Deletion of the *ATH1* gene in *Saccharomyces cerevisiae* prevents growth on trehalose

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Received 12 April 1996

Abstract **The biological function of the yeast trehalases (EC 3.2.1.28) consists of down-regulation of the concentration of trehalose via glucose formation by trehalose hydrolysis. While it is generally accepted that the cytosolic neutral trehalase (encoded by the *NTH1* gene) is responsible for trehalose hydrolysis in intact cells, very little is known about a role of the vacuolar acid trehalase and the product of the recently described neutral trehalase gene *YBR0106 (NTH2)*. We have analyzed the role of the acid trehalase in trehalose hydrolysis using the *ATH1* deletion mutant (Δ*ath1*) of *Saccharomyces cerevisiae* [M. Destruelle et al. (1995) Yeast 11, 1015–1025] deficient in acid trehalase activity under various nutritional conditions. In contrast to wild-type and a mutant deficient in the neutral trehalase (Δ*nth1*), the Δ*ath1* mutant does not grow on trehalose as a carbon source. Experiments with diploid strains heterozygous for Δ*ath1* show a gene dosage effect for the *ATH1* gene for growth on trehalose. The need for acid trehalase for growth on trehalose is supported by the finding that acid trehalase activity is induced during exponential growth of cells on trehalose while no such induction is measurable during growth on glucose. Our results show that the vacuolar acid trehalase Ath1p is necessary for the phenotype of growth on trehalose, i.e. trehalose utilization, in contrast to cytosolic neutral trehalase Nth1p which is necessary for intracellular degradation of trehalose. For explanation of the need for vacuolar acid trehalase and not cytosolic neutral trehalase for growth on trehalose, the participation of endocytosis for uptake of trehalose from medium to the vacuoles is discussed.**

Key words: *ATH1* gene; Acid trehalase; Growth on trehalose; Trehalose uptake and utilization; *S. cerevisiae*

1. Introduction

In the yeast *Saccharomyces cerevisiae*, trehalose-hydrolyzing enzyme activity was first described by Emil Fischer [1]. Since then three trehalases have been described in *S. cerevisiae*: (1) a 'neutral trehalase' with a pH optimum at 7 localized in the cytosol has been isolated, characterized and shown to be activated by a cAMP-dependent phosphorylation process [2-5]. The corresponding gene *NTH1* was cloned and sequenced by Kopp et al. [6]. (2) An 'acid trehalase' with a pH optimum at 4.5 localized in the vacuole was purified and characterized by Mittenbühler and Holzer [7]. The *ATH1* gene possibly encoding the acid trehalase has been cloned and sequenced. It was shown that its deletion leads to loss of acid trehalase activity in crude extracts however the Ath1p lacks some properties characteristic for a vacuolar protein [8]. (3) A gene called *YBR0106 (NTH2)* was discovered [9] which possibly encodes a third trehalase. The open reading frame predicts an amino acid sequence with 77% identity to the *NTH1* gene. The *YBR0106 (NTH2)* gene has been shown to be expressed to the corresponding mRNA, but it is neither responsible for neutral or acid trehalase activity nor has it any detectable influence on trehalose concentration in intact cells [10,11]. However, the *NTH1* and *YBR0106* genes were shown to be necessary for recovery of cells after heat shock, i.e. they exhibit 'heat shock protein function' consistent with their inducibility by heat stress [10,11].

The degradation of trehalose in intact cells when recovering from trehalose accumulating heat stress on fermentable carbon sources is not observed in cells carrying a deletion of the *NTH1* gene, in spite of the presence of acid trehalase activity in this mutant and expression of the *YBR0106* gene [6,10,12]. After addition of glucose to stationary cells, the trehalose level goes down in the wild type but remains high in the Δ*nth1* mutant in spite of the presence of acid trehalase activity [12]. This evidence supports the idea that the neutral trehalase is responsible for intracellular cytosolic trehalose hydrolysis in vivo.

In addition to its numerous roles in cellular metabolism, trehalose has been shown to serve as a carbon source for growth of *S. cerevisiae* cells [13,14]. Though a trehalose transport/carrier-mutant which cannot grow on trehalose has been described in *S. cerevisiae*, the exact mechanism of transport still remains unknown [13-15]. In the present study, we show that the *ATH1* gene, a deletion of which leads to loss of vacuolar acid trehalase activity, is necessary for growth of yeast cells on trehalose as sole carbon source, thereby providing in vivo evidence for participation of the acid trehalase in utilization of extracellular trehalose.

2. Materials and methods

2.1. Reagents and sources

D(+)trehalose (from *S. cerevisiae*) used for media preparation was purchased from Sigma (Deisenhofen, Germany). Glucose used was purchased from Merck (Darmstadt, Germany). Other media components: yeast extract, peptone and agar were purchased from Serva (Heidelberg, Germany).

2.2. Yeast strains and growth conditions

The diploid yeast strain YNM1 which is heterozygous for the Δ*nth1*/Δ*ath1* double mutation has been described previously [11]. Independent vegetative spores (Δ*nth1*, Δ*ath1*, Δ*nth1*/Δ*ath1* and *NTH1*/*ATH1*) resulting from the diploid YNM1 [11], served to produce the following diploid strains: YNM2, homozygous wild-type for *NTH1* and *ATH1* (has full neutral and acid trehalase activity); YNM3,

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Abbreviations: *ATH1* gene encodes the vacuolar acid trehalase; *NTH1* gene encodes the neutral trehalase; *NTH2* gene is a homologue of *NTH1* which has no clear role in trehalose hydrolysis; YEPT represents trehalose growth medium (1% yeast extract, 2% peptone and 2% trehalose) supplemented with 2% agar for solid medium

PII S0014-5793(96)00450-4

homozygous *Δnth1/Δath1* diploid (has no neutral and acid trehalase activity); YNM4, homozygous *Δnth1* diploid (has no neutral trehalase activity); YNM5, homozygous *Δath1* diploid (has no acid trehalase activity); YNM6, heterozygous for *Δath1* and heterozygous for *Δnth1*. The *YBR0106* gene deletion mutants (*Δybr0106*) have been described previously [11,16].

Yeast cells were grown on YEP medium (1% Bacto yeast extract, 2% Bacto peptone and 2% agar for solid media) supplemented with appropriate carbon source (2% glucose or 2% trehalose). For determination of the growth curves on trehalose medium, a stationary preculture of the yeast strain was prepared on YEPD and subsequently inoculated onto YEPT in an appropriate dilution to get a starting OD_{578} ~0.1. The culture was then incubated at 30°C for about 7 days. Aliquots were taken at different time intervals for spectrophotometric monitoring of growth at OD_{578}.

For growth on solid trehalose medium, the respective strains were streaked out on YEPD plates and grown at 30°C for 2 days. These cells were subsequently replica plated onto YEPT plates and incubated at 30°C for about 7 days. After this time, growth of various strains was compared.

2.3. Assay for trehalase

The activities of neutral and acid trehalase were assayed from crude extracts of cells generated in glucose or trehalose as described before [5-7].

3. Results

3.1. ATH1 mutant does not grow on trehalose as a carbon source

Two trehalose hydrolyzing enzymes, neutral trehalase with pH optimum at 7, and acid trehalase with pH optimum at 4.5, are known and well described in yeast [2-7]. The finding that the neutral trehalase is responsible for trehalose hydrolysis in intact cells [6,10,11] prompted a search for the biological function of acid trehalase. In this context we searched for conditions under which deletion of the acid trehalase gene shows any phenotype. As shown in Fig. 1, two independent wild-type vegetative spores (*NTH1/ATH1*) resulting from the diploid YNM1 showing neutral and acid trehalase activity [11] are able to grow on YEPT agar plates. Similarly, two independent *Δnth1* vegetative spores derived from YNM1 grow on the same medium. In contrast, two independent vegetative *Δath1* spores (showing no acid trehalase activity) resulting from the same diploid show no growth on this trehalose medium, similar to two independent *Δnth1/Δath1* double mutant spore clones (produced by YNM1) showing neither neutral nor acid trehalase activity.

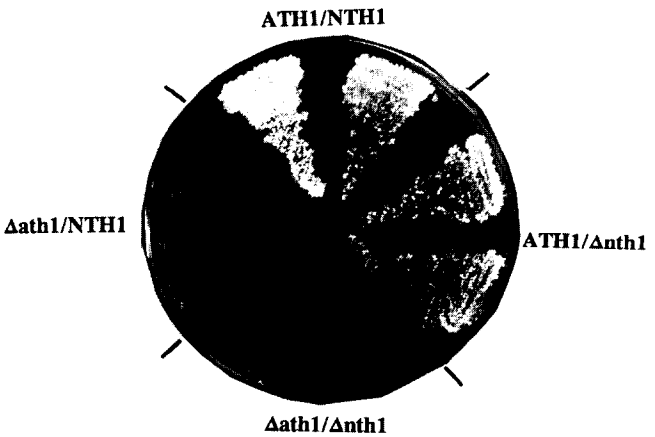

Fig. 1. Growth of haploid cells, *Δath1*, *Δnth1*, *Δath1/Δnth1* and wild type on trehalose. Cells were streaked out on a YEPD plate and allowed to grow for 2 days. After this time the cells were replica plated onto a fresh YEPT plate and allowed to grow at 30°C for about 7 days.

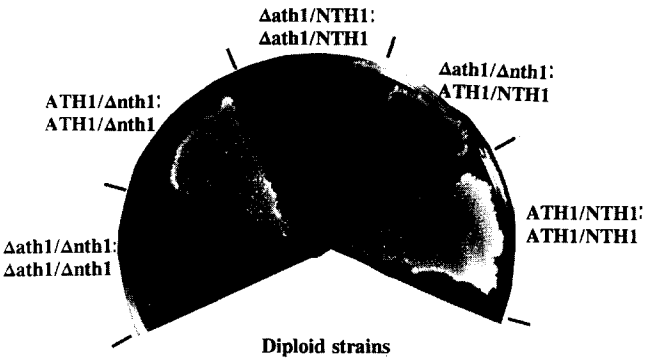

Fig. 2. Growth of diploids heterozygous or homozygous for *Δath1*, *Δnth1* compared to *ATH1/NTH1* (wild type) on trehalose. Cells were streaked out on YEPD plate and allowed to grow for 2 days. After this time the cells were replica plated onto a fresh YEPT plate and allowed to grow at 30°C for 7 days.

These results were further supported using diploids resulting from back-crosses of the above spores to study the effect of gene dosage of the *ATH1* gene during growth on trehalose. As shown in Fig. 2, a diploid homozygous for the *Δath1* mutation but wild type for *NTH1* shows no significant growth on trehalose medium, similar to a diploid homozygous for both *Δath1* and *Δnth1*. However a diploid heterozygous for the *Δath1* and *Δnth1* alleles shows a reduced growth (about half) compared to both the homozygous wild type (showing neutral and acid trehalase activity) and the diploid homozygous for the *Δnth1* mutation (Fig. 2). This result points to a gene dosage effect of the *ATH1* gene for growth on trehalose.

3.2. Growth behavior of S. cerevisiae on liquid trehalose medium

In addition to the studies on solid media, growth of the various strains on liquid trehalose medium was studied. As shown in Fig. 3, the growth rates of wild type is extremely slow with a doubling time of about 20 h, similar to the slow growth estimated on solid trehalose media (plates). The doubling time of wild type in trehalose medium is about 14 times higher than the doubling time on glucose medium, and 4 times higher than the doubling time on ethanol and glycerol (data not shown). Heterozygous diploids exhibit a doubling time of about 40–45 h, i.e. about twice the doubling time of homozygous wild type. The wild type shows a biphasic growth on trehalose medium (Fig. 3): the first phase of this growth is relatively fast and finishes at OD_{578} ~0.6. The second phase of growth on trehalose is very slow. The population reaches OD_{578}=4 in about 140 h. The growth curve of wild type is similar to that of the *Δnth1* mutant and the *Δybr0106* mutant (data not shown). For the *Δath1* mutant, the minor first phase is similar to wild type while the second major phase is absent compared to wild type (Fig. 3). The initial growth phase could be correlated with small amounts (~0.01%) of glucose contamination in the trehalose medium, as determined by the glucose oxidase/peroxidase (GOD/POD) assay method [17,18]. In a control experiment, addition of 0.01% glucose

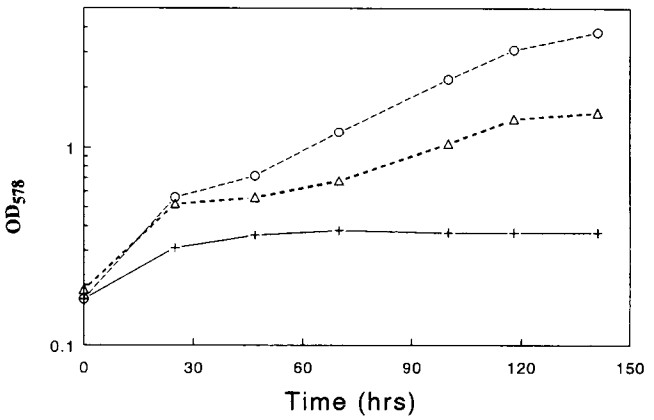

Fig. 3. Growth behavior of homozygous and herozygous Δ*ath1* compared to wild type on liquid trehalose. Pre-culture of wild type (YNM2), homozygous Δ*ath1* and heterozygous Δ*ath1* were inoculated into YEPT medium at equal concentration as described in Section 2. Aliquots were taken at different time points for optical density measurement at 578 nm. The curve was generated by a computer using slide write program. Circles (○) represent homozygous wild type, triangles (△) represent heterozygous Δ*ath1*, pluses (+) represent homozygous Δ*ath1*.

to YEP medium triggered a minor and limited growth similar to the first phase of growth in trehalose (data not shown).

3.3. *Acid trehalase activity of heterozygous strains*

Following the growth limiting effect of the Δ*ath1* deletion shown above, we assayed acid and neutral trehalase activity of a homozygous diploid (*ATH1* and *NTH1*) wild-type strains as well as a diploid heterozygous for Δ*ath1* and Δ*nth1* on glucose medium. The heterozygous strains exhibited approximately one half of homozygous wild-type strain acid trehalase activity and about one half of homozygous wild-type strain neutral trehalase activity. This observation corroborates the about half growth rate observed for this heterozygous strain on trehalose and like the experiments shown in Fig. 2 points to a *ATH1* gene dosage effect.

Following the absence of growth of the Δ*ath1* mutant on trehalose, we assayed acid and neutral trehalase activity of wild-type cells growing on trehalose or on glucose. The neutral trehalase activity is present in exponentially growing wild-type cells on glucose (14 mU/mg protein) but acid trehalase activity is not detectable in accordance with previously published results [5,7,11]. However, cells growing exponentially on trehalose show a strong induction of acid trehalase activity from not detectable up to 70 mU/mg protein. Preliminary data with cells grown on trehalose indicate an increase in Northern response, i.e. induced de novo synthesis of the enzyme in accordance with the activity measurements shown above. It is probably the induction of acid trehalase activity during growth on trehalose medium which allows growth of the cells on trehalose (see section 4).

4. Discussion

4.1. S. cerevisiae *may have two pathways for trehalose utilization*

Trehalose is a widely distributed carbohydrate in nature which is synthesized in *S. cerevisiae* in response to nutrient limitation and during certain environmental stress [19-22]. Experimental evidence has shown that trehalose biosynthesis in yeast is catalyzed by the trehalose synthase complex [23,24], while its endogenous intracellular hydrolysis is catalyzed by the neutral trehalase but not the acid trehalase nor Ybr0106p (Nth2p) [6,10,11]. The finding that *S. cerevisiae* can use trehalose as a carbon source for growth [13,14] similar to the situation in *E. coli* [25,26] raised the question what trehalase(s) out of the three known trehalases in *S. cerevisiae* participate(s) in growth on trehalose. Our results that in *S. cerevisiae* the acid trehalase deficient mutant Δ*ath1* does not grow on trehalose in contrast to the neutral trehalase mutants Δ*nth1* and *2* indicates a role for the acid trehalase in a pathway for trehalose utilization independent of the neutral trehalase. We therefore suggest that the acid trehalase is responsible for hydrolysis of the external pool of trehalose while the neutral trehalase is responsible for hydrolysis of the endogenous cytosolic trehalose pool. This may indicate that the vacuole is involved in the utilization of external trehalose. The acid trehalase acting on external trehalose may correspond to the periplasmic trehalase treA of in *E. coli* [26,27], whereas the cytosolic neutral trehalase may correspond to the cytoplasmic trehalase treF in *E. coli* (EMBL accession number S47739; W. Boos, personal communication). Interestingly, the *E. coli* treA mutant does not grow on trehalose at high osmolarity [27] similar to the yeast Δ*ath1* mutant as shown in the present work.

Trehalose transport has been described in *S. cerevisiae* [13-15] and a trehalose transporter or carrier which is assumed to move trehalose towards both sides of the yeast cell membrane has been described [14]. Interestingly, the trehalose carrier mutant can not grow on trehalose as a carbon source [14] similar to the Δ*ath1* mutant as shown in this paper. Some characteristics of the trehalose carrier described by the group of Panek, namely: low expression in exponentially growing cells on glucose due to glucose repression and high expression in stationary phase when the trehalose level in the cell is high [14,15] are similar to the metabolic regulation of acid trehalase [8,11,28] suggesting a common function or identity of acid trehalase and the trehalose carrier. In support of trehalose utilization through vacuole is the observation that the *end1* mutant which is defective in biogenesis of vacuoles [29-31] does not grow on trehalose (unpublished results).

Acknowledgements: We thank Drs. Wolfgang Heinemeyer and Dieter Wolf (University of Stuttgart) for critically reading the manuscript. We also thank Dr. Monika Destruelle for gift of the Δ*ath1* used for our crossing experiments, Dr. Howard Riezman for gift of the *end* mutants, Inge Deuchler and Markus Burgert for technical assistance, and Wolfgang Fritz for help with the figures. This work was supported by grants from the Deutsche Forschungsgemeinschaft (HO74/27-1), the Fonds der Chemischen Industrie, Frankfurt, and Wissenschaftliche Gesellschaft zu Freiburg im Breisgau.

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