+$ gene did not produce any substantial change in the size of Tre6P synthase complex and that two distinct pools containing Tps1 and Tps2 activities were observed. These findings lead to the fascinating conclusion that Tre6P phosphatase might not be part of the Tre6P synthase complex and that it is also not essential for stabilization of the Tre6P synthase complex. In addition, the total estimated molecular mass of the Tre6P synthase complex is between 250 and 450 kDa, which is much smaller than the sum of the molecular weights of Tps1, Tps2, Tps3 and Tps4 together. Based on the complex profile in the different deletion mutants, it is likely that Tps2, Tps3, and Tps4 are not part of such a complex. It can therefore be speculated that Tps1 may form a homomeric complex. As similar situation has been described for the *S. cerevisiae* enzymes glutamine synthetase and glutamate synthase involved in an alternative pathway for the formation of glutamate (Mitchell and Magasanik, 1983; Valenzuela *et al.*, 1998). It may, however, not be excluded that Tps1 is very weakly associated with the other subunits, or that Tps1 is associated with other proteins, which have not yet been identified.

Up to now, *S. pombe* has four genes which may be involved in trehalose synthesis: *tps1*$^+$, *tps2*$^+$, *tps3*$^+$, and *tps4*$^+$. Three of them are homologous to corresponding genes in *S. cerevisiae*. Analysis of the isogenic set of strains carrying deletions of *tps1*$^+$, *tps2*$^+$, *tps3*$^+$, and *tps4*$^+$ confirmed the previously suggested roles for Tps1 and Tps2 (Blàzquez *et al.*, 1994; chapter 4). Strains lacking *tps1*$^+$ lost their Tre6P synthase activity and were unable to synthesize trehalose or Tre6P. Along the same lines, strains lacking *tps2*$^+$ also lost their Tre6P phosphatase activity and accumulated large amounts of Tre6P instead of trehalose, as long as they possessed a functional *tps1*$^+$ gene. These data confirmed that *tps2*$^+$ codes for the Tre6P phosphatase (see also chapter 4). Interestingly, some trehalose accumulation was also present in

strains with a functional *tps1*$^+$ but lacking *tps2*$^+$. It was suggested that this residual trehalose was probably due to the presence of unspecific phosphatases. The gene termed *tps4*$^+$ was described at the EMBL database as a possible Tre6P phosphatase, but its deletion only caused a decrease in the *in vivo* trehalose accumulation, but no appearance of Tre6P, or decrease in Tre6P phosphatase activity (see also chapter 4). However, a substantial decrease in Tre6P synthase activity was observed in stationary phase *tps4*$^-$ strains. The *S. pombe TPS3* homologue, *tps3*$^+$, was submitted to EMBL GenBank (accession number Z98850). Its deletion caused a strong reduction in the Tre6P synthase activity *in vitro* but did not affect the *in vivo* accumulation of trehalose in stationary phase.

Possibly, the formation of trehalose by a complex in *S. cerevisiae* allows a tighter control of the cellular Tre6P levels, which otherwise might cause too much inhibition of hexokinase activity. This could explain why the *S. cerevisiae* Tps1 occurs both in a complex and as monomeric protein. *S. pombe* cells might control the first steps of glycolysis in a different way since its hexokinase is not inhibited by Tre6P. The fact that no monomeric Tps1 could be detected in *S. pombe* is in agreement with this hypothesis. If Tps2 is not present in the complex there is no channelling of Tre6P inside the complex and Tre6P would leak out into the cytosol which would not cause a problem in *S. pombe* because of its hexokinase being insensitive to Tre6P.

Summing up, the results presented here showed for the first time that Tps1 is also part of a multimeric complex in the fission yeast *S. pombe*. Importantly, however, neither Tps2, nor Tps3, nor Tps4 are part of this Tre6P synthase complex. It is therefore possible that the complex is composed of multiple Tps1 subunits (from 4 to 8 subunits), or of additional, not yet identified, subunits. Interestingly, the Tps2 protein is also part of a protein complex with a molecular mass around 200 kDa in which neither Tps3 nor Tps4 seemed to be present. Accordingly, Tps2 may also be functioning as a homodimer or be associated with other unknown proteins. Such results are very promising and the purification of the Tre6P synthase complex and characterization of the actual components of this complex would be extremely valuable in future experiments.

CHAPTER 6

GENERAL DISCUSSION

Temperature is one of the most important environmental parameters influencing all activities of microorganisms. In their natural habitats yeasts often experience temperatures considerably above or below the temperature limits for growth. When *S. cerevisiae* cells growing exponentially on glucose are exposed to temperatures much higher than those permiting growth, they rapidly lose viability. However, when these cells are first submitted to a mild heat shock prior to the lethal heat shock, they display stress responses mechanisms which increase their ability to survive the injuries caused by a subsequent lethal heat shock. The increase in survival of the lethal treatment, induced by exposure to mild temperatures has been defined as acquired thermotolerance (Lindquist, 1986).

There is substantial evidence from studies in *S. cerevisiae* that both trehalose and hsp synthesis play important roles in the development of heat-induced thermotolerance. However, unequivocal proof that trehalose can contribute to increased thermotolerance could not be obtained with *S. cerevisiae tps1* mutants because of their pleiotropic phenotype. *S. pombe tps1⁻* mutants do not show the pleiotropic defects observed in the *S. cerevisiae tps1* deletion mutant, and are therefore able to grow on glucose. This finding provided the basis for one of the central questions in this thesis: what is the relative contribution of trehalose to development of induced thermotolerance in *S. pombe*?

IS TREHALOSE AN ELEMENT OF THE HEAT-SHOCK RESPONSE IN *S. POMBE*?

To shed light on the specific role of general protein synthesis for thermotolerance, the upper temperature limit for protein synthesis was first determined. The optimum temperature for induction of hsp synthesis was shown to be around 37.5°C. Temperatures higher than 40°C completely prevented their synthesis. However, the optimum temperature for trehalose accumulation was shown to be at 42.5°C. At lower temperatures (35°C) trehalose was produced, but in lower amounts. Analysis of acquisition of thermotolerance in *tps1⁻* mutant cells showed that during a mild heat shock at temperatures higher than 40°C and lower than 47.5°C, *tps1⁻* mutant cells were extremely thermosensitive, even in the absence of the protein translational inhibitor, cycloheximide. However, they were quite thermotolerant to mild heat shocks at temperatures lower than 40°C. Addition of cycloheximide prior to the mild heat shock reduced, but did not abolish, acquisition of thermotolerance at these lower temperatures. These findings suggest that the relative importance of hsp and trehalose synthesis for the acquisition of thermotolerance is strongly influenced by temperature. However, additional,

unknown, hsp- and trehalose-independent factors contribute substantially to the acquired thermotolerance.

ARE TREHALOSE FORMATION AND ACQUISITION OF THERMOTOLERANCE UNDER THE CONTROL OF THE *S. POMBE* Ras/cAMP PATHWAY?

A second question which emerged was also based on studies in *S. cerevisiae* with respect to the Ras/cAMP pathway. In *S. cerevisiae*, trehalose metabolism and acquisition of thermotolerance were shown to be under the negative control of cAPK (Toda *et al.*, 1985; Hottiger *et al.*, 1989; Schüller *et al.*, 1994). *S. cerevisiae* cells with low constitutive cAPK activity were remarkably thermotolerant, while cells with high constitutive cAPK activity were thermosensitive (Ruis and Schüller, 1995).

Since the Ras/cAMP pathways in *S. cerevisiae* and in *S. pombe* differ profoundly (Maeda *et al.*, 1994b), it could be expected that both yeasts may differ in the regulation of trehalose metabolism and thermotolerance acquisition. Interestingly, *S. pombe* *pka1$^-$* cells were highly thermotolerant even in the absence of a mild heat shock. It was found that only *pka1$^-$* cells carrying a functional *tps1$^+$* gene were able to accumulate trehalose and to develop higher thermotolerance levels during the challenging heat shock, whereas *pka1$^-$tps1$^-$* cells showed no trehalose accumulation and lower thermotolerance levels. Despite the lack of trehalose formation the double mutants were still more thermotolerant than wild-type cells. These findings could be due to elevated levels of hsps resulting from deletion of the *pka1$^+$* gene. An alternative way of controlling the cAMP intracellular levels *in S. cerevisiae* cells can be achieved through Ssa1, which seems to bind to Cdc25. Recruitment of hsps to denatured proteins in response to stress conditions would reduce interaction with Cdc25 and, hence, lead to reduced activity of the Ras/cAMP pathway, which would enhance the general stress response (Geymonat *et al.*, 1998). An elevated level of hsps could, for instance, positively influence general translation by stabilizing ribosomes, as several members of the Hsp70 family are known to be associated with ribosomes. The exact levels of various hsps could be determined by the use of specific antibodies. Alternatively, hsps could more specifically stabilize factors directly required for acquisition of thermotolerance, *e.g.*, they could stabilize Tre6P synthase, or factors in stress signalling, like components of the Wis1/Spc1 MAPK cascade. It seems, therefore, important to focus on transduction pathways involved in acquisition of stress resistance in order to obtain a more detailed picture of the factors that contribute to thermotolerance in *S. pombe*.

WHAT IS THE IMPORTANCE OF TRE6P PHOSPHATASE FOR DEVELOPMENT OF STRESS RESISTANCE IN *S. POMBE*?

In order to unravel the role of trehalose accumulation in heat shocked cells it was important to clone both the gene coding for Tre6P synthase (*tps1*$^+$, Blázquez *et al.*, 1994), as well as the gene coding for Tre6P phosphatase (*tps2*$^+$).

The *S. pombe TPS2* homologue, *tps2*$^+$, was isolated and found to encode a 99 kDa protein, deletion of which caused heat-induced accumulation of Tre6P and loss of Tre6P phosphatase activity. Deletion of the *tps2*$^+$ gene also caused temperature-sensitive growth at elevated temperatures. This may be due to the excessive accumulation of Tre6P under such conditions, which may be toxic due to a shortage of intracellular P_i that is fixed in Tre6P. Importantly, heat shock induced an increase in the *S. pombe* Tre6P phosphatase activity which was preceded by accumulation of *tps2*$^+$ transcript, suggesting that *tps2*$^+$ is probably subject to transcriptional regulation under heat-shock conditions. Increase in the mRNA levels resemble the pattern described for members of the heat-shock gene family. This observation supports the earlier results that trehalose accumulation is an important step in the heat-shock response in *S. pombe* cells.

Analysis of the *tps2*$^-$ mutants revealed that loss of Tps2 caused rapid cell death upon entry into stationary phase and decreased the ability of cells to form asci and to undergo meiosis. Furthermore, under osmotic stress conditions (growth on YES plates containing 1 M KCl) strains lacking the *tps2*$^+$ gene grew very poorly when compared to wild type strains. Taken together, these observations point towards an important role of *tps2*$^+$ under adverse conditions such as osmotic stress, and nutrient depletion. Again, a link between trehalose formation and stress conditions was confirmed.

STRUCTURAL ANALYSIS OF TREHALOSE FORMATION IN *S. POMBE*

Characterization of the *tps2*$^+$, *tps3*$^+$ and *tps4*$^+$ genes brought new insights into trehalose formation in *S. pombe*. It was surprising that the *in vitro* activity of Tre6P synthase was not affected by deletion of *tps2*$^+$, but was notably affected by deletion of either *tps4*$^+$ or *tps3*$^+$, indicating that Tps4 and Tps3, but not Tps2, are required for Tre6P synthase activity. This suggests that, if *S. pombe* is, like *S. cerevisiae*, also able to form trehalose with a multimeric complex, Tps4 and Tps3, but not Tps2, would possibly be involved in stabilization and/or regulation of the complex.

It was shown that also *S. pombe* cells form trehalose via a multimeric complex comprising Tre6P synthase activity. Interesting, however, was the observation that Tre6P phosphatase activity was not part of this Tre6P synthase complex. Surprisingly, deletion of the other two known genes involved in trehalose formation in *S. pombe*, *tps3*$^+$ and *tps4*$^+$, did not affect the molecular weight of the Tre6P synthase complex, which was estimated to be around

250 to 450 kDa. However, deletion of *tps3*$^{+}$ or *tps4*$^{+}$ caused a significant decrease in the *in vitro* activity of Tre6P synthase. Therefore, it may be that, despite not being present in the *S. pombe* Tre6P synthase complex, Tps4 and Tps3 are important for the regulation of the complex. It is worth recalling the situation in *S. cerevisiae* where deletion of the *TPS3* gene was shown to cause only a limited effect on the *in vitro* Tre6P synthase activity, but when combined with deletion of the *TSL1* gene a strong reduction in trehalose accumulation *in vivo* and a strong decrease in the Tre6P synthase activity *in vitro* could be observed. Furthermore, the *tps3 tsl1* double deletion mutant in *S. cerevisiae* had a defective Tre6P synthase/phosphatase complex with lower molecular weight and monomeric Tps1 could be observed (Reinders *et al.*, 1997; Bell *et al.*, 1998). Taken together, the results suggest that the *S. pombe* Tps1 may form a homomeric structure, or that Tps1 may be associated with other unknown proteins. However, a weak association of Tps1 with Tps2, Tps3 and Tps4 it can not be excluded.

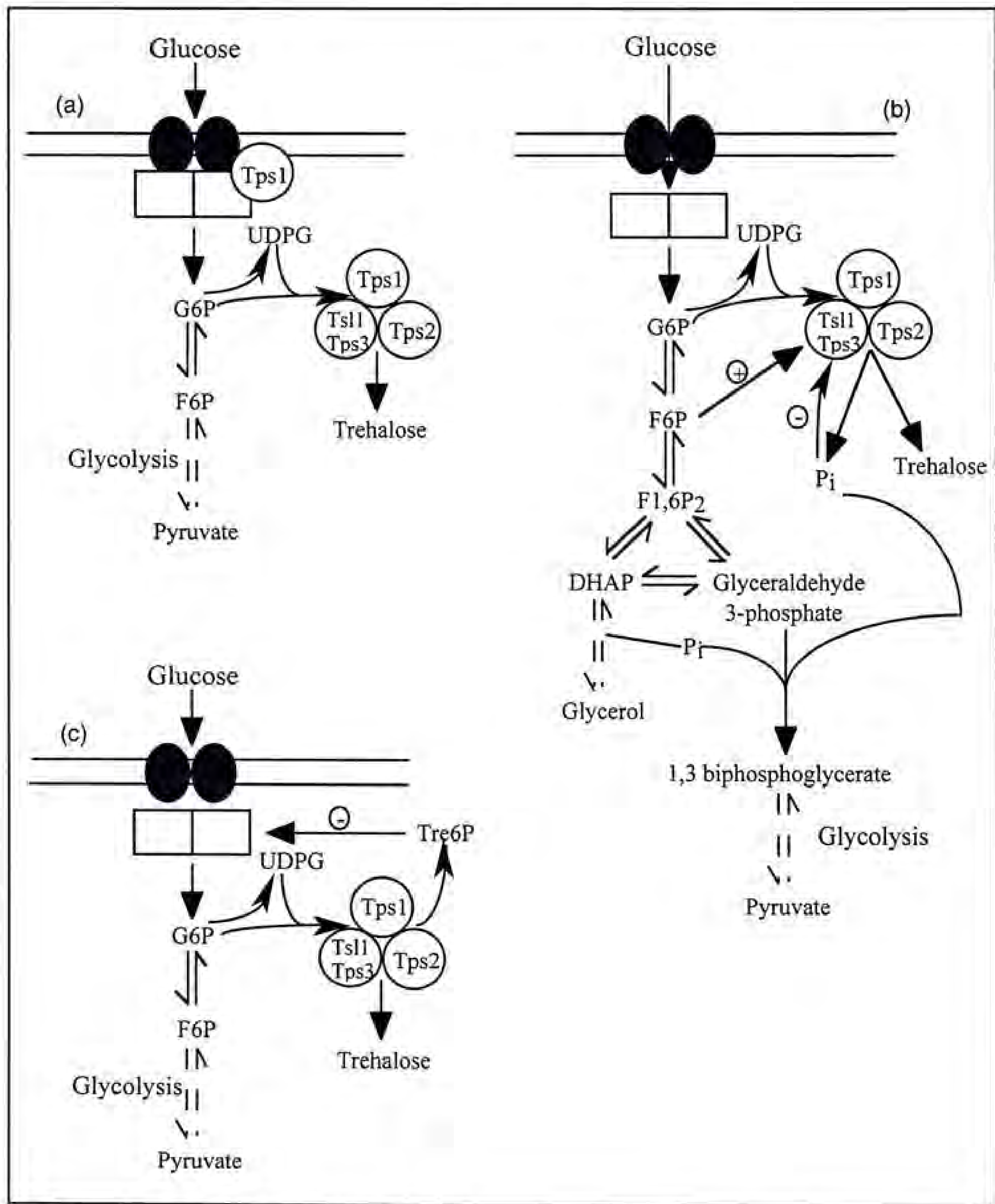
SIMILARITIES AND DIFFERENCES IN THE REGULATION OF TREHALOSE FORMATION BETWEEN *S. POMBE* AND *S. CEREVISIAE*

Based on the results presented in this work, it can be concluded that *S. pombe* shares some properties with *S. cerevisiae* with respect to the regulation of trehalose formation. Heat-shock treatment induced, in both *S. cerevisiae* and *S. pombe*, activation of the key enzymes involved in trehalose metabolism, Tre6P synthase, Tre6P phosphatase, and trehalase and also induced trehalose accumulation. Trehalose accumulation and hsp synthesis play major roles in the acquisition of thermotolerance. Thermotolerance in *S. pombe* is also negatively controlled by the Ras/cAMP pathway. Deletion of the *pka1*$^{+}$ gene in *S. pombe* caused an increase in the *tps1*$^{+}$ transcript (Fernández *et al.*, 1997) and strong accumulation of trehalose. Deletion of the *tps1*$^{+}$ gene in a *pka1*$^{-}$ background reduced the acquisition of thermotolerance, but these strains were still more thermotolerant than wild-type strains. It can be suggested that, as observed in *S. cerevisiae*, *S. pombe pka1*$^{-}$ strains possess high constitutive levels of hsp, which could protect these strains in the absence of trehalose.

However, *S. pombe* also differs profoundly from *S. cerevisiae*. Despite the fact that both *S. pombe* and *S. cerevisiae* Tre6P synthases appear to be negatively controlled by cAPK, this regulation apparently requires different pathways. Unlike in *S. cerevisiae*, cAPK activation in *S. pombe* is not mediated via a Ras protein. Deletion of the *tps1*$^{+}$ gene in *S. pombe* does not cause the pleiotropic effects observed in *S. cerevisiae* and these mutants are able to grow on glucose. Possibly this effect could be explained by the different behaviours of the hexokinases in these two yeast species with respect to Tre6P. *S. cerevisiae* hexokinase was shown to be inhibited by Tre6P, whereas no inhibition by Tre6P was observed for *S. pombe* hexokinase. Addition of glucose to the *S. cerevisiae tps1* mutant causes rapid ATP depletion and accumulation of fructose-1,6-biphosphate ($F1,6P_2$), suggesting that the early steps of glycolysis

exceed the capacity of the glycolytic pathway. A link between Tre6P synthase and the control of glycolytic influx was confirmed by deletion of the *HXK2* gene, encoding hexokinase II. Deletion of *HXK2* in an *S. cerevisiae tps1* mutant restored growth on glucose. Three models were proposed to explain the role of Tre6P synthase in the control of glucose influx into glycolysis and they are depicted in Figure 1. The first model suggests that Tre6P synthase could possess a dual role: one as a subunit of the Tre6P synthase/phosphatase complex and one as a direct regulator of glucose transport and phosphorylation, mediated by the "General Glucose Sensor" (GGS), a complex comprising a glucose carrier, a sugar kinase, and Tps1 (Figure 1a). The second model is the so-called "Phosphate recovery model" and is based on the assumption that trehalose formation is required as a metabolic buffer system to release inorganic phosphate from Tre6P. Glycolysis overflow would lead to high amount of F6P, an activator of the Tre6P synthase/phosphatase complex, and a drop in P_i levels, an inhibitor of the complex. Trehalose formation would sustain the P_i requirement for glyceraldehyde-3-phosphate dehydrogenase, which seems to be the bottleneck reaction in the glycolysis pathway (Figure 1b). The last model is based on the observation that Tre6P directly inhibits hexokinase activity in *S. cerevisiae*. To avoid inhibitory effects of free Tre6P it would be favourable to pass this product of Tre6P synthase directly to Tre6P phosphatase to produce free trehalose. This could be best achieved in a complex structure that links the enzymatic functions of Tre6P synthase and Tre6P phosphatase (Figure 1c).

None of these models seem to be relevant in *S. pombe*: first, because *S. pombe* does not require the entrapment of Tre6P in a complex structure containing both Tre6P synthase and Tre6P phosphatase, since its hexokinase is not inhibited by Tre6P; and second, because *S. pombe tps1* mutants have no growth defect on glucose. A somewhat intermediate situation, concerning *TPS1* deletion, has been reported for *A. niger* and *C. albicans*, in which the loss of Tre6P synthase(s) caused an apparent growth defect only at very high glucose concentrations and/or at elevated temperatures, respectively (Wolschek and Kubicek, 1997; Zaragoza *et al.*, 1998). It seems possible that a control of the glycolytic flux by Tre6P synthase only occurs in fungi with a high fermentative capacity such as *S. cerevisiae* and *K. lactis*, and not in fungi with prevailingly respiratory metabolisms, such as *A. niger*, *C. albicans*, *S. pombe*, and as has been recently shown, *H. polymorpha* (Reinders *et al.*, 1999). Thus, the importance of Tre6P synthase for the control of glycolysis in fungi may be determined not only by susceptibility of hexokinases to Tre6P-mediated inhibition but also by the general (fermentative or respirative) nature of the carbohydrate metabolism. Further investigations concerning the actual composition of the Tre6P synthase in *S. pombe* would be also valuable to understand the different regulatory mechanisms governing trehalose formation in *S. pombe* cells in comparison to *S. cerevisiae* cells.



Adapted from Thevelein and Hohmann, 1995.

Figure 1. The three proposed models for the role of Tre6P synthase in the control of glycolysis in *S. cerevisiae.* In (a) GGS model, (b) the phosphate recovery model, and (c) the Tre6P inhibition model. The abbreviation DHAP means Dihydroxyacetone phosphate. For further details, see text.

CHAPTER 7

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Curriculum Vitae

Maria de Jesus de Souza Ribeiro

June 11, 1967	Born in Niterói-Rio de Janeiro, Brazil.
1974-1978	Primary school in Niterói.
1979-1984	Progymnasium and Gymnasium in Niterói.
1985-1989	Enrolled at the Federal University of Rio de Janeiro (UFRJ), Brazil to study chemistry.
1990-1993	MSc at the Department of Biochemistry, Institute of Chemistry at the UFRJ, Brazil. Thesis under the supervision of Prof. Dr. Anita Dolly Panek on "Trehalose metabolism during heat shock at 36°C in *Saccharomyces cerevisiae*".
1993-1995	Assistant at the Department of Biochemistry, Institute of Chemistry at the UFRJ, Brazil, at the lab of Prof. Dr. Anita Dolly Panek.
1995-1999	Ph.D. course at the Botanical Institute of the University of Basel under the supervision of Prof. Dr. Andres Wiemken, Prof. Dr. Thomas Boller, and PD Dr. Claudio De Virgilio. Defence of a Ph.D. thesis on "Trehalose metabolism and its role in the fission yeast *Schizosaccharomyces pombe*".

During my studies I visited lectures of the following scientists:

J. Oetiker, R. Vögeli-Lange, P. Mathias, R.G. Clerc; C. De Virgilio, K. Ballmer-Hofer, A. N. Eberle, M. N. Hall, U. Otten, R. C. Skoda, N. Kralli, T. J. Resink, H. Müller and W. Krek.