Small heat-shock protein Hsp12 contributes to yeast tolerance to freezing stress

A. Pacheco, C. Pereira, M. J. Almeida and M. J. Sousa

The HSP12 gene encodes one of the two major small heat-shock proteins of Saccharomyces cerevisiae and is induced under different conditions, such as low and high temperatures, osmotic or oxidative stress and high sugar or ethanol concentrations. However, few studies could demonstrate any correlation between HSP12 deletion or overexpression and a phenotype of sensitivity/resistance, making it difficult to attribute a role for Hsp12p under several of these stress conditions. We investigated the possible role of Hsp12p in yeast freezing tolerance. Contrary to what would be expected, the hsp12 null mutant when subjected to prolonged storage at −20 °C showed an increased resistance to freezing when compared with the isogenic wild-type strain. Because the mutant strain displayed a higher intracellular trehalose concentration than the wild-type, which could mask the effect of manipulating HSP12, we overexpressed the HSP12 gene in a trehalose-6-phosphate synthase (TPS1) null mutant. The tps1Δ strain overexpressing HSP12 showed an increase in resistance to freezing storage, indicating that Hsp12p plays a role in freezing tolerance in a way that seems to be interchangeable with trehalose. In addition, we show that overexpression of HSP12 in this tps1Δ strain also increased resistance to heat shock and that absence of HSP12 compromises the ability of yeast cells to accumulate high levels of trehalose in response to a mild heat stress.

INTRODUCTION

Because freezing is one of the major abiotic stresses, the adaptation mechanisms that preserve cells at subzero temperatures are extremely important in the development of technology for the cryopreservation of life. Preservation of cell activity is also a very important issue in frozen-dough technology. This process is well established in the modern baking industry, as it can more easily supply oven-fresh bakery products to consumers while improving labour conditions. Nevertheless, storage of frozen bread dough may lead to the loss of cell viability of baker’s yeast as well as of its baking capacity, and consequently to economic losses (Alves-Araújo et al., 2004; Randez-Gil et al., 1999). In spite of their importance, limited information is available about the mechanisms and determinants of freezing resistance and cold responses in yeast (Kandror & Goldberg, 1997; Kandror et al., 2004; Odani et al., 2003; Zarka et al., 2003). When yeast cells are cultured at 4 °C for a long period, several heat-shock proteins (HSPs) are induced (Homma et al., 2003), suggesting that the induction of these genes might be necessary for adjustment to cold resistance. Considerable evidence indicates that the intracellular level of trehalose may determine the survival response of yeasts under extreme environmental conditions (Diniz-Mendes et al., 1999; Hottiger et al., 1987; Singer & Lindquist, 1998; Van Dijck et al., 1995; Wiemken, 1990). In general, there is wide consensus that trehalose can serve as a stress protectant when yeast cells are confronted with high or low temperatures (Attfield, 1987; Hottiger et al., 1987). Nevertheless, no direct correlation was observed between trehalose accumulation and freezing resistance, above a certain intracellular concentration (Alves-Araújo et al., 2004).

In plants, LEA (late embryogenesis abundant)-like proteins have been described to play a role in the cold acclimation process and their expression and accumulation is increased in response to cold and freezing temperatures (Borovskii et al., 2002; NDong et al., 2002). In yeast these proteins also seem to contribute to stress tolerance, since heterologous expression of tomato, wheat and barley LEA proteins conferred increased resistance to osmotic or freeze stresses (Zhang et al., 2000). Small heat-shock protein 12 (Hsp12) has been described as a LEA-like protein in Saccharomyces cerevisiae (Mtwisha et al., 1998) and it has been reported to be localized both at the plasma membrane, protecting membranes from desiccation (Sales et al., 2000), and in the cytoplasm and cell wall, enhancing barotolerance of the yeast (Motswene et al., 2004). HSP12 is induced massively in yeast cells exposed to heat shock, osmostress, oxidative stress or high concentrations of alcohol, as well as in early

Abbreviations: HSP, heat-shock protein; LEA, late embryogenesis abundant; PI, propidium iodide; RE, relative expression.

Received 17 November 2008
Revised 11 March 2009
Accepted 12 March 2009
stationary-phase cells (Praekelt & Meacoak, 1990; Stone et al., 1990). It is also induced at 0 °C as part of the near-freezing response (Kandror et al., 2004) and by 4 °C exposure (Murata et al., 2006). However, to date few studies could demonstrate any correlation between HSP12 disruption or overexpression and a phenotype of sensitivity/resistance, making it difficult to attribute a role for Hsp12p under most of these stress conditions. In this study we investigated the possible role of Hsp12p in freezing tolerance, using the yeast S. cerevisiae as a model. We report here that Hsp12p plays a role in cryoresistance, even though the hsp12 null mutant was revealed to be more resistant to freezing than the wild-type strain. We also show that stationary-phase cells of the hsp12Δ mutant have a higher intracellular trehalose concentration than wild-type cells, even though heat-induced trehalose accumulation is impaired in this mutant. Overexpression of HSP12 in a tps1Δ strain allowed us to demonstrate a clear increase in resistance to freezing storage and also to heat stress.

METHODS

Strains. The Saccharomyces cerevisiae BY4742 wild-type strain and isogenic mutants used in this study are listed in Table 1. The plasmid vector pRS 41H (Taxis & Knop, 2006) was kindly provided by Christof Taxis and Michael Knop from the EMBL Cell Biology and Biophysics Unit, Heidelberg, Germany.

Media and growth conditions. YPD (1 % yeast extract, 2 % Bactopeptone, and 2 % glucose) and LB medium were prepared as previously described (Sambrook et al., 1989). For the selection of hphNT1, 300 mg hygromycin B L−1 was added to standard YPD plates or liquid YPD medium after autoclaving and cooling to 60 °C (Duchefa Biochemical). The hygromycin B stock solution was used as provided by the manufacturer. When necessary, 100 μg ampicillin ml−1 was added to standard LB plates or liquid medium, after autoclaving and cooling to 60 °C. A sterile filtered stock solution of the vector pRS41H (Promega) was used in a 50 μl reaction mixture. The 300 bp PCR product was cloned into pGEM T-easy cloning vector (Promega). The EcoRI-restricted fragment was then cloned into the EcoRI-restricted and dephosphorylated p26GPD vector (Mumberg et al., 1995). The 1240 bp KpnI-GPD promoter-HSP12-CYC terminator–SacI fragment was subcloned on pRS41H centromeric plasmid vector, creating pRSHSP12, which contains the HSP12 gene under the regulation of the S. cerevisiae GPD promoter from p26GPD. DNA fragments resolved in agarose gels were purified by use of a QIAquick gel extraction kit (Qiagen).

Transformation and DNA sequencing

E. coli transformation. All the vectors constructed were first established in E. coli XL1-Blue, according to the ‘SEM’ method (Inoue et al., 1990). The correct plasmid constructs were verified by restriction map analysis followed by DNA sequencing with an ABI PRISM 310 Genetic Analyzer, using the method of Sanger & Coulson (1975).

Yeast transformation. All yeast transformations were performed using the lithium acetate protocol as previously described (Schiestl & Gietz, 1989). Correct yeast transformations were verified by plasmid DNA isolation using the Chargeswitch plasmid yeast mini kit (Invitrogen) and subsequent transformation in E. coli according to the ‘SEM’ method. DNA cloning and manipulation were performed according to the standard protocols as described by Sambrook et al. (1989).

Yeast freezing. Freezing assays were performed as previously described (Alves-Araújo et al., 2004) with minor changes. Stationary-phase cells (25 ml of 24 h culture) were washed twice with deionized water and suspended in a quarter of the initial volume in sterile water. Aliquots (500 μl) of cells were transferred into 1.5 ml tubes, centrifuged and the pellet suspended in 100 μl LF (liquid fermentation) medium (Hino et al., 1990). The samples were frozen at −20 °C for different time periods.

Extraction and assay of trehalose. Trehalose extraction was performed for the yeast suspensions prepared as described above, immediately before freezing, and sampled for dry weight. Cells were harvested by centrifugation and washed twice with cold deionized water. Trehalose was extracted from cold cell pellets with 5% (w/v) trichloroacetic acid (Merck) for 45 min with occasional shaking. Cells were then centrifuged at 735 g for 10 min. Extraction was repeated once more, and supernatants from the two extractions were combined and used for the determination of trehalose by HPLC. The apparatus used was a Gilson chromatograph (132–RI Detector), equipped with a carbohydrate H+ column (SS-100, H+, Hypersil) that was main-

Table 1. S. cerevisiae strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>BY4742 (wt)</td>
<td>MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>hsp12Δ</td>
<td>BY4742; YFL014w::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>tps1Δ</td>
<td>BY4742; YBR126c::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>drr1Δ</td>
<td>BY4742; YBR208c::kanMX4</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>tps1Δ-Pemppy</td>
<td>tps1Δ-pRS41H</td>
<td>This study</td>
</tr>
<tr>
<td>tps1Δ-Prshp2</td>
<td>tps1Δ-pRSHSP12</td>
<td>This study</td>
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</table>
Hsp12p contributes to yeast freezing tolerance

Measurement of cell viability. Cell viability was analysed by flow cytometry using the membrane exclusion dye propidium iodide (PI), as previously described (Alves-Araújo et al., 2004). In these assays, cells with conserved membrane integrity are not permeated by PI (PI− cells), while those that have lost their membrane integrity do incorporate the fluorochrome (PI+ cells) (Prudencio et al., 1998). Previous results showed that in cells subjected to freezing stress, the loss of cell proliferative capacity (expressed as c.f.u. counts) perfectly correlates with loss of membrane integrity (Alves-Araújo et al., 2004).

Expression analysis by quantitative real-time PCR. Relative expression (RE) was calculated as \(C_t^c{\text{gene of interest}} - C_t^{\text{housekeeping gene}}\). The Ct values of both the calibrator and the samples of interest were normalized to the endogenous housekeeping 18S rRNA gene. Target gene expression was considered increased if relative expression was \(2^\Delta C_t\)–(housekeeping gene) 18S Ct, once values of RE in each experiment were normalized with respect to the calibrator sample, with a RE value set to 1. Both actin (ACT1) and 18S rRNA gene transcript levels were used to control for amount of RNA.

Reproducibility of the results. All the experiments were repeated at least three times, and the data reported are mean values ± SD. When statistical analyses were performed, the significance was tested by ANOVA and t-test (GraphPad Prism 5).

RESULTS

The hsp12Δ strain is more resistant to freezing at −20 °C than the wild-type strain

Aiming at the investigation of the possible role of Hsp12p in yeast freezing tolerance, we subjected stationary-phase cells of *S. cerevisiae* BY4742 (wild-type) and of a hsp12-deleted isogenic strain to prolonged storage at −20 °C. Since we have previously shown that in cells subjected to freezing stress, loss of cell proliferative capacity perfectly correlated with the loss of membrane integrity (Alves-Araújo et al., 2004), viability of the cells during freezing was assessed by flow cytometry using the fluorochrome PI. In both strains, we analysed cell viability for a storage period of 8 days. Contrary to what would be expected taking into account that HSP12 is highly induced by cold stress (Kandror et al., 2004; Murata et al., 2006), viability loss during freezing storage at −20 °C was lower for the hsp12 null mutant than for the wild-type strain (Fig. 1a). A decrease of 80% in cell viability was observed for the wild-type strain after 8 days storage, whereas the hsp12Δ strain showed a loss in cell viability of only about 40% after the same period, indicating that the absence of Hsp12p resulted in a higher resistance to freezing. To further support that the observed differences were due to absence/presence of Hsp12p in the cells, the same experiments were performed with cells harvested in the exponential growth phase. At this culture stage, HSP12 expression is repressed. As a consequence, Hsp12p is absent or vestigial in both hsp12Δ and wild-type strains (Praefke & Meacock, 1990). As expected, the two strains showed a similar behaviour, no significant differences being observed in freezing resistance at −20 °C (Fig. 1b).

The HSP12 deletion strain displays an increase in intracellular trehalose content

Trehalose is widely recognized as one of the most effective compounds in the protection of cellular structures against the damage caused by freezing. The accumulation of trehalose in fungi is associated in general with periods of starvation and reduced growth rate (Kuenzi & Fiechter, 1972; Lillie & Pringle, 1980; Panek, 1975). For our freezing experiments, cells were harvested in stationary phase, when trehalose synthesis is particularly intensive (Kuenzi & Fiechter, 1972; Lillie & Pringle, 1980; Panek, 1975). In addition, it has been previously described that another HSP-disrupted strain, *hsp104Δ*, displayed increased intracellular trehalose concentrations (Fujita et al., 1998). In order to ascertain if the hsp12A mutant, similarly to *hsp104Δ*, could have an increase in intracellular trehalose concentration, which could be masking any freezing sensitivity caused by HSP12 deletion, we assessed the trehalose content of this strain. The results showed that when compared to the wild-type strain, the *hsp12Δ* mutant presented a 50% higher intracellular trehalose concentration (Table 2). We further confirmed that this increase in trehalose content was specific for the disruption of the HSP12 gene, since no differences in the trehalose content were observed for the same wild-type strain (BY4742) disrupted in another gene, *dur1,Δ* (results not shown). The results supported our hypothesis that an increase in intracellular trehalose concentration could be responsible for the higher freezing resistance observed, not allowing differentiation of the possible effects of HSP12 deletion. In addition, they suggest that under these circumstances trehalose may in fact be more critical for survival than Hsp12p, as reported by some other authors (Hottiger et al., 1989; Kandror et al., 2002), or at least it may replace the functions of Hsp12p.

Hsp12p contributes to yeast freezing resistance

We next wanted to check whether the overexpression of HSP12 might also have an effect on freezing tolerance. The fact that HSPs and trehalase both contribute to yeast stress tolerance (Iwahashi et al., 1997; Sales et al., 2000) is an obstacle to the assessment of their individual role in the acquisition of freezing tolerance by cells. To be able to evaluate any effect of HSP12 overexpression, minimizing trehalase influence, we used a trehalose-6-phosphate synthase *TPS1* null mutant, lacking the enzyme trehalose-6-phosphate synthase, which is responsible for the first step in trehalose synthesis. Mutations in the *TPS1* gene are reported to result in lack of ability to synthesize trehalose. Nevertheless, since previous studies (Plourde-Owobi et al., 1999, 2000), and more recently Jules et al. (2008), reported that accumulation of trehalose may arise from the active.
uptake of exogenous trehalose (which comes from the yeast extract used to make the culture medium; Plourde-Owobi et al., 1999) by the AGT1-encoded transporter (De Hertogh et al., 2006), we also determined the trehalose content of the transformed tps1Δ−pempty and tps1Δ−pHSP12 strains. Intracellular trehalose content was residual and similar in both strains [7.7 and 5.4 mg (g cells)−1 respectively, Table 2], therefore effectively removing influences of trehalose on strain properties. The expression of HSP12 in this strain was confirmed by real-time RT-PCR. As can be seen in Fig. 1(d), the tps1Δ−pHSP12 strain showed a significant increase in expression of the HSP12 gene when compared with the tps1Δ−pempty strain.

Stationary-phase cells of tps1Δ−pempty and tps1Δ−pHSP12 strains were frozen at −20 °C and viability was determined over time. The tps1Δ strain overexpressing HSP12 showed an increase in resistance to freezing when compared to the strain harbouring the empty vector (Fig. 1c).

**Table 2.** Intracellular trehalose concentration in stationary phase cells of the strains under study

The values represent mean ± SD from five experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>[Trehalose]IN (mg (g cells)−1)</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>35.3 ± 3.4</td>
</tr>
<tr>
<td>hsp12Δ</td>
<td>54.2 ± 5.6</td>
</tr>
<tr>
<td>tps1Δ−pHSP12</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>tps1Δ−pempty</td>
<td>7.7 ± 0.6</td>
</tr>
</tbody>
</table>

Heat-stress tolerance is also increased in an HSP12-overexpressing strain

Previous work has indicated that mutations in the HSP12 gene did not cause a detectable phenotype under several stress conditions, including heat stress (Prackelt & 2006)
defects in trehalose mobilization upon different stimuli. For that purpose, we examined the intracellular trehalose concentration in stationary-phase cells of both strains, after a glucose pulse or a temperature shift to 50 °C. Trehalase is activated under both conditions, originating a rapid decrease of intracellular trehalose concentration (Carrillo et al., 1992; Thevelein, 1984, 1991). The results showed that there were no significant differences in trehalase degradation over time, either after a glucose pulse or during incubation at 50 °C (Fig. 3a, b and c).

To determine if deletion of HSP12 would also increase trehalose accumulation during a mild heat treatment, we grew wild-type and hsp12Δ cells at 25 °C until stationary phase and then subjected them to incubation at 37 °C. As shown in Fig. 3(d), the temperature shift from 25 to 37 °C caused a rise of about fourfold in the wild-type strain, in accordance with previous research reported by Parrou et al. (1997). In contrast, the hsp12Δ strain displayed only a 1.5-fold increase in trehalose content (Fig. 3e). The results show that, contrary to what happens upon entrance into stationary phase, where the trehalose accumulation is increased, the hsp12Δ strain shows a decreased capacity to accumulate trehalose upon mild heat stress.

**DISCUSSION**

In this paper we report that disruption of the cold-stress-inducible HSP12 gene does not result in increased sensitivity to freezing storage. Our results point to a redundant role for Hsp12p under freezing conditions and identify trehalose as the cell component that is able to replace its functions under such conditions. In fact, when the intracellular trehalose content was decreased to residual

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**Increase of intracellular trehalose content in the hsp12Δ strain is not due to increased expression of TPS1**

We next addressed whether HSP12 could directly affect TPS1 expression, which could be responsible for the observed differences in the intracellular trehalose levels. In order to determine if increase in trehalose content in the hsp12Δ strain was due to an induction of TPS1 expression, we measured TPS1 mRNA levels by real-time RT-PCR in wild-type and hsp12Δ strains. The data showed no significant differences in the expression of TPS1 in the two strains (Fig. 2b).

**HSP12 deletion does not affect trehalose mobilization but reduces the heat-induced increase of intracellular trehalose concentration**

The higher trehalose concentration in the hsp12Δ strain could also result from a decreased removal by trehalase, as found for the hsp104Δ strain (Iwahashi et al., 1998). We next tried to evaluate if HSP12 deletion could originate

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**Fig. 2.** Heat-shock tolerance at 50 °C of tps1Δ-transformed strains. (a) Effect of heat shock on cell viability of tps1Δ strains transformed with the empty plasmid (□) and HSP12-expressing plasmid (■). The cells of both strains were grown in YPD supplemented with 300 mg hygromycin L⁻¹ at 25 °C until stationary phase (OD₆₅₀ ≈ 3–4). Cells were then diluted to OD₆₅₀ 1 and subjected to a temperature shift from 25 to 50 °C. Viability of the cells was determined by c.f.u., at the indicated times. Two-way ANOVA analysis revealed that differences between strains were extremely significant (P<0.0001). (b) Real-time RT-PCR measuring expression of TPS1. Expression was measured in wild-type and hsp12Δ and relative expression (RE) values are shown. Differences between wild-type and hsp12Δ strains were not significant (P=0.13). Comparisons between strains were performed by an unpaired t-test.
levels by TPS1 deletion, an increase in freezing resistance was observed after HSP12 overexpression. Hsp12p has been described to localize at both the cytosol and the plasma membrane (Sales et al., 2000), and extracellularly at the cell wall (Motshwene et al., 2004). Using a model liposome system, it was also shown that Hsp12p acts in a similar way to trehalose in the protection of membrane integrity against desiccation (Sales et al., 2000). Taking into account that loss of cell viability under freezing conditions depends directly on the capacity of yeast cells to preserve their membrane integrity (Alves-Araújo et al., 2004), the results suggest that the protection role of Hsp12p during freezing storage is most probably exerted at the plasma membrane level.

Using the tps1Δ-pHSP12 strain, we could also show an increase in heat-shock resistance by HSP12 overexpression, demonstrating that Hsp12p can also in fact contribute to heat resistance.

Deletion of HSP12 led to an increase in intracellular trehalose content, which was not accompanied by a significant induction of the TPS1 gene, or by a decreased capacity for trehalose mobilization. Our results support the interpretation that such an increase may result from activation of trehalose-6-phosphate synthase. This is different from what was found for HSP104 deletion, where the observed increase of trehalose content was attributed to a decrease in both neutral trehalase and trehalose-6-phosphate synthase activities (Iwahashi et al., 1998).

Although absence of HSP12 led to higher induction of trehalose accumulation upon entry into stationary phase, it decreased the ability of the yeast cells to accumulate high levels of trehalose in response to stress by mild heat shock. These results suggest that Hsp12p may have a role in the induction of trehalose accumulation in response to heat shock.

In conclusion, by overexpressing HSP12 in a tps1Δ strain, we could show that Hsp12p contributes to both freezing and heat resistance, and that its protective role during freezing storage is interchangeable with that of trehalose. Moreover, the tps1Δ-pHSP12 system developed may prove useful to assess the individual contribution of Hsp12p in other conditions where it shares roles with trehalose.

ACKNOWLEDGEMENTS

The authors are grateful to Christof Taxis and Michael Knop from EMBL, Cell Biology and Biophysics Unit, Meyerhofstr, Heidelberg.
Germany, for kindly providing the plasmid vector pRS41H, and to Peter Piper for helpful discussion and suggestions. A.P. was supported by PhD fellowships from BD/13282/2003, Fundação para a Ciência e Tecnologia, Portugal.

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Edited by: D. Burke