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Is thermotolerance of yeast dependent on trehalose accumulation?

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Abstract

During heat stress, trehalose concentration increases in yeast cells in parallel to thermotolerance. This parallelism suggested that trehalose mediated thermotolerance. We show in this work that, under certain conditions, trehalose accumulation and increase in thermotolerance do not go in parallel. A mutant deficient in the trehalose-degrading neutral trehalase shows, after shift from 40°C to 30°C, low thermotolerance in spite of a high trehalose concentration. When glucose is added to stationary yeast cells with high trehalose concentration and high thermotolerance, trehalose concentration decreases while thermotolerance remains high. A mutant deficient in ubiquitin-conjugating genes, *ubc4ubc5*, shows during exponential growth a low trehalose concentration, but a high thermotolerance, in contrast to wild-type cells. Because the *ubc4ubc5* mutant synthesizes heat-shock proteins constitutively, it is proposed that, under these conditions, accumulation of heat-shock proteins, and not trehalase, mediates thermotolerance.

Key words: Thermotolerance; Trehalose; Heat-shock protein; Heat stress; Neutral trehalose deletion mutant

1. Introduction

In exponentially growing cells of *Saccharomyces cerevisiaea*, heat stress (treatment for about 20–60 min at 39–45°C) induces, parallel to the accumulation of trehalose, the appearance of thermotolerance, i.e. the ability to survive heat shock (10–20 min treatment at 50–52°C) [1–4]. This phenomenon is reversible: when the temperature is lowered again to the physiological growth temperature, the trehalose concentration decreases and thermotolerance is reduced [1–3]. The parallelism of an increase and decrease in trehalose concentration with the appearance and disappearance of thermotolerance suggested that a high concentration of trehalose might be the protectant against over-heating at 50–52°C. In contrast to this idea, however, we had shown with a heat-shock protein 104 deletion mutant (*hsp104Δ*) constructed by Sanchez and Lindquist [5], that heat stress causes trehalose accumulation exactly as in the corresponding wild-type, but does not induce thermotolerance [6]. In the present paper we describe additional experiments where trehalose accumulation and thermotolerance do not go in parallel. In agreement with the results from our previous experiments with the *hsp104Δ* strain we conclude that, under certain conditions, it is not the accumulation of trehalose but very probably the heat stress-induced synthesis of heat-shock proteins which leads to the acquisition of thermotolerance.

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2. Materials and methods

2.1. Reagents

Restriction enzymes, auxiliary enzymes and biochemicals were purchased from Boehringer-Mannheim (Germany), Merck (Darmstadt, Germany) and Sigma (Deisenhofen, Germany). Random priming kit and [α-^{32}P]dCTP for Southern analysis of the *nth1* deletion mutant strain YSN1 (*nth1Δ*) were purchased from United States Biochemical Corp. and Amersham Buchler, Germany.

2.2. Strains and growth conditions

The *nth1Δ* construct in a yeast integrative plasmid was made by precise deletion of the open reading frame reported by Kopp et al. [8] using an inverse PCR method (Van Dijck et al, manuscript in preparation). The *nth1* deletion mutant strain YSN1 (*nth1Δ*) made in our laboratory and confirmed by Southern blot for correct integration in the genome (data not shown) has no neutral trehalase activity. The isogenic wild-type strain of the *nth1Δ* strain, called YS18, was a kind gift from D.H. Wolf (University of Stuttgart). The *ubc4ubc5* double mutant and its isogenic wild-type strain was a kind gift from S. Jentsch (University of Heidelberg). The genotypes of these strains are listed below.

YS18: *Matα his3-11,15 leu2-3,112 ura3Δ5 canR gal*$^-$
YSN1: *Matα his3-11,15 leu2-3,112 ura3Δ5 canR gal*$^-$ *nth1ΔLEU2*
DF5: *Matα lys2-802 leu2-3,2-112 ura3-52 his2Δ200 trp1-1 (am)*
ubc4ubc5: *Matα lys2-802 leu2-3,2-112 ura3-52 his2Δ200 trp1-1 (am)-ubc4ΔHIS3ubc5ΔLEU2*

Yeast cells were grown on YEPD medium (1% Bacto yeast extract, 2% Bacto peptone and 2% glucose) at 30°C for 16 h (exponential phase) or for 24 h (stationary phase). However, the *ubc4ubc5* double mutant was grown at 30°C for 36 h (exponential phase) because this mutant grows slowly.

2.3. Heat treatment

Heat stress was performed by shifting the exponentially growing cells to 40°C for 40 min. Heat shock treatment was conducted for 10–20 min at 50°C. In some cases the heat-stressed cells were returned to 30°C for 40 min before heat-shock treatment (cf. Table 1). Heat shock was given to stationary phase cells 30 min after addition of 100 mM glucose (cf. Table 2). Before heat-shock treatment the stationary phase cells were washed and resuspended in YP medium.

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2.4. Trehalose assay and viability determination

An aliquot was withdrawn after each temperature shift or before and after the addition of glucose. The sample was divided into two parts and used for the trehalose assay and cell viability determination. Trehalose was determined in the supernatant using purified acid trehalase, a sensitive and specific method developed in our laboratory [7]. Cell viability was determined by diluting an aliquot and plating equal dilutions on YEPD plates followed by incubation for 3 days at 30°C. The cells were counted and the percentage survival was calculated using the cell number obtained at normal growth temperature before heat stress or heat shock as a reference.

3. Results

3.1. Trehalose concentration and cell survival in wild-type cells and cells carrying a deletion of the NTH1 *gene after heat shock*

The steady-state concentration of trehalose in *Saccharomyces cerevisiae* is controlled by the rate of synthesis and the rate of degradation. Degradation by hydrolysis of trehalose is catalysed by neutral trehalase which has been cloned and sequenced in our laboratory [8]. A neutral trehalase deletion strain (*nth1Δ*) shows drastically higher steady-state concentrations of trehalose at exponential growth and after stress at 40°C (see columns 1 and 2 in Table 1) when compared with the corresponding wild-type. When the stress temperature of 40°C is shifted down again to the normal growth temperature of 30°C the concentration of trehalose falls in the wild-type to the low value characteristic of exponentially growing yeast; however, in the *nth1Δ* mutant strain, the trehalose concentration remains high (cf. columns 2 and 3 in Table 1). When cells returned to 30°C were subjected to heat shock, i.e. 10 or 20 min treatment at 50°C, both types of cells showed survival rates that had no correlation to the different trehalose levels (cf. column 3 in Table 1). In the case of 20 min treatment at 50°C, both cell types showed very low survival rates despite the fact that trehalose concentration remained high in the mutant strain and low in the wild-type during the 50°C treatment.

3.2. Change in trehalose concentration by addition of glucose to stationary phase cells

Stationary phase wild-type and *nth1Δ* cells exhibit high concentration of trehalose and a high survival rate after 50°C treatment (cf. column 1 in Table 2). Thirty minutes after addition of glucose to stationary phase wild-type cells the trehalose concentration decreases to 2.6 mM, i.e. about 5% of the initial value, but the survival rate after 10 min at 50°C remains high, i.e. at 74% of the rate before addition of glucose (Table 2). It may be seen from Table 1 that exponentially growing *nth1Δ* cells exhibit a similar low concentration of trehalose (2.5 mM) as the wild-type cells after glucose addition, however, they have a survival rate at 50°C of less than 0.1%. The stationary phase *nth1Δ* cells, deficient in neutral trehalase, show no decrease in trehalose concentration after addition of glucose, indicating that neutral trehalase is the enzyme catalyzing degradation of trehalose after addition of glucose. Activation of neutral trehalase after addition of glucose is possibly mediated via a cAMP-dependent phosphorylation process (reviewed by Thevelein [9]).

3.3. Constitutive heat-shock protein synthesis in the mutant strain, ubc4ubc5

Exponentially growing cells of the mutant strain, *ubc4ubc5*, defective in the conjugation of ubiquitin [12], exhibit a low steady-state concentration of trehalose similar to exponentially growing wild-type cells (cf. Table 3, row 1). When the exponentially growing cells are submitted to heat shock at 50°C, the survival rate of the *ubc4ubc5* mutant cells is high in spite of the very low concentration of trehalose. Because *ubc4ubc5* cells synthesize heat-shock proteins constitutively, i.e. without heat stress [12], it is very probable that the accumulated heat-shock proteins are responsible for the high survival rate in the absence of an elevated trehalose concentration.

4. Discussion

The paralellism between trehalose accumulation and desiccation tolerance demonstrated by Attfield [1] and Hottiger et al. [2,3] after heat stress was discussed as an indication that trehalose is the substantial protective agent against over-heating and desiccation. Doubts in the general validity of this relationship arose, however, when Winkler et al. [6] demonstrated that a mutant defi-

Table 1
Trehalose concentration and percentage cell survival of exponentially growing cells of wild type and *nth1Δ* after 10 and 20 min at 50°C

		Exponential cells at 30°C	40 min stress at 40°C	Subsequent return to 30°C/40 min
Wild-type	Trehalose (mM)	< 0.1	10	< 0.1
	% survival 10 min 50°C	< 0.1%	59%	32%
	% survival 20 min 50°C	< 0.1%	48%	< 0.1%
nth1Δ	Trehalose (mM)	2.5	45	36
	% survival 10 min 50°C	< 0.1%	40%	24%
	% survival 20 min 50°C	< 0.1%	9%	< 0.1%

For growth conditions, heat treatment, trehalose assay and determination of percentage survival, see section 2.

Table 2
Trehalose concentration and percentage cell survival after 10 min at 50°C of stationary phase cells of wild type and *nth1Δ* before and 30 min after addition of glucose

		Stationary phase cells	30 min after glucose addition
Wild-type	Trehalose (mM)	40	2.6
	% of cells surviving 10 min at 50°C	100%	74%
nth1Δ	Trehalose (mM)	40	40
	% of cells surviving 10 min at 50°C	100%	100%

For growth conditions, heat treatment, trehalose assay and determination of precentage survival, see section 2.

cient in the synthesis of the heat-shock protein, 104 [5], does not exhibit thermotolerance, even though the accumulation of trehalose was as high as in the corresponding wild-type strain. In the present paper three more examples for the antiparallelism of trehalose accumulation and thermotolerance are presented.

Wild-type cells that are returned from the heat stress temperature of 40°C (high trehalose concentration) to the normal growth temperature of 30°C (low trehalose concentration) exhibit a low survival rate after treatment for 20 min at 50°C. As shown in Table 1, the survival rate decreases from 48% to 0.1%. As reported previously [8], in mutant cells lacking neutral trehalase activity, trehalose concentration does not or decreases very little after returning from 40°C to 30°C, whereas thermotolerance drops as steeply as it does in the wild-type cells (Table 1). Because the concentration of heat-shock proteins 70 and 104 drops on returning from the stress temperature of 40°C to the normal growth temperature at 30°C ([13]; Nwaka, unpublished results from this laboratory), the thermotolerance in this case may be controlled by heat-shock proteins and not by trehalose, which does not drop in the trehalase deletion mutant.

After preparation of this manuscript we saw a paper of De Virgilio et al. [14] which reports experiments with a neutral trehalase deletion mutant with results similar to those described in Table 1 of the present paper. A quantitative difference to our results is that De Virgilio et al. found a slow decay of thermotolerance in their trehalase mutant after recovery from heat stress while we do not find any significant difference in the rate of decay of thermotolerance in our wild-type and *nth1Δ* strain after recovery from heat stress. In both sets of experiments the concentration of trehalose in the *nth1* mutant remained high during recovery (as previously described by Kopp et al. [8]). De Virgilio et al. [14] conclude from the experiments with the phenotypic neutral trehalase defective mutant, that the high concentration of trehalose after recovery from heat stress protects the cells from heat damage by 20 min treatment at 50.4°C. As shown in column 3 of Table 1 using the *nth1Δ* mutant, we found no difference to the corresponding wild-type and therefore conclude that the high trehalose concentration (36 mM) is not thermoprotective under these conditions. Some of the differences in the report from De Virgilio et al. [14] and in the present paper are difficult to explain. Unfortunately the sequence of the gene from which the deletion mutant has been constructed by De Virgilio et al. and Schoppink, Klaassen and Osinga (chapter Yeast Strains in [14]) has not been clearly shown to be identical to the *NTH1* gene published by Kopp et al. [8] and available at the EMBL Data Library with accession number X65925. Therefore, De Virgilio et al. [14] should not call their constructs *NTH1* and *nth1Δ*.

When glucose is added to stationary phase wild-type cells exhibiting high concentrations of trehalose, the latter drops rapidly to a very low value [9], whereas thermotolerance remains high, i.e. does not parallel the decrease in the trehalose concentration. In the case of trehalase minus cells, trehalose concentration does not decrease after addition of glucose, and as expected, thermotolerance also does not change. The lack of a decrease in the trehalose concentration in the experiments shown in Tables 1 and 2 with the neutral trehalase deletion mutant points strongly to neutral and not to acid trehalase being the catalyst of trehalose degradation in yeast cells.

In the ubiquitin double mutant *ubc4ubc5*, described by Seufert and Jentsch [12], the trehalose concentration during exponential growth is as low as in the wild-type (Table 3). Nevertheless, thermotolerance is very high in the mutant when compared to the wild-type. According to Seufert and Jentsch [12] the *ubc4ubc5* double mutant is defective in the control of heat-shock protein synthesis and synthesizes heat-shock proteins constitutively. Therefore, the experiment depicted in Table 3 not only demonstrates the antiparallelism of thermotolerance and trehalose accumulation, but also emphasizes that a high level of heat-shock proteins may cause high thermotolerance.

The accumulation of both trehalose and heat-shock proteins under heat stress conditions, i.e. treatment for 20–60 min at 39–45°C [4,13] suggests a role for both events in producing thermotolerance. Based on experiments with the *hsp104Δ* mutant, we suggested a protective function of elevated trehalose levels on the machin-

Table 3
Trehalose concentration and percentage cell survival of exponentially growing cells of a *ubc4ubc5* mutant and the wildtype (*DF5*) after 10 min at 50°C

	Wild-type	*ubc4ubc5*
Trehalose concentration (mM)	0.3	0.2
% survival after 10 min at 50°C	< 0.1%	48.8%

For growth conditions, trehalose assay and determination of survival, see section 2.

ery which synthesizes heat-shock proteins [6]. The rapid heat stress response, measured as an increase in the synthesis of trehalose after transfer to the stress temperature, suggests such a protective 'fire brigade function' of trehalose. The final long-term protective effect might then be mediated by the accumulated heat-shock proteins.

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