The three trehalases Nth1p, Nth2p and Ath1p participate in the mobilization of intracellular trehalose required for recovery from saline stress in *Saccharomyces cerevisiae*

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INTRODUCTION

During saline stress, as in other types of stress, the budding yeast *Saccharomyces cerevisiae* accumulates large amounts of the non-reducing disaccharide trehalose (Parrou *et al.*, 1997). This molecule possesses particular features as a structural protector, without reaching concentration levels that could have notable impact on the osmotic properties of the cells. Trehalose can interact with phospholipids in membrane bilayers and also with polar residues in proteins, protecting them during different stress situations (Crowe *et al.*, 1992). Trehalose accumulation is achieved by the induction of gene expression, and also regulation of the catalytic activity of enzymes involved in its synthesis (Winderickx *et al.*, 1996; Parrou *et al.*, 1997; Blomberg, 2000). Also, the enzymes involved in trehalose degradation show salt-stimulated expression (Parrou *et al.*, 1997; Zahringer *et al.*, 2000; Garre *et al.*, 2009). The behaviour of different trehalase mutants under saline stress has been recently reported for exponentially growing (Mahmud *et al.*, 2009) and stationary-phase (Garre *et al.*, 2009) cells. All these data together indicate that both trehalose accumulation and trehalose turnover are important in salt adaptation. Several studies based on genetic experiments and phenotypic behaviour of mutants have revealed the necessity of trehalose mobilization to allow for an appropriate recovery when the stress condition disappears (Nwaka *et al.*, 1995b; Singer & Lindquist, 1998b; Pedreño *et al.*, 2002), although no biochemical analyses have been performed to study trehalose content and growth profile after stress.

Two kinds of trehalase activity have been described in *S. cerevisiae* (Nwaka & Holzer, 1998). One of them is the neutral trehalase activity encoded by the NTH1 gene. Nth1p is localized in the cytosol and possesses maximal activity at neutral pH. The principal function of this hydrolase is the breakdown of intracellular trehalose into two molecules of glucose (San Miguel & Argüelles, 1994), although its participation in extracellular trehalose assimilation, together with the Agt1p H\(^+\)-disaccharide symporter, has been described (Jules *et al.*, 2004). This enzyme is regulated at the transcriptional level by glucose repression and also by the general stress-response pathway through the interaction of Msn2/Msn4 transcriptional factors with stress-response elements (STREs) present in the NTH1 gene promoter (Zahringer *et al.*, 1997, 2000). Post-translational regulatory mechanisms have also been described, such as cAMP-dependent phosphorylation and interaction with the inhibitory protein Dcs1p (Zahringer *et al.*, 1998; De Mesquita *et al.*, 1997, 2003). The other trehalase activity was named acid trehalase because it has a lower optimal pH. The localization of the enzyme responsible for this activity, Ath1p, has been controversial. Classically it was considered vacuolar (Alizadeh & Klionsky, 1996), but other data point to its localization in the periplasmic space (Jules *et al.*, 2004). Recently, subcellular localization experiments have been developed.
by gene fusion to the green fluorescent protein GFP, which have determined that Ath1p resides and functions in the vacuole (Huang et al., 2007). This enzyme has been implicated in the degradation of extracellular trehalase as carbon source for growth (Nwaka et al., 1996; Jules et al., 2004; Basu et al., 2006); its participation in the degradation of intracellular trehalose was not ruled out (Nwaka et al., 1995a), and this was only recently demonstrated experimentally (Garre et al., 2009). Regulation of the enzymic activity of Ath1p is not clear, and apparently the protein needs to be synthesized 'de novo' to be active (San Miguel & Argüelles, 1994). Acid trehalase activity has been classically considered not to be regulated by stress because of the lack of STRE sequences at the ATH1 gene promoter. However, we recently showed the Hog1-dependent transcriptional induction of the ATH1 gene under saline stress, and also the parallel increase in acid trehalase activity and the effects of ATH1 gene deletion on trehalose levels (Garre et al., 2009). A third gene related to trehalase degradation has been described, NTH2, which shares a high degree of sequence homology (77%) with the NTH1 gene (Wolfe & Lohan, 1994). The function of Nth2p is not clear; changes in transcript levels have been detected, but no effect on trehalase levels or neutral trehalase activity was associated with these changes (Nwaka et al., 1995a). However, recently an Nth2p-related trehalase activity in the presence of extracellular trehalose was described (Jules et al., 2008).

In this work, we performed salt-stress experiments and analysed growth and trehalose mobilization during recovery of yeast cells in a set of single, double, triple and quadruple mutants, in the same genetic background, in genes related to trehalose metabolism. We found that all known trehalases can participate in the intracellular mobilization and influence yeast growth rate; we also propose the existence of other as yet unknown mechanisms for trehalose mobilization during recovery after saline stress.

METHODS

Yeast strains and construction of null mutants. Strains and oligonucleotide primers used in this study are summarized in Table 1 and Table 2, respectively. Disruptions of the NTH2 and AGT1 genes were performed in laboratory strain MCY1264 (S288c background) or its previously constructed trehalase mutants (Garre et al., 2009) through homologous recombination with a deletion cassette as described elsewhere (Gündener et al., 1996). S1 and C2 primers were used for the synthesis of deletion cassettes. PCR conditions were as follows: 2 min at 94°C; 30 cycles of 15 s at 94°C, 30 s at 50°C and variable time (1 min kb−1) at 72°C; 5 min at 72°C. Purified PCR products were used for yeast transformation by the lithium acetate method (Gietz et al., 1995) and transformants were selected by plating on YPD medium with geneticin (200 mg l−1).

Cultivation, stress and recovery conditions. Cells were grown in liquid YPD medium (2 % glucose, 2 % peptone, 1 % yeast extract) to OD600 6.0 with vigorous shaking at 30°C. For salt-stress experiments, cells were harvested by centrifugation, resuspended in fresh YPD containing 1.2 M NaCl and incubated for 3 h. For recovery experiments in liquid medium, cells were first exposed to saline stress as just described, and then they were harvested and transferred to fresh YPD for the recovery phase and incubated at 30°C for between 5 and 9 h. During the recovery period, samples were taken for determination of trehalose content and measurement of OD600. Viability after stress was assayed by viable cell count in a Neubauer chamber with the methylene blue method and by plating serial dilutions (1 × 10−2, 5 × 10−3, 2.5 × 10−3, 1.25 × 10−3, 6.25 × 10−4, 3.125 × 10−4) of cells in YPD and checking growth after 24–49 h at 30°C.

Determination of trehalose. For intracellular trehalose determination, cell-free extracts from 10 mg yeast cells were obtained as described elsewhere (Parrou & François, 1997). Trehalose was determined in 150 μl cellular extracts or in extracellular medium (diluted 1/5) by enzymic degradation with commercial trehalase (Sigma). Released glucose was determined by a glucose oxidase/peroxidase assay. The amount of trehalose is expressed as μg trehalose (mg cell dry weight)−1.

Glycerol assay. For intracellular glycerol determination, cell-free extracts from 5 mg yeast cells were obtained as described elsewhere (Panadero et al., 2006). Cells were collected by filtration and transferred to a cold tube containing 1 ml distilled water. The yeast suspension was boiled for 10 min, cooled on ice, and centrifuged at 13000 r.p.m. for 10 min (4°C). Glycerol was determined in the supernatant with a commercial kit (Scil Diagnostics) following the manufacturer’s instructions. The amount of glycerol is expressed as μg glycerol (mg cell dry weight)−1.

RESULTS

Deletion of the ATH1 gene improves the recovery after saline stress

Numerous studies have shown that yeast accumulates high quantities of trehalose in response to some suboptimal conditions.
conditions, included saline stress (Parrou et al., 1997; González-Hernández et al., 2005). When the stress condition is removed the mobilization of this metabolite seems to be essential to recover normal cell functions. The main enzymic activities involved in mobilization of intracellular trehalose are the neutral and acid trehalases, encoded by the NTH1 and ATH1 genes respectively, so we decided to test the involvement of both trehalase activities during stress recovery. For this purpose, we used mutant strains previously constructed by deletion of nth1 during stress recovery. For this purpose, we used mutant strains previously constructed by deletion of nth1 and ath1 genes, in both the control and the nth1 backgrounds, enabled the mutant strains Δath1 and Δnth1Δath1 to reach higher cell densities, to even twice that of the control and the nth1 strains during the time-course of the experiments (Fig. 1). The Δath1 strain showed additional advantage at the very beginning of growth, displaying the shortest lag phase.

**Novel activities involved in trehalose mobilization**

To investigate the relationship between yeast fitness and trehalose content, we quantified this metabolite during recovery after saline stress (Fig. 2). As expected, the level of trehalose that had accumulated during the salt stress in the MCY1264 control strain declined quickly and no trehalose was found after only 30 min incubation in fresh YPD. The behaviour of the Δath1 strain, lacking acid trehalase, showed an identical pattern of trehalose mobilization, despite the higher trehalose level accumulated during the salt stress, up to threefold higher than the control strain. On the other hand, the Δnth1 mutant strain, which accumulated amounts of trehalose during the stress similar to the Δath1 strain ([about 140 μg trehalose (mg cell dry weight)⁻¹]), displayed a slower mobilization, and trehalose

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
</tr>
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<tbody>
<tr>
<td>AGT1 S1</td>
<td>AACATTTCGAGTCGTTGGATATGATTGTAATGTTTAAACCACAGGTACGGTACGGGTTC</td>
</tr>
<tr>
<td>AGT1 C2</td>
<td>CCCCCTCTACATTTCATCTACGTGCCATTTAAATTTCTGGACCTTGATATC</td>
</tr>
<tr>
<td>NTH2 S1</td>
<td>ATTTGACGGGATCTGACCTAGCAGCTATAGGACGTACGGTACGGGTTC</td>
</tr>
<tr>
<td>NTH2 C2</td>
<td>TTACACAACCTTGATTAATATATTTGGACCTTGATATC</td>
</tr>
</tbody>
</table>

*Underlined sequences represent the 5’ end (S1) or 3’ end (C2) of the corresponding gene. The end of the primer sequence is homologous to the pUG6 template (no underlined sequence).
concentration decreased to 25% in 6 h. The highest trehalose accumulation after 3 h incubation in 1.2 M NaCl was measured for the double mutant \(\text{Δnth1Δath1} \). This strain reached up to 380 mg trehalose (mg cell dry weight)\(^{-1}\), eightfold more than the control strain. The \(\text{Δnth1Δath1} \) mutant behaviour was surprising because, despite the lack of the described functional trehalases, the level of intracellular trehalose declined to 60 mg trehalose (mg cell dry weight)\(^{-1}\) after 4 h incubation in fresh YPD, a value similar to that of the \(\text{Δnth1} \) mutant. These data indicate that other novel mechanisms are involved in the mobilization of trehalose in the absence of the major trehalases in the \(\text{Δnth1Δath1} \) mutant strain.

A role for Nth2p in the mobilization of intracellular trehalose during salt-stress recovery

Nth2p was described as a putative trehalase due to its high sequence homology with the \(NTH1 \) gene (Wolfe & Lohan, 1994). Although no evidence was available about an associated trehalase activity and no effects in trehalose levels have been previously described, changes in \(NTH2 \) gene expression have been detected (Nwaka et al., 1995a, b), so its functionality had never been completely ruled out. Recently, an Nth2p-related trehalase activity was described in a \(\text{tps1} \) mutant yeast strain forced to grow on galactose in the presence of extracellular 1% trehalose, demonstrating that the \(NTH2 \) gene codes for an active trehalase (Jules et al., 2008). Furthermore, it has been proposed that the Agt1p H\(^+\)-disaccharide symporter participates in trehalose utilization by transporting this metabolite inside the cell, where it can be degraded (Plourde-Owobi et al., 1999; Malluta et al., 2000; Jules et al., 2004). In order to identify new activities involved in physiological intracellular trehalose mobilization in strains lacking the main neutral and acid trehalases, we carried out the sequential deletion of the \(NTH2 \) and \(AGT1 \) genes in the \(\text{Δnth1Δath1} \) mutant strain and studied the involvement of these genes in controlling trehalose levels.

Growth-recovery experiments after saline stress were performed as described above to compare the fitness of \(\text{Δnth1Δnth2Δath1} \) and \(\text{Δnth1Δnth2Δath1Δgt1} \) mutants with mutant \(\text{Δnth1Δath1} \) and the control strain MCY1264. Fig. 3(a) shows the growth profiles for the new mutants compared to the control and the double mutant \(\text{Δnth1Δath1} \). Both the triple and the quadruple mutants showed the same growth rate during the 5.5 h

**Fig. 2.** Trehalose mobilization after saline stress in the control strain and trehalase mutants. The cellular trehalose content was measured during the incubation in liquid YPD for 8.5 h. The data are given as the mean±SE of at least three independent experiments.

**Fig. 3.** Effect of \(NTH2 \) and \(AGT1 \) gene deletions on cell growth during recovery after saline treatment. (a) After 3 h of saline stress, cells were transferred to fresh liquid YPD medium and incubated with shaking at 30 °C for 5.5 h. Growth was measured as the OD\(_{600}\) increment with respect to the inoculated OD\(_{600}\) in recovery medium. The data are given as the mean±SE of three independent experiments. (b) Growth recovery on solid medium. Dilutions of MCY1264 and trehalase mutant strains were spotted on YPD plates after incubation for 3 h in liquid YPD containing 1.2 M NaCl or without NaCl (control), and then incubated at 30 °C for 25 h. A representative result from two independent experiments is shown.
analysed, but the rate was lower than for the Δnth1Δath1 mutant or the control strain, and they reached a lower cell density. The identical behaviour of Δnth1Δnth2Δath1 and Δnth1Δnth2Δath1Δagt1 mutants suggested that deletion of the NTH2 gene, added to the previous NTH1 and ATH1 gene deletions, was the reason for the slower growth during the recovery in fresh YPD after saline treatment. To rule out a direct effect of the NTH2 deletion alone, we constructed a single Δnth2 mutant strain and compared its behaviour to the control and double Δnth1Δath1 mutant strains. The growth recovery profile for the single Δnth2 mutant strain was identical to that of the control strain (Fig. 3a). When growth recovery after salt stress was assayed on YPD plates for the whole set of strains (Fig. 3b), the behaviour of the single Δnth2 mutant was again indistinguishable from that of the control strain growth, whereas the better growth after saline stress of the double Δnth1Δath1 mutant was evident, and the worst performance was displayed by the triple Δnth1Δath1Δnth2 mutant strain.

To gain more information on the effects of the NTH2 gene, intracellular trehalose level was measured for the Δnth1Δnth2Δath1 and Δnth1Δnth2Δath1Δagt1 multiple mutants, and also for the single Δnth2 mutant, during the time-course of recovery after saline stress as previously done for the single Δnth1 and Δath1 and the double Δnth1Δath1 mutants. As can be seen in Fig. 4, the trehalose content after salt stress and its mobilization profile for the single Δnth2 mutant are very similar to those of the control strain. However, the two new strains lacking the NTH1 and ATH1 genes and also the NTH2 gene accumulated approximately the same amount of trehalose during salt stress and significantly less than the double Δnth1Δath1 strain, although twice the amount found in the control strain. The profile of trehalose mobilization is also very similar for both strains, and during the first 30 min the intracellular trehalose concentration in these two mutants decreased at a similar rate as that in the double Δnth1Δath1 mutant. However, trehalose mobilization was almost arrested after 30 min and a high level of the disaccharide, slowly decreasing, was maintained for the next 5 h, when significant growth still occurred (see Fig. 3a). These results indicate that the NTH2 gene participates in the mobilization of the accumulated trehalose in response to saline stress, at least in the absence of functional Nth1p and Ath1p trehalases. They also show that the H+*-trehalose symporter encoded by the AGT1 gene does not contribute to trehalose mobilization for cell recovery under these stress conditions.

Intracellular trehalose mobilization could be associated with the export of the disaccharide and also with glycerol biosynthesis, so growth recovery experiments in SD medium were also performed for all the strains in order to measure extracellular trehalose, and intracellular glycerol was determined in cells from growth-recovery experiments in liquid YPD. Quantification of extracellular trehalose indicated low levels of the disaccharide in the medium without significant differences between strains (data not shown). Oscillations in the very low level of extracellular trehalose did not correlate with the amount of intracellular trehalose mobilized by the different strains, supporting the contention that, quantitatively, trehalose export is not the main event in mobilization of intracellular trehalose after saline stress. Quantification of intracellular glycerol (Fig. 5) indicated that all the strains had a high content of this metabolite just after the salt stress and showed a fast decrease during growth recovery, without significant differences between them. Even more challenging is the finding that the absence of the three trehalases and the trehalose transporter did not completely impair trehalose mobilization during cell growth recovery, as a small but significant decrease in the trehalose pool and a slow growth were still observed in the multiple Δnth1Δnth2Δath1 and Δnth1Δnth2Δath1Δagt1 mutants.

**DISCUSSION**

The presence of saline and other stresses provokes the accumulation of trehalose in *S. cerevisiae* (Parrou *et al.*, 1997; González-Hernández *et al.*, 2005; Mahmud *et al.*, 2009). The increase in the intracellular pool of trehalose in response to stress is in agreement with the high mRNA levels for the genes coding for the subunits of the trehalose synthase complex, TPS1, TPS2, TPS3 and TSL1 (Winderickx *et al.*, 1996), and also Tps1p protein accumulation (Parrou *et al.*, 1997), described in cells exposed to NaCl. However, the increased transcription of these biosynthetic genes is accompanied by induction of genes coding for degradative enzymes and activation of the corresponding trehalase activities, as has been described for the NTH1 (Zahringer *et al.*, 2000) and ATH1 genes (Garre *et al.*, 2009). The role of this simultaneous expression and functioning of biosynthetic and degradative machineries in...
metabolism is still controversial, and such futile cycles have been proposed both as metabolic mistakes and as key points in metabolic control (Hottiger et al., 1987; Mahmud et al., 2009). In the case of trehalose metabolism, having both machineries ready to use under the control of the very fast mechanisms of enzymic regulation could be the energetic payment for a suitable system to quickly accumulate trehalose when protection is needed, and then efficiently eliminate it, allowing fast recovery of cell growth after stress (Hottiger et al., 1987; Van Dijck et al., 1995; Singer & Lindquist, 1998b; Wera et al., 1999; Sebollela et al., 2004). Trehalose elimination is a determining factor in the resumption of normal growth due to its ability to interact with membranes and proteins. This not only protects the structure of biomolecules, but can also block enzymic activities (Sebollela et al., 2004) and/or obstruct access of chaperone to partially denatured proteins to restore functionality (Singer & Lindquist, 1998a).

Evidence from genetic experiments supported the importance of trehalose mobilization for stress recovery and the term ‘poor stress recovery’ was proposed from the behaviour of the first nth1 mutant described in the literature (Nwaka et al., 1995b). In this work we combined a genetic strategy with biochemical analysis to determine the contribution of the different trehalase enzymes, not only in the phenotypic behaviour of the constructed mutants, but also in the trehalose profiles when yeast cells recover growth after saline stress in a rich medium. To our knowledge, these are the first biochemical and physiological data obtained from cells recovering growth after stress. Our results are in agreement with the genetic data from other authors (Parrou et al., 1997; Mahmud et al., 2009), showing that the deletion of the NTH1 gene was linked to high trehalose accumulation during saline stress (Fig. 2, zero time point). However, according to our previous description of Ath1p involvement in the response to saline stress (Garre et al., 2009), salt-stressed cells of the Δnth1 mutant strain also contained high amounts of trehalose, in fact a similar content to the Δnth1 mutant. Accordingly, deletion of both trehalases caused higher overaccumulation of this metabolite in the double Δnth1Δath1 strain. The elevated trehalose levels at the end of saline stress, shown by the Δath1 and Δnth1Δath1 mutants, point to the participation of Ath1p in mobilization of intracellular trehalose. Although these appear to be the first observations showing a relationship between mobilization of intracellular trehalose and acid trehalase activity, early data from Nwaka et al. (1995b) previously suggested this function for Ath1p in experiments showing poor glycerol growth in ath1 mutants. Participation of Ath1p in trehalose accumulation during saline stress could be typical of stationary-phase cells, as no clear effects of Ath1 deletion were observed when the saline stress is applied to exponentially growing cells (Mahmud et al., 2009), in agreement with the previously described profile of acid trehalase activity along the yeast growth curve (San Miguel & Argüelles, 1994; Garre et al., 2009). Despite these indirect early data, traditionally the only described function for acid trehalase has been the hydrolysis of extracellular trehalose in agreement with the previously described profile of acid trehalase activity along the yeast growth curve (San Miguel & Argüelles, 1994; Garre et al., 2009). Despite these indirect early data, traditionally the only described function for acid trehalase has been the hydrolysis of extracellular trehalose in agreement with the previously described profile of acid trehalase activity along the yeast growth curve (San Miguel & Argüelles, 1994; Garre et al., 2009).
et al., 1995; Alizadeh & Klionsky, 1996) and better fits our model of Ath1p function in the mobilization of intracellular trehalose. However, a dual and likely regulated subcellular localization of the acid trehalase is also possible, as the different results from different groups have been obtained under very different growth conditions. Acid trehalase might be mainly periplasmic when yeast cells are forced to grow without glucose, due to the tps1 mutation, and in the presence of trehalose (Jules et al., 2008), but might be localized in the vacuole under normal growth in glucose or during the salt-stress response (Garre et al., 2009) and subsequent growth recovery in rich medium, as found in GFP gene fusion experiments (Huang et al., 2007). Alternatively, acid trehalase could be present at both locations simultaneously and only change its percentage distribution under different environmental conditions.

We have shown that ATH1 deletion caused a fast growth recovery after saline stress, the mutant reaching a higher cell density than the control strain (Fig. 1). In contrast, NTH1 gene deletion was not advantageous in our experimental conditions, but neither did it cause the deep poor recovery phenotype described under several other experimental conditions, but neither did it show any growth improvement. The fast recovery phenotype we observed for the Δnth1 mutant could be due to a rise in fermentative metabolism in concordance with previously published data (Jung & Park, 2005).

The most unexpected finding was the significant trehalose mobilization in the double mutant Δnth1Δath1, because it lacked both trehalase activities described to date, although published data showing NTH2 gene expression did not allow us to completely rule out the existence of an associated trehalase activity (Nwaka et al., 1995a, b). Previous enzymic analysis with the single Δnth1 and the double Δnth1Δath1 (Garre et al., 2009) mutants showed a low level of neutral trehalase activity we always named ‘residual’, which likely corresponds to Nth2p, as suggested recently (Jules et al., 2008). In our experiments, the involvement of Nth2p in the mobilization of intracellular trehalose for yeast cell recovery from saline stress and its influence on the growth rate suggest the physiological relevance of this function, at least when the action of the main trehalases Nth1p and Ath1p is impaired. In addition to the interest of this finding, it was even more unexpected to discover the ability to mobilize trehalose of the triple Δnth1Δnth2Δath1 and quadruple Δnth1Δnth2Δath1Δagt1 mutants, which quickly mobilized some of the disaccharide, at approximately the same rate as the control strain during the first 30 min. It is worth noting that the trehalose level decreased to approximately one-half during the time-course of the recovery experiments in the absence of Nth2p, and that the main decrease occurred during the first 30 min, when no cell growth was yet detectable, so the decrease in trehalose content cannot be explained by dilution. Moreover, these strains recover growth, although very slowly. The analysis of extracellular trehalose during growth recovery in the different mutants indicates that mobilization of this disaccharide is mainly achieved by intracellular trehalose degradation, as was also suggested by the identical behaviour in growth and trehalose accumulation for the triple Δnth1Δnth2Δath1 and quadruple Δnth1Δnth2Δath1Δagt1 mutants. The analysis of intracellular glycerol during the same process points to the use of the glucose resulting from trehalose hydrolysis mainly to obtain energy for growth, as no differential accumulation of this osmolyte was observed between strains.

The next challenge will be to investigate the activities or mechanisms accounting for the fast trehalose mobilization during the first 30 min on fresh rich medium after the salt-induced growth arrest by the trehalase mutants. Global gene expression analyses are under way in order to identify new regulatory circuits and mechanisms involved in trehalase mobilization for yeast cell recovery after salt stress.

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