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Phenotypic features of trehalase mutants in *Saccharomyces cerevisiae*

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Abstract In the yeast *Saccharomyces cerevisiae*, some studies have shown that trehalose and its hydrolysis may play an important physiological role during the life cycle of the cell. Recently, other studies demonstrated a close correlation between trehalose levels and tolerance to heat stress, suggesting that trehalose may be a protectant which contributes to thermotolerance. We had reported lack of correlation between trehalose accumulation and increase in thermotolerance under certain conditions, suggesting that trehalose may not mediate thermotolerance [Nwaka, S., et al. (1994) FEBS Lett. 344, 225–228]. Using mutants of the trehalase genes, *NTH1* and *YBR0106*, we have demonstrated the necessity of these genes in recovery of yeast cells after heat shock, suggesting a role of these genes in thermotolerance (Nwaka, S., Kopp, M., and Holzer, H., submitted for publication). In the present paper, we have analysed the expression of the trehalase genes under heat stress conditions and present genetic evidence for the 'poor-heat-shock-recovery' phenotype associated with *NTH1* and *YBR0106* mutants. Furthermore, we show a growth defect of neutral and acid trehalase-deficient mutants during transition from glucose to glycerol, which is probably related to the 'poor-heat-shock-recovery' phenomenon.

Key words: Trehalase gene; *NTH1*, *YBR0106* and *ATH1*; Trehalose hydrolysis; 'Poor-heat-shock-recovery' phenotype; Thermotolerance; Stress response element CCCCT (STRE); Growth on glycerol

1. Introduction

The biological function of the yeast trehalases consists of the control of trehalose concentration, which is assumed to play a role as a reserve carbohydrate, in thermotolerance, germination of spores, and other life functions of yeast (reviewed in [1–3]). Two kinds of trehalases are known; a 'neutral' trehalase and an 'acid' trehalase. The neutral trehalase is a cytosolic protein; it has a pH optimum at 7 and is activated by cAMP-linked phosphorylation as shown by Londesborough and Varimo [4]. The protein was purified and characterised by App and Holzer [5], and the corresponding neutral trehalase-encoding gene was cloned and sequenced in *Saccharomyces cerevisiae* by Kopp et al. [6]. The acid trehalase is a vacuolar protein with a pH optimum at 4.5. The protein was purified and characterised by Mittenbühler and Holzer [7]. Little is known about the regulation of acid trehalase, however, compartmentation between this enzyme (vacuole) and its substrate (cytosol), as well as catabolite repression, has been discussed as a possible regulatory mechanism [1,7]. A putative acid trehalase-encoding gene called *ATH1* has been cloned and sequenced [8,9]. A deletion of the *ATH1* gene leads to loss of acid trehalase activity, however, it is not clear if *ATH1* is indeed the structural gene for acid trehalase or if it is a regulatory gene [8,9]. In this study we shall refer to the *ATH1* deletion mutant strain as an acid trehalase-deficient strain (*Δ*ath1), as in [8,9]. The degradation of trehalose in intact cells when recovering from heat stress is not observed in cells carrying a disruption or deletion of the *NTH1* gene, in spite of high acid trehalase activity in this mutant and expression of the *YBR0106* gene [6,10,11], suggesting that the neutral trehalase is responsible for trehalose hydrolysis in vivo.

The *YBR0106* gene (located on chromosome II), sharing 77% identity to the predicted amino acid sequence level of the *NTH1* gene (located on chromosome IV) [12] is expressed via the corresponding mRNA [11]. Using disruption mutants of the *YBR0106* gene as well as its overexpression strain, we do not find any trehalose hydrolysing activity of the *YBR0106* gene product in vitro or in vivo. In the present study, we present genetic evidence for the necessity of the trehalase genes, *NTH1* and *YBR0106*, in recovery of cells after heat shock. It is also shown that a deletion of the *ATH1* gene leading to loss of acid trehalase activity has no effect on this phenotype of 'poor-heat-shock-recovery' observed with *NTH1* and *YBR0106* mutants. Consistent with the idea of necessity of the two trehalase genes (*NTH1* and *YBR0106*) for recovery from heat shock is the observation that the *NTH1* and *YBR0106* genes are induced at heat stress (cf. Fig. 1). Furthermore, we show that the neutral and acid trehalases are engaged in growth transition from glucose to glycerol (cf. Fig. 3).

2. Materials and methods

2.1. Yeast strains, plasmid construction and growth conditions

The *NTH1* deletion strains, YSN1 (*Mata his3-11,15leu2-3,112ura3Δ5 CanR gal-nth1ΔLEU2*) and YSN1A (*Mata ura3-52 leu2-3,112his2-Δ200trp1-Δ901 ade2-101 suc2Δ9 nth1ΔLEU2*), as previously described [10,11], were obtained by integrative transformation of their respective isogenic wild-type strains YS18 [13] and SEY6211, respectively [14]. The strain YSN1/pNTH resulted from transformation of YSN1 with the plasmid pNTH [6] containing the *NTH1* and its flanking sequences as a 6 kb *Sal*I fragment. The *ATH1* deletion strain YMA1 (*Matα ura3-52 leu2-3,112 suc2-Δ9 his3-Δ200 trp1-Δ901 lys2-801 Δath1 :: URA3*) [8,9] was crossed with YSN1A to get the diploid strain YNM1, from which independent spores were generated as follows; spores carrying (i) the *Δath1* (has no acid trehalase activity), (ii) *nth1Δ* (has no neutral trehalase activity), (iii) both mutations *Δath1/nth1Δ* (has no neutral and acid trehalase activity) and (iv) spores which are wild-type for *ATH1* and *NTH1* genes (has both neutral and acid trehalase activity).

For the construction of the *YBR0106* deletion strain (*ybr0106ΔURA3*), the method described by Rothstein [15] was used. The *YBR0106* ORF (2.34 kb) was amplified by PCR from strain YSN1 as described in [11]. The PCR product was then subcloned into the *Bam*HI site of pTZ18R (Pharmacia, Freiburg), giving rise to pTZ18R/YBR-ORF. The resulting plasmid was digested with *Bst*EII having only two sites in the plasmid at positions 360 and 1662 bp of the *YBR0106* ORF, thereby eliminating a 1.3 kb fragment of the *YBR0106* ORF and leaving 360 and 670 bp from the 5′ and 3′ coding regions, respectively, for homologous replacement of the *YBR0106* wild-type gene. The

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URA3 gene isolated as a *Bam*HI fragment from plasmid YDpU [16] was used to replace the 1.3 kb *Bst*EII fragment by ligation after Klenow filling both ends to generate blunt ends. The resulting deletion plasmid was linearised with *Bam*HI prior to transformation into a wild-type yeast strain, YS18, to generate strain YSN011 (*ybr0106ΔURA3*). The strain YSN011 (*ybr0106ΔURA3*), resulting from the elimination of the 1.3 kb fragment of the *YBR0106* ORF showed the same phenotype as the previously described *YBR0106* disruption strain YSN01 (*ybr0106::URA3*), which carries a disruption of the *YBR0106* gene at the middle of the gene [11]. To generate double mutants of the *YBR0106* and *NTH1* genes, called strain *nth1Δ/ybrΔ*, the strain YSN011 (Mata) was crossed with an *nth1Δ* spore (Matα) resulting from the diploid strain YNM1, as described [17].

Yeast cells were grown on YEP medium (1% Bacto yeast extract, 2% Bacto peptone and 2% agar for solid media) and supplemented with an appropriate carbon source (2% glucose, 2% galactose or 3% glycerol) at 30°C for about 16 h for exponential growth or 24 h (stationary phase). For growth on plates, cells were incubated at 30°C for 3 days.

2.2. Molecular biology methods

Standard molecular biology techniques were adapted as described in Sambrook et al. [18]. Total RNA for Northern blot analysis was prepared according to the method of Elder et al. [19] except that three organic extractions were done with water-equilibrated phenol/chloroform/isoamyl alcohol (25:24:1). Random priming kit, [α-^{32}P]dCTP, Hybond N$^+$ for DNA labelling and for blot analysis were purchased from Amersham Buchler, Germany and USB, and the manufacturers' instructions were followed.

2.3. Heat shock treatment and determination of cell survival: growth transition from glucose to glycerol

The yeast strains to be tested were streaked out on YEPD plates and incubated at 30°C for 2–3 days. The cells were then replica plated onto a fresh YEPD or YEPGalactose or YEPGlycerol plate as required. In heat shock treatment, plates were transfered to a temperature of 50–55°C for 6–10 h. The cells treated at 45°C for 2 days gave a similar effect. After this time the cells were shifted back to 30°C and growth was monitored.

2.4. Assay for trehalase and trehalose

The neutral and acid trehalases were assayed from crude extracts of yeast as described [5–7]. Trehalose was assayed from intact cells as described by Kienle et al. [20].

2.5. Heat stress induction of the NTH1 gene, the YBR0106 gene and neutral trehalase activity

200 ml YEPD cell culture was grown exponentially at 30°C and about 100 ml of these cultures was chilled on ice and harvested at 4°C before extraction of total RNA or preparation of extract for trehalase assay. The rest of the 200 ml culture was shifted to 40°C for 40 min and after this time the cells were immediately chilled on ice and harvested at 4°C before being used for extraction as required.

3. Results

3.1. Increased expression of NTH1 and YBR0106 genes at heat stress

Exponentially growing yeast cells on glucose show low mRNA expression of the *NTH1* and *YBR0106* genes, as shown in Fig. 1A (lane 1) and Fig. 1B (lane 1), respectively. However, the low mRNA expression of the two genes increased at heat stress, when the exponentially growing cells at 30°C were shifted to 40°C for 40 min: Fig. 1A (lane 2) and Fig. 1B (lane 2), respectively. The expression of the *NTH1* gene is increased about 10 times in strain YSN01 (*ybr0106::URA3*), while the expression of the *YBR0106* gene increased about 3 times in strain YSN1 (*nth1ΔLEU2*), as determined using a PhosphoImager (from Molecular Dynamics). Both strains YSN01 and YSN1 resulted from the same parental strain YS18. These data support a role of the trehalase genes, *NTH1* and *YBR0106*, in heat protection, similar to HSP genes, the expression of which also shows a heat stress response [21,22]. We have also found that the *YBR0106* gene is expressed at high levels in stationary phase compared to exponentially growing cells [11], similar to the *NTH1* gene, indicating regulation by catabolite repression [23] and/or nutrient limitation [24]. In all cases studied, we have found that the *NTH1* gene is more expressed compared to *YBR0106* under similar experimental conditions.

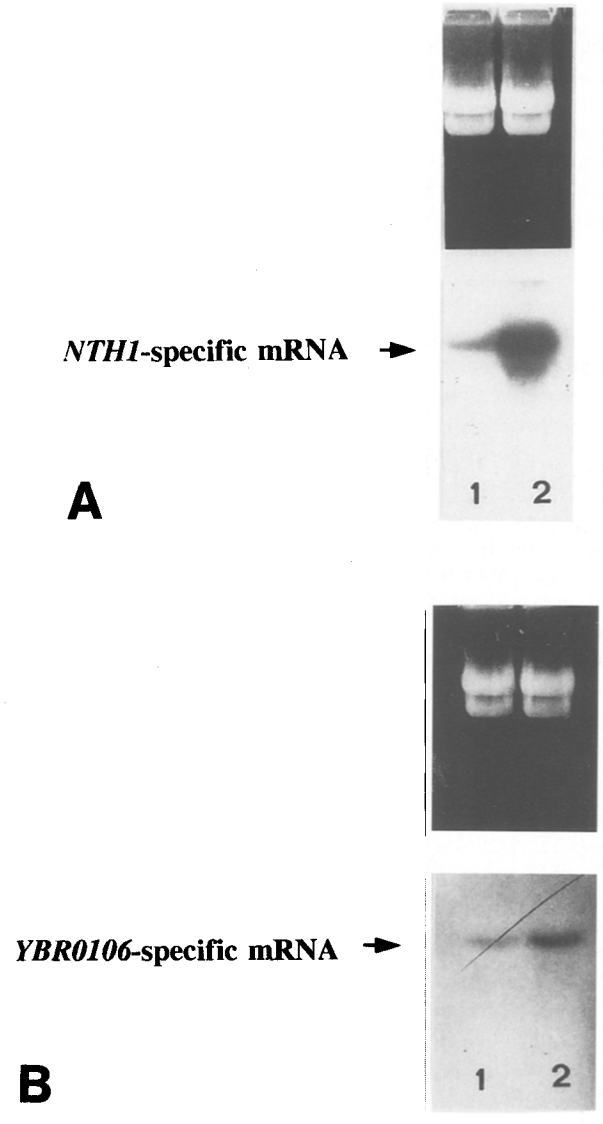

Fig. 1. Northern blot analysis of *NTH1* and *YBR0106* genes at heat stress. Total RNA was prepared from exponentially growing and heat-stressed exponential cells on YEPD and equal amounts of RNA per lane was separated on a 1% agarose/formamide gel as shown on the upper panels of A and B. Using a 1.7 kb (*Bam*HI–*Hin*dIII) fragment from the *NTH1* ORF [11] as a radiolabelled probe, an *NTH1*-specific mRNA was detected from exponentially growing cells (lower panel of A, lane 1) and heat-stressed cells (lower panel of A, lane 2) of strains YSN01 (ybr0106 mutant). Similarly, a *YBR0106*-specific signal was detected in exponentially growing cells (lower panel of B, lane 1) and heat-stressed exponential cells (lower panel of B, lane 2) of strain YSN1 (nth1*Δ*) using a 728 bp (*Sfu*I–*Bst*EII) fragment from YBR0106 gene as a radiolabelled probe.

3.2. Neutral and acid trehalase activity at heat stress

The findings on increased mRNA expression of the *NTH1* gene at heat stress prompted studies on activity of neutral and acid trehalases in different strains under heat stress conditions. As shown in Table 1, we found an about three-fold increase in neutral trehalase activity after shifting exponentially growing cells on glucose at 30°C to heat stress at 40°C for 40 min, similar to the result we obtained for the mRNA. Surprisingly, these exponential cells on glucose (whether heat stressed or not) did not show any increase in neutral trehalase activity after phosphorylating the crude extract with an ATP/cAMP mixture (Table 1), in contrast to a 2–4 times increase in neutral trehalase activity in stationary cells grown on glucose or exponential cells grown on galactose [17]. We do not have a definite explanation for this, however, it could be related to the finding that cAMP levels of exponential cells growing on glucose are high when compared to stationary cells [25,26]. Furthermore, as shown in Table 1, we could not detect any significant acid trehalase activity either from heat stressed or non-heat stressed exponentially growing cells, thereby supporting our finding that the *ATH1* gene is not involved in the recovery of cells after heat shock in contrast to the *NTH1* and *YBR0106* genes. Winkler et al. [27] have previously recorded a two-fold increase at heat stress in neutral trehalase activity but could also not detect acid trehalase activity in exponentially growing cells of strain M1 either before and after heat stress at 45°C for 20 min.

3.3. Acid trehalase activity is not necessary for the 'poor-heat-shock-recovery' phenotype

We have demonstrated the necessity of the trehalase genes, *NTH1* and *YBR0106*, in recovery of yeast cells on glucose after heat shock [11]. Using spores resulting from a cross of YMA1 (*ath1ΔURA3*) and YSN1A (*nth1ΔLEU2*), we have analysed a possible role of acid trehalase in the recovery of yeast cells after heat shock. As seen in Fig. 2a, the wild-type spores (indicated by C) carrying wild-type activities of the neutral and acid trehalases recover in 2 days at 30°C after treatment at 53°C for 6 h. The spores which are carrying Δath1 (has no acid trehalase activity) but are wild-type for *NTH1* and *YBR0106*, recover to a similar extent as the wild-type cells (Fig. 2a, indicated by B). As expected, spores which carry nth1Δ (has no neutral trehalase activity) but are wild-type for the *ATH1* gene show a defect in

Table 1
Neutral and acid trehalase activity at heat stress

		NTH activity without ATP/cAMP mixture (mU/mg protein)	NTH activity with ATP/cAMP mixture (mU/mg protein)	ATH activity (mU/mg protein)
YS18	30°C	12	14	n.d.
(wild-type)	40°C/40′	40	43	<2
YSN011	30°C	12	16	n.d.
(ybr0106Δ)	40°C/40′	37	36	<2
YSN1/pNTH	30°C	22	24	n.d.
(nth1Δ/pNTH)	40°C/40′	52	54	<2

NTH (neutral trehalase) and ATH (acid trehalase) specific activity in exponentially growing cells of strains YS18, YSN011 and YSN1/pNTH on YEPD and at heat stress (40°C for 40 min). The NTH activity was determined both with and without phosphorylation with an ATP/cAMP mixture. The exponentially growing cells and heat-stressed cells were harvested, washed, and crude extract prepared using glass beads. Neutral and acid trehalases were assayed as described in section 2. n.d., not detectable.

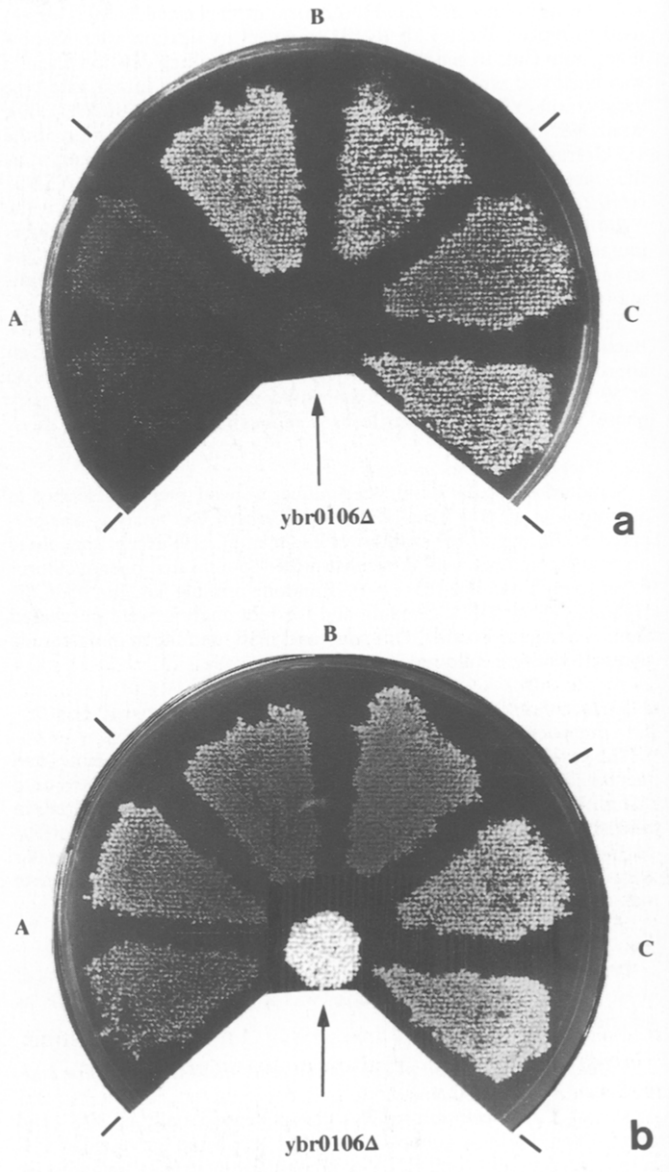

Fig. 2. (a) The *ATH1* gene is not necessary for the 'poor-heat-shock-recovery'. Strains represented by A are two independent spores carrying nth1Δ (showing no neutral trehalase activity) but are wild-type for *ATH1* and *YBR0106* genes. Strains represented by B are two independent spores carrying *Δath1* (showing no acid trehalase activity) but are wild-type for *NTH1* and *YBR0106* genes. Strains represented by C are two independent wild-type spores (wild-type for *NTH1*, *ATH1* and *YBR0106* genes). The strain *ybr0106Δ* is shown with an arrow. These cells were streaked out on YEPD and incubated at 30°C for 2–3 days. After this time the cells were replica plated onto a fresh YEPD plate and immediately transfered to 53°C for 6 h. At the end of this time, the cells were shifted back to 30°C and growth compared after 2 days at 30°C. (b) The 'poor-heat-shock-recovery' phenotype is not seen on glycerol. Strains A, B and C are as described in a. Cells grown for 2–3 days on a YEPD plate at 30°C were replica plated onto a YEPGlycerol plate and transfered to 53°C for 6 h. After this time the cells were shifted back to 30°C and growth compared after 5 days. The *ybr0106Δ* strain is shown with an arrow.

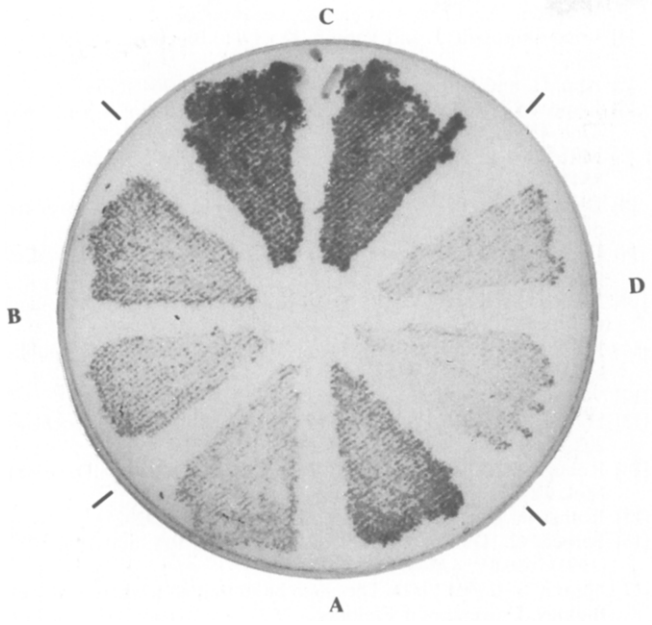

Fig. 3. Poor growth of the *nth1*, *ath1* and *nth1/ath1* double mutants on glycerol. Strains represented by the letter A are two independent *nth1Δ* spores (have no neutral trehalase activity) but are wild-type for the *ATH1* gene. Strains represented by the letter B are two independent *Δath1* spores (have no acid trehalase activity) but are wild-type for the *NTH1* gene. Strains represented by the letter C are two independent spores wild-type for the *NTH1* and *ATH1* genes (have both neutral and acid trehalase activity). Strains represented by the letter D are two independent spores with the *nth1Δ/ath1Δ* double mutation (have neither neutral nor acid trehalase activity). The cells were grown on the YEPD plate for 2–3 days at 30°C, replica plated onto a YEPGlycerol plate and then incubated at 30°C for 5 days. After this time the growth on YEPGlycerol was compared.

recovery (Fig. 2a, spores represented by A). The YSN011 strain (*ybr0106Δ*) indicated by an arrow in the middle of Fig. 2a, was used as a control and it shows, as expected, a defect in recovery after heat shock. The finding that the *ATH1* gene is not involved in recovery of cells after heat shock is further supported by the result in Table 1, which shows that acid trehalase activity does not increase at heat stress. The experiments from spores provide genetic evidence for the necessity of the *NTH1* and *YBR0106* genes but not the *ATH1* gene for the 'poor-heat-shock-recovery' phenotype.

3.4. *Growth of the* nth1, ath1, nth1/ath1 *double mutants and wild-type on transition from glucose to glycerol*

While studying the role of the trehalase genes *NTH1* and *YBR0106* in the recovery of cells after heat shock in various growth media, we found that the *NTH1* deletion strain (*nth1Δ*) grows poorly on YEPGlycerol compared to wild-type at a normal temperature of 30°C (Fig. 2b, strains represented by A). We therefore extended the study of growth on glycerol to *NTH1*, *ATH1* and *YBR0106* genes. As shown in Fig. 3, the wild-type strains (represented by C) show normal growth on glycerol when transfered from YEPD medium to YEPGlycerol medium, similar to strain *ybr0106Δ* shown with an arrow in Fig. 2b. However, mutants of the *NTH1* and *ATH1* genes (Fig. 3, represented by A and B, respectively) show a growth defect on glycerol at 30°C. Double mutants of the *NTH1* and *ATH1* (*nth1Δ/ath1Δ*) are even more affected (Fig. 3, represented by D). Glycerol is a poor non-fermentable carbon source which yeast can use for growth and as an energy supply. It is known that the ATP level of glycerol-grown cells is low when compared to other carbon sources such as glucose and ethanol [28]. The poor growth on glycerol might suggest that cells need additional internal sources of energy. One possibility is the breakdown of trehalose to glucose, which the cells can use as an energy source. This might explain why the cells carrying a deletion of the *nth1* or *ath1* genes show a defect of growth on glycerol.

Our results also indicate that spores of the *nth1Δ/ath1Δ* double mutant can germinate (at least on fermentable carbon sources), in contrast to the idea that trehalose hydrolysis and the trehalases are important for germination of fungal spores [1,29]. We have also found that an *nth1Δ/ybr0106Δ* spore can germinate [17].

4. Discussion

In the yeast *Saccharomyces cerevisiae*, temperature and other stress conditions induce the expression of genes through at least two elements, the heat shock element (HSE) and the stress regulated element (STRE) [30,31]. The expression of some of these genes are also induced when cells enter stationary phase, or when cells are grown with a limited carbon or nitrogen source, which can be considered as a stress due to nutrient limitation [24,32]. The transcriptional induction of many of the heat and other stress-induced genes is mediated by the heat shock factor, HSF [33]. Recently, a novel element that mediates stress-induced transcriptional activity has been identified having the consensus sequence CCCCT, called STRE [30,31,34,35]. This element is known to mediate the stress-induced expression of the *CTT1* gene, encoding the cytosolic catalase T [35], and the *TPS2* gene, encoding the 102 kDa subunit of the trehalose synthase complex [31]. It is also present in the promotor region of other stress-induced genes such as *UB14*, *HSP12*, *HSP26*, *HSP104*, *DDR2* and *PTP2* [30]. We have found the STRE element in the promotor region of the *NTH1* and *YBR0106* gene. In the *NTH1* gene it is located at positions −347 to −343, −339 to −335 and −152 to −148 of the promotor region (EMBL accession number: X65925; see [6,36]) while in the *YBR0106* gene it is located at positions −687 to −683 and −463 to −459, and two other similar sequences are located at position −458 to −454 and −271 to −267 upstream of the 5′ region (EMBL accession number: Z26494). In addition, a single heat shock element HSE (nGAAnnTTCn) is also found at position −348 to −339 of the *YBR0106* gene promotor. The presence of these motifs in the promotor region of the *NTH1* and *YBR0106* gene may explain our finding of heat stress-induced expression of these genes. We are planning site-specific mutagenesis of the stress response sequences to check whether they are linked to the heat-induced expression and the 'poor-heat-shock-recovery' phenotype associated with mutants of the *NTH1* and *YBR0106* genes. Although the exact roles of all the stress-induced proteins are not well understood, it is evident that the stress response requires the coordinated activity of a number of gene products involved in diverse cellular functions. Furthermore, the non-reducing disaccharide, trehalose, is also believed to be a factor of the stress response because its concentration increases in response to certain stressful conditions such as heat stress, dessication, nutrient limitation [3,37]. However, some

workers have suggested that an increased amount of trehalose may not be sufficient for stress tolerance [10,27,31,38]. Gounalaki and Thireos [31] have demonstrated that the induction of the *TPS2* gene (and possibly other genes participating in trehalose synthesis) and accumulation of trehalose at heat stress is mediated by the *YAP1* gene (a yeast transcriptional activator that mediates multiple drug resistance) through the C4T element (STRE). The regulation of the *NTH1* and *YBR0106* genes by heat stress and the presence of the STRE element in their promotor may suggest that not only trehalose synthesis but also trehalose degradation, i.e. trehalose metabolism as a whole, may participate in the stress response.

A possible mechanism for the phenotypes associated with the trehalase mutants, namely 'poor-heat-shock-recovery' and poor growth on glycerol, could be due to the inability of the cell to deliver glucose from trehalose or another trehalase-degradable carbohydrate for energy. In addition to the regulation of the *NTH1* and *YBR0106* genes by temperature, severe heat shock may damage a component of the cell membrane [1,39] and disturb the uptake of glucose from the medium. Then glucose can be supplied to the cell by the breakdown of trehalose via the trehalases, thereby providing the cell with the energy needed for recovery. In support of the sugar uptake defect hypothesis is the finding that the 'poor-heat-shock-recovery' phenotype of the *NTH1* and *YBR0106* mutants is not clearly seen on glycerol medium, probably because glycerol is taken up independently of glucose. Furthermore, if this hypothesis is true, one can ask the question why does *ATH1* not play a role in the heat shock phenotype? One possible explanation could be the compartmentation of the acid trehalase in the vacuole and trehalose in the cytosol [1]. A second possible explanation could be the lack of induction of acid trehalase activity by heat stress (Table 1) and absence of C4T in its promotor [9]. Although we do not have any in vivo evidence for acid trehalase hydrolysis of trehalose, due probably to the problem of compartmentation, it has recently been proposed that during nitrogen or carbon starvation, the vacuole can come into contact with the cytosol, resulting in autophagocytosis [32]. During this period the acid trehalase could come into contact with the trehalose in the cytosol and lead to formation of glucose necessary for optimal growth on glycerol. This would in part explain the poor growth we get with the *ATH1* mutant on glycerol.

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