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Disruption of the Yeast *ATH1* Gene Confers Better Survival after Dehydration, Freezing, and Ethanol Shock: Potential Commercial Applications

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The accumulation of trehalose is a critical determinant of stress resistance in the yeast *Saccharomyces cerevisiae*. We have constructed a yeast strain in which the activity of the trehalose-hydrolyzing enzyme, acid trehalase (ATH), has been abolished. Loss of ATH activity was accomplished by disrupting the *ATH1* gene, which is essential for ATH activity. The Δ*ath1* strain accumulated greater levels of cellular trehalose and grew to a higher cell density than the isogenic wild-type strain. In addition, the elevated levels of trehalose in the Δ*ath1* strain correlated with increased tolerance to dehydration, freezing, and toxic levels of ethanol. The improved resistance to stress conditions exhibited by the Δ*ath1* strain may make this strain useful in commercial applications, including baking and brewing.

The disaccharide trehalose serves important physiological functions in a variety of organisms, including bacteria, fungi, and plants (9, 38). In *Saccharomyces cerevisiae*, trehalose serves as a major reserve carbohydrate and can constitute up to 23% or more of the dry weight of the cell, depending on the growth conditions and stage of the life cycle (6, 39). In times of nutrient limitation, trehalose accumulates in *S. cerevisiae* and is subsequently utilized as a reserve energy source should starvation conditions persist (23). Trehalose also acts as a critical membrane-protecting agent during environmental stress conditions such as dehydration (3, 4, 10, 11, 20) and freezing (12, 16, 21, 34) and confers increased cell viability in the presence of high concentrations of ethanol (27).

Trehalose must be present on both sides of the plasma membrane to confer stress resistance to yeast cells. This is accomplished by a trehalose-specific transporter on the plasma membrane, which translocates trehalose from the cytosol to the extracellular environment (5, 7, 10). The transporter is inactive during the exponential phase of cell growth when the supply of fermentable sugars is abundant and cellular trehalose levels are low. During respiratory and stationary phases of the life cycle, the trehalose transporter is synthesized in response to the exhaustion of fermentable sugars in the growth medium, which also coincides with an increase in cellular trehalose levels (5, 7). Mutant strains lacking transporter activity show significantly lower stress resistance (10). Spectroscopic studies indicate that the hydroxyl groups of trehalose form hydrogen bonds with the polar head groups of the lipid bilayer and stabilize the membrane against phase transitions which could otherwise result in displacement of membrane proteins and/or leakage of cytosolic contents (3, 4).

Cellular levels of trehalose are controlled by a balance between its synthesizing and hydrolyzing enzymes. The bifunctional enzyme trehalose-6-phosphate-synthase/trehalose-6-phosphate phosphatase synthesizes trehalose in the cytosol through the condensation of glucose-6-phosphate and UDP glucose to form trehalose-6-phosphate, which is then dephosphorylated to yield the glucose disaccharide trehalose (33). Two enzymes are capable of hydrolyzing trehalose: a neutral cytosolic trehalase (NTH) and an acidic vacuolar trehalase (ATH) (17, 24). NTH has been purified from *S. cerevisiae* (1), and the corresponding gene has been cloned and sequenced (19). The enzyme was shown to be most active at pH 6.7 to 7.0 (1, 24). The activity of NTH is regulated by the *RAS*/adenylate cyclase signal transduction pathway, which converts the inactive enzyme to its phosphorylated, active form (1). The role of NTH in protecting cells against heat shock has also recently been demonstrated (30, 31).

The vacuolar ATH, while not yet characterized at the molecular level, has been analyzed biochemically (17, 24, 28). Optimal ATH enzyme activity occurs between pH 4.0 and 5.0 (24). ATH activity is blocked in *sec* and *pep4* mutants, suggesting transport to the vacuole via the secretory pathway and maturation by a vacuolar processing enzyme, proteinase A, respectively (14, 15). ATH is also glycosylated, providing further evidence that ATH is targeted to the vacuole by the secretory pathway (28). The regulatory pathway controlling ATH activity is unknown.

ATH and NTH show opposite patterns of activity during the yeast life cycle (35). The activity of NTH is high when cells are growing exponentially on fermentable sugars and decays rapidly as cells enter respiratory and stationary phases (22, 35). By contrast, ATH activity is detected only when cells enter respiratory and stationary phases or when they are grown on a respiratory substrate such as ethanol or glycerol (35). Therefore, high ATH activity corresponds to stages of the yeast life cycle when its substrate, trehalose, accumulates to appreciable levels, while NTH is active only during the exponential growth phase, when trehalose levels are low. How ATH gains access to its substrate and the physiological role of the enzyme are not known. In addition, the functional coordination of these two trehalases during the various stages of the yeast life cycle is unknown but suggests an interdependence on the nutrient-sensing pathways.

Trehalose accumulation has important consequences for various commercial applications. Accumulation of trehalose correlates with increased survival of yeast cells following dehydration or freezing (11, 16, 32). In practical terms, higher

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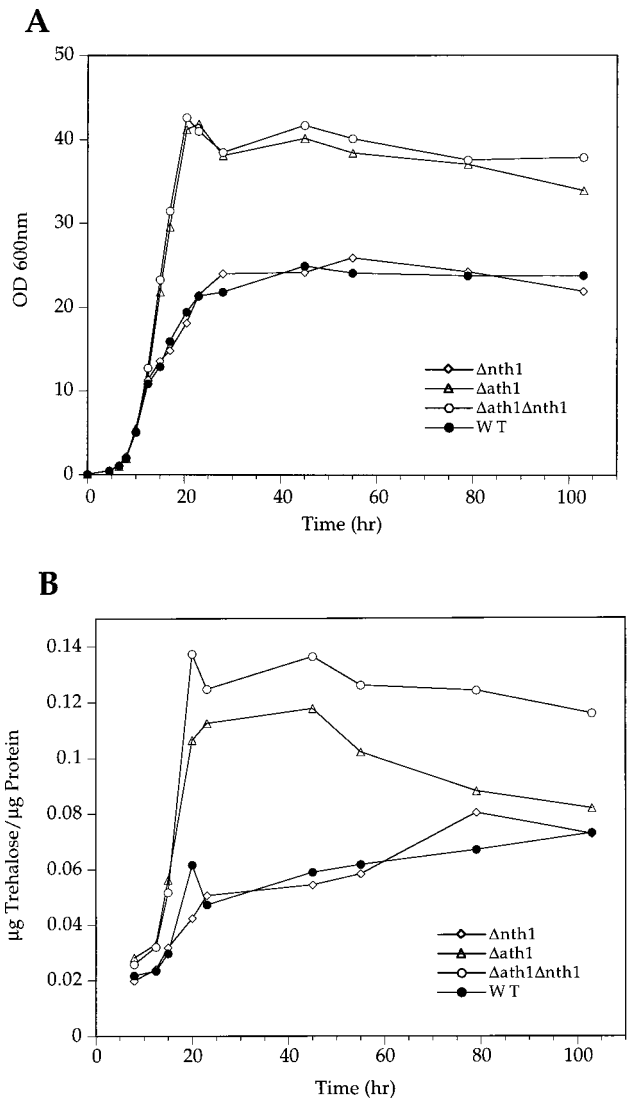

FIG. 1. The *ath1* mutation results in higher cell densities and trehalose accumulation. Wild-type (WT) and trehalase-deficient yeast strains were grown in YP–20% glucose. At the indicated times, aliquots were removed to determine the cell density (A) and the trehalose concentration (B), as described in Materials and Methods. The data shown are from a single experiment but are representative of three separate replicates.

trehalose concentrations mean that lower levels of yeast cells are required for dehydrated or frozen dough preparations, resulting in reduced cost without compromising proofing times (12, 16, 25, 34). Similarly, ethanol resistance is a critical feature of yeast strains used for brewing; alcohol levels in wine and sake, for example, are in part determined by the toxicity of ethanol (2, 27). In addition, yeast strains that yield higher levels of ethanol during fermentation may be exploited to produce ethanol as an alternative fuel source. Commercial yeast strains have been genetically selected for centuries. Having the ability to precisely manipulate resistance to stress conditions may allow the generation of strains having combinations of useful features, including stress resistance and particular sensory characteristics.

To determine the role of ATH during growth under stress conditions, we have examined the behavior of strains carrying disruptions in the recently cloned *ATH1* gene, which has been shown to be essential for ATH activity (8). We show that a Δ*ath1* strain accumulates higher levels of trehalose relative to its isogenic wild-type strain. Furthermore, this trehalose accumulation correlates with increased cell densities and greater resistance to various stress conditions.

MATERIALS AND METHODS

Strains and culture conditions. The wild-type strains used in this study were SEY6210 (*MAT*α *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2Δ9*) and SEY6211 (*MAT***a** *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 suc2Δ9*). The Δ*ath1* strain used in this study, MDY3 (*MAT*α *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2Δ9 Δath1::URA3*) in the SEY6210 genetic background, was from Destruelle et al. (8), and the Δ*nth1* strain YSN1A (*MAT***a** *ura3-52 leu2-3,112 his2-Δ200 trp1-Δ901 ade2-101 suc2Δ9 Δnth1::LEU2*), in the SEY6211 background, was from Nwaka et al. (29). The Δ*ath1*Δ*nth1* strain THY2 (*MAT*α *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2Δ9 Δath1::URA3 Δnth1::LEU2*) and the Δ*nth1* strain THY1 (*MAT*α *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-801 suc2Δ9 Δnth1::LEU2*) were constructed by crossing MDY3 and YSN1A to each other or YSN1A to SEY6210, respectively. Diploids were selected on the appropriate auxotrophic plates, and the resultant strains were sporulated and tetrads were dissected as described previously (13). Strains exhibiting the appropriate phenotypes were backcrossed to SEY6210. Yeast cells were grown in 1% yeast extract–2% peptone (YP) or synthetic minimal medium (SM; 0.67% yeast nitrogen base [YNB], auxotrophic amino acids) containing 2 to 40% glucose as indicated.

Reagents. YNB, Bacto Tryptone, Bacto Peptone, Bacto Yeast Extract, and Bacto Agar were from Difco Laboratories (Detroit, Mich.). Trehalose was from Pfanstiehl Laboratories Inc. (Waukegan, Ill.). Biochemical reagents were from Sigma (St. Louis, Mo.).

Growth conditions. For the growth analysis of the strains, cells were grown in YP supplemented with 2, 5, or 20% glucose. The initial cell density upon inoculation was 0.05 OD_{600} unit of cells per ml. We define 1 OD_{600} unit as the number of cells per milliliter that give an optical density (OD) of 1.0 at 600 nm. For the strains used in the present study, an OD_{600} of 1.0 corresponds to 10^7 cells for both the Δ*ath1* and wild-type strains. The cultures were incubated in an orbital shaker (200 rpm) at 30°C. Cell growth was determined by OD_{600} measurements with a Beckman DU-70 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Additional samples of 5 OD_{600} units of cells were removed for trehalose and protein determinations at each time point.

Trehalose and protein determinations. Culture samples of 5 OD_{600} units of cells were washed twice in cold YNB (0.67%), resuspended in 200 μl of cold H_2O, and divided into two 100-μl aliquots in 1.7-ml microcentrifuge tubes. One aliquot was lysed with glass beads: acid-washed glass beads (0.45 to 0.55 mm in diameter) were added to a level 80% of the resuspended cell volume. The suspension was mixed by vortexing at maximum speed for 1 min. In control experiments, we found that maximal protein extraction as measured by the Lowry method was achieved after approximately 30 s of vortexing. After glass bead lysis, the protein concentration of the crude lysate, at an appropriate dilution, was measured by the Lowry method (26, 36), using the commercial Pierce bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin fraction V as a standard. From the second aliquot, cellular trehalose was extracted with 0.5 M trichloroacetic acid on ice for 30 min as previously described (23). The trehalose content was determined by the anthrone method (18, 37). The trehalose extracted from approximately 1.5 OD_{600} units of cells gave a signal within the linear range of the anthrone assay. The trehalose concentration was normalized and expressed as micrograms of trehalose per microgram of total protein.

Colony survival after dehydration. The Δ*ath1* and wild-type strains were grown in either YP–2% glucose or SM–2% glucose. Culture samples of 15 OD_{600} units of cells were removed at various times, washed twice, resuspended in 300 μl of cold YNB, and divided into three aliquots in 1.7-ml microcentrifuge tubes. Two aliquots (one serving as a backup sample) from each time point were subjected to dehydration in a Speed-Vac SVC100 (Savant Instruments, Inc., Farmingdale, N.Y.) for 5 h. Following dehydration, the microcentrifuge tubes were capped and stored in a closed light-proof container at room temperature for 1 to 3 weeks; all samples from a particular experiment were stored for an equivalent length of time. The third aliquot was used for the trehalose and protein assays. The dried pellets (5 OD_{600} units of cells each) were rehydrated in 500 μl of YNB at 40°C for 10 min. Cell viability was determined after appropriate dilutions of the rehydrated samples and spreading on YP–2% glucose plates. Viable colonies were counted after 48 h of growth at 30°C. All platings were done in triplicate.

Colony survival after freezing. Wild-type and Δ*ath1* cultures were grown to respiratory or stationary phase in SM–2% glucose. Approximately 5 OD_{600} units of cells were collected in microcentrifuge tubes and washed in YNB. The cell pellets were resuspended in 500 μl of YNB. Samples were placed in a model 1010 Micro Computer Programmable Freezing Controller (CryoMed, Mt. Clemens, Mich.) and cooled to 4°C at 4°C/min and then to 2°C at 1°C/min. The samples were subsequently frozen by lowering the temperature to −20°C at 0.5°C/min and were held at that temperature for 10 min. Frozen samples were rapidly

thawed to 24°C in a water bath. After 2 min of thawing, the cells were mixed twice by inversion. Thawed cells were diluted in YNB and spread in triplicate on solid YP–2% glucose plates. Viable colonies were counted after 48 h at 30°C.

Ethanol measurements in culture supernatant. The supernatants from 1-ml culture samples of the Δ*ath1* and wild-type strains grown in YP–0 to 40% glucose were removed every day for 5 days and frozen prior to subsequent analysis. In addition, cell density was determined at each time point. Culture supernatant samples were then assayed for ethanol content with a Hewlett-Packard series II gas chromatograph (Hewlett-Packard Co., Wilmington, Del.) with a flame ionization detector; 0.1% isopropanol was used as the internal standard.

Colony survival after ethanol shock. The Δ*ath1* and wild-type strains were cultured in SM–2% glucose for 41 h at 30°C. Culture samples of 20 OD_{600} units of cells were washed twice in YNB at room temperature. The cell pellets were resuspended in YNB containing 18% ethanol at a cell concentration of 5 OD_{600} units of cells per ml and incubated at 30°C. Samples were removed every hour, for 5 h, and serial dilutions of the ethanol-treated samples were spread in triplicate on YP–2% glucose plates. Viable colonies were counted after 48 h at 30°C.

RESULTS

Strains with a chromosomal disruption of *ATH1* exhibit increased cell densities and accumulate higher levels of trehalose. Strains lacking the activity of ATH (Δ*ath1*), NTH (Δ*nth1*), or both enzymes (Δ*ath1*Δ*nth1*) were examined under various nutrient conditions to discern differences in their growth characteristics. In rich media, strains lacking ATH activity grew up to a twofold-higher cell density than the wild-type and Δ*nth1* strains (Fig. 1A). In YP–20% glucose, both the wild-type and Δ*nth1* strains shifted from exponential-phase growth to the diauxic lag phase earlier than the Δ*ath1* and Δ*ath1*Δ*nth1* strains, which continued to grow exponentially for an additional 10 h before entering the diauxic lag phase (Fig. 1A). The consequent disparity in cell density between strains lacking ATH activity (Δ*ath1* and Δ*ath1*Δ*nth1*) and those with functional ATH (wild type and Δ*nth1*) was maintained throughout subsequent periods. Therefore, disrupting the activity of ATH, but not NTH, improved growth relative to the wild-type strain.

We wanted to determine if the increased cell density seen in the Δ*ath1* strains correlated with an increase in the cellular trehalose levels. At various times during growth, samples were removed and assayed for trehalose as described in Materials and Methods. The Δ*ath1* mutants accumulated substantially higher trehalose levels than the wild type and the Δ*nth1* mutant in YP–20% glucose (Fig. 1B). Increased cell density of the Δ*ath1* mutants and its correlation with elevated trehalose levels were also observed in YP–2% and YP–5% glucose, although the difference was not as dramatic (data not shown). In all cases, detectable trehalose accumulation appeared after a short lag during exponential growth phase and reached a maximum level as the cells entered the diauxic phase of growth, consistent with previous findings (21). The rapid accumulation of trehalose, however, was sustained over a longer period in the Δ*ath1* mutant strains than in the wild-type and Δ*nth1* strains, resulting in higher overall cellular trehalose levels.

During diauxic lag and early respiratory phases (between 20 and 60 h after inoculation), trehalose content of the Δ*ath1* mutants cultured in YP–20% glucose was at its highest, constituting over a twofold increase in trehalose levels over those of the wild-type and Δ*nth1* strains (Fig. 1B). Thereafter, the cellular trehalose levels in the Δ*ath1* mutants slowly decreased. The concentration of cellular trehalose in the wild-type and Δ*nth1* strains continued to gradually increase after the initial rapid accumulation observed during the diauxic lag phase. In all cases, however, trehalose levels began to slowly decrease as the cells continued in stationary phase (data not shown), presumably due to the mobilization of trehalose as a reserve energy source (23, 38). Because disrupting *NTH1* did not appear to offer any advantage over the wild type in cell growth and trehalose levels, subsequent examination of the strains under stress conditions focused on the behavior of the Δ*ath1* and the wild-type strains only.

The Δ*ath1* mutant survives dehydration conditions better than the wild-type strain. Trehalose accumulation correlates with increased survival following dehydration (10, 11). We wanted to determine if the increase in trehalose seen in the Δ*ath1* mutant conferred greater resistance to stress conditions. Dehydration survival and cellular trehalose levels of the Δ*ath1* and wild-type strains were determined for cells grown in SM–2% glucose medium as described in Materials and Methods (Fig. 2). Cells harvested during the exponential growth phase showed no viability after dehydration in either the Δ*ath1* or the wild-type strain (Fig. 2A and B). Survival was only detected during the diauxic lag phase and continued through much of the respiratory or stationary period. The Δ*ath1* strain viability after dehydration was consistently higher than that of the wild-type strain throughout the period of growth examined. Colony survival of the Δ*ath1* strain over that of the wild-type strain ranged from a twofold to several thousandfold increase in viability (cf. 37- and 48-h time points in Fig. 2B). However, in both the Δ*ath1* and wild-type strains, the survival of the colonies fell precipitously after approximately 60 to 70 h of growth.

The Δ*ath1* and wild-type strains exhibited similar cell densities when grown in SM–2% glucose (Fig. 2A). We examined whether a difference in cellular trehalose levels between these strains, prior to desiccation, correlated with the significant disparity in dehydration resistance. Samples were removed at each time point prior to dehydration to determine trehalose levels, as described in Materials and Methods. Similar to the accumulation patterns observed in rich medium (Fig. 1B), appreciable trehalose levels were only detected as the cells exited from the exponential phase of growth in minimal medium (Fig. 2C). In the wild-type strain, the highest accumulation of trehalose occurred after only 15 h of incubation; this level was roughly maintained for 50 to 60 h, after which the trehalose levels began to decrease gradually (Fig. 2C). However, the rise in trehalose levels was sustained for a substantially longer time in the Δ*ath1* strain; trehalose accumulated rapidly to levels twice that seen in the wild-type strain, achieving a peak level after 37 h of growth. The Δ*ath1* trehalose levels then decreased in a pattern similar to that of the wild-type strain at the 70-h time point.

The trehalose levels of the wild-type and Δ*ath1* strains also exhibited an oscillatory pattern, with transient peaks of accumulation occurring every 15 to 20 h (Fig. 2C). Interestingly, these periods of local elevated trehalose roughly corresponded to the peaks in dehydration survival (cf. Fig. 2B and C at 10 to 20, 30 to 40, and 50 to 60 h), and during the period in which both strains showed a decrease in trehalose levels (after 70 h of culture), a concomitant decrease in dehydration survival was observed. In summary, the Δ*ath1* strain accumulated up to twice as much trehalose as the wild-type strain in minimal medium, and this correlated with increased survival under dehydration stress conditions.

Similar but less dramatic survival differences were observed with cells grown in rich YP–2% glucose medium (Fig. 3). Under these conditions, the Δ*ath1* mutant was more resistant to dehydration than the wild-type strain over a significant period of the yeast life cycle. The greatest difference in viability was recorded between 20 and 40 h after inoculation, when the Δ*ath1* strain showed a relative increase of one- to threefold in viability over the wild-type strain (Fig. 3B). Thereafter, dehy-

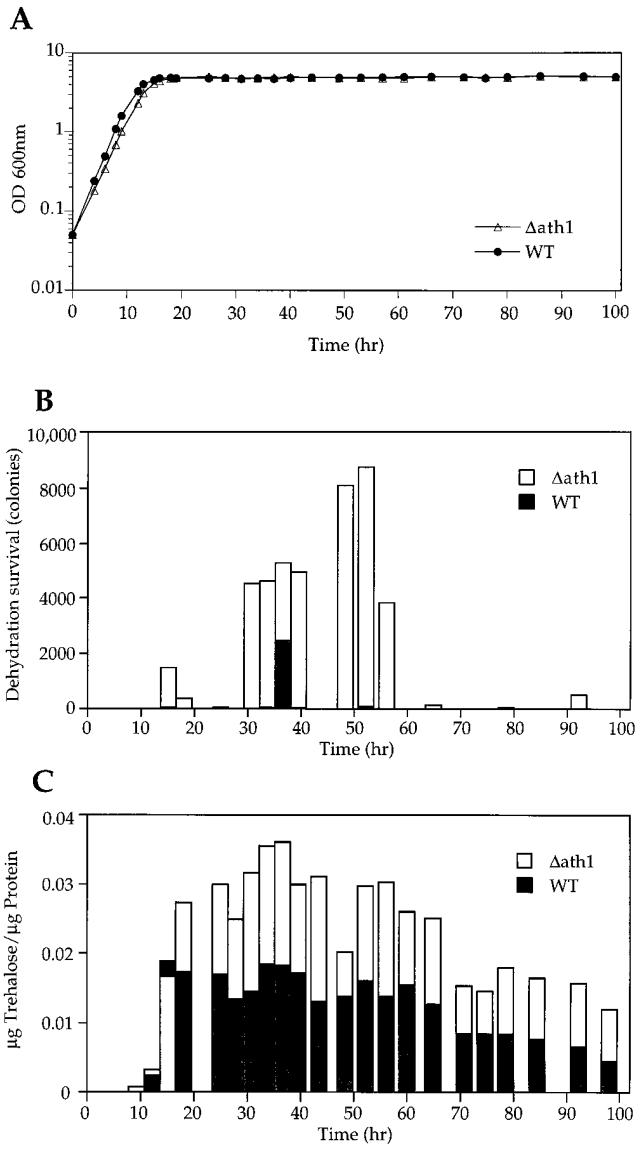

FIG. 2. The Δ*ath1* mutant survives dehydration better than the wild-type strain. (A) Wild-type (WT) and Δ*ath1* yeast strains were grown in SM–2% glucose, and the cell density was monitored. (B) At the indicated times, aliquots were removed and dehydrated. The dried yeast cells were rehydrated at 40°C, diluted, and spread onto YPD plates as described in Materials and Methods. Dehydration survival was calculated as the average number of colonies from triplicate platings and was corrected for differences in cell density prior to dehydration. (C) Additional aliquots of cells were removed at each time point and assayed for total cellular trehalose and protein as described in Materials and Methods. The data shown are from one experiment. Similar results were seen in replicates of this experiment, with some variations in the magnitude of the differences between the two strains.

dration survival rates of the Δ*ath1* and wild-type strain grown on rich medium remained relatively equal.

The Δ*ath1* mutant survives freezing better than the wild-type strain. Similar to its protective role during dehydration, trehalose accumulation confers resistance to freezing (12, 16, 21). We therefore examined the effect of the Δ*ath1* mutation on survival of frozen yeast cells, as described in Materials and Methods. The wild-type cells showed 79, 77, 41, and 18% survival relative to the Δ*ath1* cells following freezing for cultures at 72, 93, 116, and 138 h of growth, respectively (data not shown). With frozen cells the magnitude of the difference in viability was less substantial than that seen with dehydrated cells. Freezing cells in suspension is less relevant than dehydration to commercial procedures; frozen yeast cells are used in preparation of dough, while dried yeast cells are prepared from suspension. For this reason, we did not pursue comparisons of freeze survival more extensively.

The Δ*ath1* mutant produces higher concentrations of ethanol in its culture supernatant than the wild-type strain. Another stress condition that is relevant particularly to the brewing industry is a yeast strain's tolerance to ethanol. Initially, we examined whether the Δ*ath1* strain would produce higher levels of ethanol than the wild-type strain. Both strains were grown in rich YP medium supplemented with varying concentrations of glucose (0 to 40%). Samples were removed at various times to measure cell density and ethanol levels in the culture supernatant, as described in Materials and Methods. Under rich-medium culture conditions, the Δ*ath1* strain produced higher cell densities than the wild-type strain at glucose concentrations between 5 and 35% (Fig. 4A). The percentage of ethanol in the culture supernatant also showed that the Δ*ath1* strain was able to produce and/or tolerate higher concentrations of ethanol than the wild-type strain over a significant range of glucose concentrations (Fig. 4B). However, this experiment did not explicitly indicate whether the higher concentrations of ethanol in the Δ*ath1* culture supernatant were due to an inherently better fermentation ability of the Δ*ath1* strain or to a higher resistance to ethanol.

The Δ*ath1* mutant is more resistant to exogenous ethanol shock than the wild-type strain. To determine directly whether the Δ*ath1* strain could tolerate higher levels of ethanol, Δ*ath1*

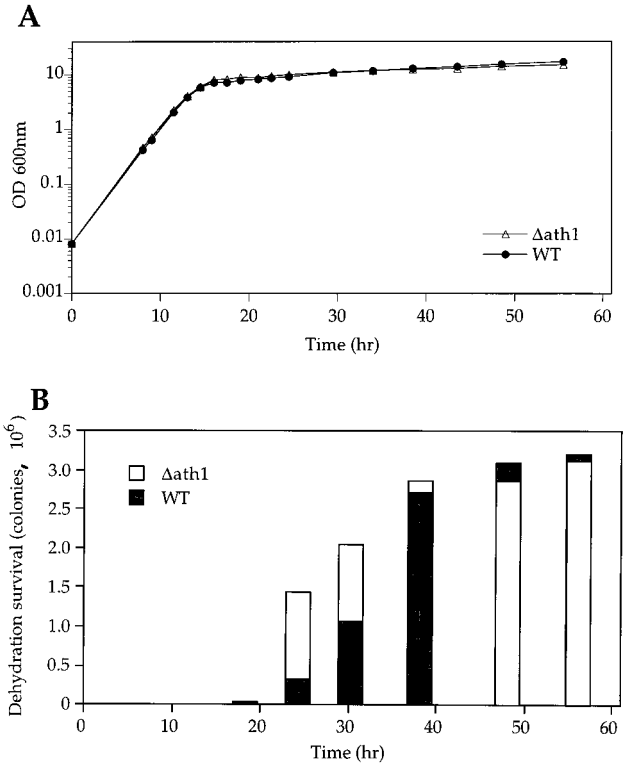

FIG. 3. Colony survival after dehydration of the Δ*ath1* and wild-type (WT) strains grown in rich glucose medium. (A) Yeast cells were grown in YP–2% glucose and the cell density was monitored. (B) Dehydration survival was determined as described in the legend to Fig. 2.

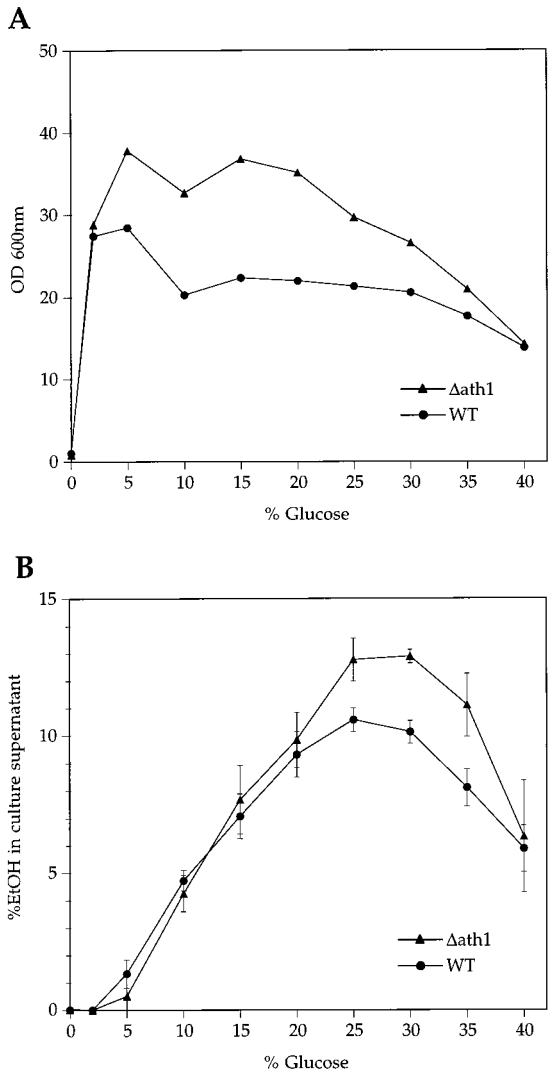

FIG. 4. Cell density and ethanol content in the culture supernatant of the Δ*ath1* and wild-type (WT) strains grown in YP–0 to 40% glucose. (A) Growth of the Δ*ath1* and wild-type strains over a wide range (0 to 40%) of glucose concentrations in YP medium. The growth curves represent cell density at 49 h after inoculation. (B) At each time point, aliquots were removed and the ethanol concentration in the medium was determined by gas chromatography, as described in Materials and Methods. The data shown in panel A are from one experiment. Essentially identical results were seen in multiple replicates of this experiment. The data in panel B are the averages of two independent experiments.

and wild-type strains were grown in SM–2% glucose. At the 41-h time point, which was determined to be a period of high cellular trehalose levels in both the wild-type and Δ*ath1* strains (Fig. 2C), samples were removed and exposed to 18% ethanol as described in Materials and Methods. After 1 h of ethanol exposure, the Δ*ath1* strain showed a fivefold increase in the number of surviving colonies over the wild-type strain (Fig. 5). By the second hour of incubation, the number of viable Δ*ath1* colonies decreased by eightfold, while the viability of the wild-type strain was nearly abolished. While a few Δ*ath1* colonies still survived after 3 h of ethanol exposure, no surviving colonies were detected after 4 h of incubation in the YNB–18% ethanol medium. Therefore, the Δ*ath1* strain exhibited a substantial increase in survival, relative to the wild-type strain, under direct exposure to toxic levels of ethanol, and this increased ethanol tolerance correlated with the elevated cellular trehalose content.

DISCUSSION

We have examined, for the first time, the phenotypic effects of a Δ*ath1* mutation on yeast growth and stress responses. The *ATH1* gene is essential for ATH enzyme activity (8), although its role in ATH activity is uncharacterized; specifically, it is not known if *ATH1* is the structural gene for ATH. In this study, we have demonstrated that the Δ*ath1* strain exhibited greater resistance to dehydration, freezing, and exposure to toxic levels of ethanol than the wild-type strain. In addition, the Δ*ath1* strain maintained up to a nearly twofold increase in cell density over the wild-type strain in rich-medium conditions. The improved stress resistance and growth exhibited by the Δ*ath1* strain strongly correlated with its ability to accumulate higher levels of trehalose when grown in both rich and minimal media. Interestingly, disrupting the activity of the cytosolic NTH did not confer similar increased growth capabilities on the Δ*nth1* strain, nor did it cause elevated accumulation of cellular trehalose. Therefore, disrupting *ATH1*, but not *NTH1*, resulted in elevated accumulation of trehalose and greater stress resistance.

It is worth noting that the studies on dehydration and freezing resistance were carried out with cells grown in 2% glucose. At higher glucose concentrations there are substantial differences between the Δ*ath1* and wild-type strains with regard to final cell density. This difference makes it highly problematic to examine both strains at equivalent phases of growth. Because growth phase is a critical factor in stress resistance, we wished to eliminate this variable from the experiment. Accordingly, we chose the 2% glucose level, at which the growth rates and final cell densities are nearly indistinguishable between the two strains (Fig. 2A and 3A). Differences in survival then would more likely be due to changes in intracellular trehalose levels and not to growth phase. In addition, these conditions are more in line with industrial growth conditions in which the glucose levels are kept at less than 1% to limit fermentation which would otherwise lead to lower trehalose levels. Higher glucose concentrations may be relevant to sweet or pastry

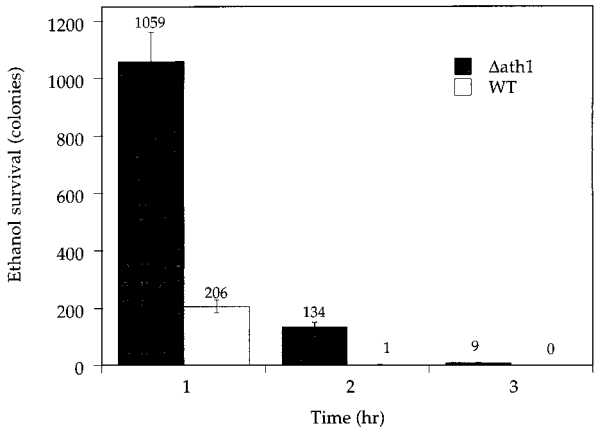

FIG. 5. Colony survival of the Δ*ath1* and wild-type (WT) strains after an 18% exogenous ethanol shock. Cells were grown in SM–2% glucose for 41 h, pelleted, and resuspended in YNB containing 18% ethanol, as described in Materials and Methods. The number of surviving colonies after 1, 2, and 3 h of ethanol shock is indicated above each graph bar. The data shown are from one experiment. Similar results were seen in replicates of this experiment, with some variations in the magnitude of the differences between the two strains.

dough preparations, however, in which fivefold-higher sugar levels are used (20% sugar based on bakers' percent compared with 4% for standard dough). We have demonstrated that the Δ*ath1* mutant showed greater survival even when grown in 2% glucose. We expect the magnitude of the difference would be greater for cells grown in 20% glucose.

The accumulation of trehalose on both sides of the plasma membrane offers yeast cells greater protection during environmental assaults such as dehydration, freezing, and ethanol shock. A basal level of trehalose appeared to be necessary to provide a protective function to yeast plasma membranes. For example, our dehydration experiments showed that the concentrations of intracellular trehalose must reach a minimum level (~0.015 to 0.025 μg of trehalose per μg of total cellular protein [cf. Fig. 2B and C]) before significant survival can be detected, consistent with previous observations (11). In addition, the degree of trehalose accumulation above this basal level appeared to correspond to the degree of survival after dehydration; the greatest viability of the strains roughly correlated with the periods of highest cellular trehalose content. Similarly, the Δ*ath1* sample that was exposed to ethanol shock had a twofold-higher trehalose concentration than the wild-type strain and displayed survival rates that were at least five times greater than those of the wild-type strain.

Since abolishing ATH activity appears to result in a hardier yeast strain, an obvious question remains concerning the role of this enzyme in yeast physiology. It must be kept in mind, however, that we have only examined the phenotypic features of the Δ*ath1* and wild-type strains under a limited number of nutritional conditions and only during the relatively early stages of the yeast life cycle. The mobilization of trehalose as a reserve energy source may be physiologically important in the later phases of the life cycle, when nutritional resources are scarce (23, 38). In addition, when stationary cells are reinoculated into rich medium at lower cell densities, they rapidly degrade accumulated trehalose and resume growth in an exponential manner (35, 38). ATH may be necessary for this rapid degradation of accumulated trehalose. Whether the mobilization of trehalose is a requisite step in resuming exponential growth is unknown. If ATH is inactive, this transition out of stationary phase may be retarded. Although the accumulation of trehalose is critical for yeast survival under various stress conditions, it may have undesirable effects if it is not degraded. For example, depositing trehalose on the plasma membrane increases the rigidity of the membrane, thus potentially inhibiting various physiological functions for which a more fluid membrane is necessary.

The functional and temporal coordination of the two trehalases in yeast cells is not well understood. The cytosolic NTH is active only during the exponential phase of the life cycle, when cells are growing on fermentable sugars (35) and trehalose is rapidly hydrolyzed by NTH. The activation of NTH is controlled by the *RAS*/adenylate cyclase pathway, which phosphorylates the enzyme to its active form. It is not clear why the hydrolysis of trehalose cannot be regulated solely by controlling the phosphorylation and dephosphorylation of NTH. A possible need for a second trehalase, ATH, may be explained by the relative instability of NTH compared with ATH. Thus, as the cells exit the exponential phase and enter the respiratory or stationary phase, cyclic AMP and fermentable sugar levels decrease, leading to NTH dephosphorylation and subsequent degradation. During the same period, ATH is synthesized and the accumulation of trehalose begins. However, since ATH is sequestered in the vacuole, away from its cytosolic substrate, trehalose continues to accumulate. A possible reason why ATH is synthesized long before it is needed may be because general protein synthesis is severely down-regulated later in the stationary phase due to limited nutritional resources (39). Since ATH is the only trehalase active during the late stages of the life cycle, when trehalose is mobilized as an energy source, there must be a mechanism by which trehalose is delivered to the vacuole. This may be accomplished by a trehalose transporter residing on the vacuolar membrane (similar to the one found on the plasma membrane) or perhaps by an autophagic mechanism. Therefore, the point of regulation of this enzyme may be at the level of the delivery of its substrate to the vacuole and not by any direct modifications to the protein.

The improved growth characteristics and stress resistance of the Δ*ath1* strain make it an attractive candidate for a number of commercial applications. More than 500 million pounds (226,796,185 kg) of compressed yeast are produced in the United States each year (33a), and more than 2 million tons are produced worldwide (34), for use in a variety of baked products. Exploiting the superior growth of the Δ*ath1* strain may decrease commercial growth times and reduce production costs. The increased survival exhibited by the Δ*ath1* strain after freezing and dehydration may be utilized to decrease the amount of yeast used in frozen dough preparations as well as in the preparation of instant active dry yeast, thus reducing consumer costs. Furthermore, greater dehydration resistance may also endow the Δ*ath1* strain with a longer storage life without compromising its viability upon rehydration. The ability of the Δ*ath1* strain to tolerate higher levels of ethanol may be useful for the brewing industry or in fuel production. We are currently examining the *ATH1* gene to determine whether it is the structural gene for ATH.

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