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Roles of Glycerol and Glycerol-3-Phosphate Dehydrogenase (NAD^+) in Acquired Osmotolerance of *Saccharomyces cerevisiae*

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In a cell culture of *Saccharomyces cerevisiae* exponentially growing in basal medium, only 0.02% of the cells were osmotolerant, i.e., survived transfer to medium containing 1.4 M NaCl. Short-time conditioning in 0.7 M NaCl medium transformed the whole population into an osmotolerance phenotype. During this conditioning, the rate of formation of glycerol, the main compatible solute in *S. cerevisiae*, increased threefold and the specific activity of glycerol-3-phosphate dehydrogenase (NAD^+) (GPDH) (EC 1.1.1.8) was enhanced sixfold. The apparent flux control coefficient for GPDH in the formation of glycerol was estimated to be 0.6. Glycerol production was also favored by regulated activities of alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase $[NAD(P)]^+$ (EC 1.2.1.5). About 50% of the total glycerol produced during conditioning in 0.7 M NaCl was retained intracellularly, and the increased glycerol accumulation was shown to be not merely a result of enhanced production rate but also of increased retention of glycerol. Washing the cells with solutions of lower salinities resulted in loss of glycerol, with retained levels proportional to the concentration of NaCl in the washing solution. Cycloheximide addition inhibited the development of acquired osmotolerance and conditioned cells washed free of glycerol retained a high degree of osmotolerance, which indicate that protein synthesis was required to establish the osmotolerance state.

The response of *Saccharomyces cerevisiae* to a sudden osmotic dehydration is closely linked to the physiological state of the cell culture. Exponentially growing cells are the least tolerant, with only a minor fraction of the population being capable of forming colonies on low-water-potential agar media (3, 11). This heterogeneity of the population has been shown to be physiological and not genetic (11). Other environmental stresses to which the response of *S. cerevisiae* is influenced by the physiological state of the cell culture are exposure to heat (19, 21), ionizing radiation (13), and chemical mutagens (15). The osmosensitivity phenotype of exponentially growing cells is altered and the osmotolerance is improved when cells: (i) enter the transition phase between fermentation and respiration, (ii) enter the stationary phase, (iii) grow exponentially in media containing low levels of salt, and (iv) are transferred to and conditioned for a short time in solutions containing low levels of salt (3, 11). Alterations in osmotolerance by short-time conditioning at low levels of salt are one example of a more general biological phenomenon, improved tolerance to an extreme stress by short-time conditioning to an intermediate stress. Other examples are the acquired thermotolerance of *S. cerevisiae* (12, 16) and the acquired osmotolerance phenotype of *Escherichia coli* (7).

The involvement of protein synthesis in the development of acquired thermotolerance of *S. cerevisiae* has attracted much attention. Some investigators report a high correlation between the synthesis of heat shock proteins and thermotolerance (12, 17), while others present opposing results (9, 22). The necessity for protein synthesis upon acquisition of osmotolerance was therefore of interest.

Extensive studies have been undertaken to examine the production and accumulation of osmotica by yeast cells in response to growth under osmotic stress. While some yeasts osmoregulate by accumulating more than one type of polyol, *S. cerevisiae* exclusively uses glycerol (4). The selective advantage of glycerol in osmoregulation could be attributed to the compatible nature of this solute (4, 6), but glycerol may also have a specific role as a protective agent for some osmosensitivity cellular function.

Enhanced intracellular levels of glycerol are not the only salt-induced feature observed for cells grown in medium containing salt. The specific activity of the cytoplasmic glycerol-3-phosphate dehydrogenase (NAD^+) (GPDH) (EC 1.1.1.8), one of the two enzymes constituting the glycerol-producing pathway, increases markedly, with salt-induced activities being as much as 30-fold higher than those in the uninduced state (8). Cells in the transition between fermentation and respiration or entering the stationary phase have all been shown to contain raised levels of GPDH (3).

Glycerol accumulation and induced levels of GPDH are factors that may contribute to the capacity of the cells to withstand and recover from a severe osmotic shock. This study was undertaken in an attempt to evaluate the contribution of these two factors to the osmotolerance phenotype and to characterize the response of the cells to short-time conditioning.

MATERIALS AND METHODS

Yeast strain. Baker's yeast *Saccharomyces cerevisiae* Y41 (1) (ATCC 38531) was maintained on YEPD agar (yeast extract, 1% [wt/vol]; peptone, 2% [wt/vol]; glucose, 2% [wt/vol]; agar, 2% [wt/vol]).

Media and growth conditions. The defined medium that was used, yeast nitrogen base without amino acids (YNB) (Difco Laboratories, Detroit, Mich.), was supplemented with 0.5% glucose, and when indicated, with appropriate amounts of NaCl. YNB (10× strength) was filter sterilized, while glucose and salt solutions were autoclaved separately. The pHs of all media were adjusted to 6.0. In all cases, the flasks were incubated at 30°C on a rotary shaker (110 rpm). Primary cultures were grown for 24 to 48 h in 300-ml flasks containing 50 ml of medium and then used as the inoculum for 500 ml of medium, contained in 2.8-liter Fernbach flasks,

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to yield an initial concentration of 2×10^4 CFU/ml. Short-time conditioning experiments were performed with cells grown for about 15 h to an optical density at 610 nm of 0.50 (equivalent to 5×10^6 CFU/ml), as seen with a spectrophotometer (model B; Beckman Instruments, Inc., Fullerton, Calif.); these cells were growing at a specific growth constant of 0.26.

Dry weight determinations were performed in triplicate on samples finally washed twice in distilled water. Cells conditioned in 0.35 or 0.7 M NaCl were first washed in stepwise declining salt concentrations before the final washings in distilled water.

Short-time conditioning procedure. The cell culture was divided into 250-ml portions and centrifuged (4,000 × *g*, 5 min, 20°C). The cell pellets were suspended in 250 ml of YNB medium with 0.35 or 0.7 M NaCl or without NaCl (control). The cultures were incubated for 60 min, which is referred to as the period of conditioning.

Viability measurements. Viability was measured in YEPD agar, and when indicated, 1.4 M NaCl was added. Pour plates were used to circumvent the problem of NaCl diffusion under the sample drop. Before agar and sample were mixed, the agar was thermally equilibrated to 42°C. The plates were incubated at 30°C in plastic bags, and the 1.4 M NaCl plates were counted after 6 days. Longer incubation gave no rise in colony number. The plates without NaCl were counted after 2 days of incubation.

Rate of protein synthesis. The rate of protein synthesis was measured by adding 0.30 μCi of [^{35}S]methionine in 10 μl (specific activity of 240 Ci/mmol; Amersham Corp., Amersham, England) to 200 μl of cell suspension in Eppendorf tubes. After 5 min of incubation at 30°C, the suspension was transferred into 5 ml of ice-cold 5% (wt/vol) trichloroacetic acid. The trichloroacetic acid suspension was filtered through 0.2-μm-pore-size filters (Nuclepore Corp., Pleasanton, Calif.) after 30 min of incubation on ice. The filters were washed three times with 3 ml of ice-cold 5% (wt/vol) trichloroacetic acid, dried for 1 h at 50°C, and counted in a Tri-Carb liquid scintillation spectrometer (model 3255; Packard Instrument Co., Inc., Rockville, Md.) after addition of 7 ml of Aquassure scintillation cocktail (Dupont, NEN Research Products, Boston, Mass.). The values (in disintegrations per minute) were calculated by using the counting efficiency determined by the external standard ratio method.

Analyses of glycerol, acetate, and ethanol. Analyses of glycerol, acetate, and ethanol were carried out by enzymatic methods, as described previously (3).

Preparation of cell extracts for enzyme activity measurements. Cells (0.25 to 0.5 g [wet weight]) were suspended in 0.5 ml of homogenization buffer (10 mM triethanolamine, 1 mM dithioerythritol, 1 mM EDTA [pH 7.5]). The cell suspension was homogenized at 4°C for 5 min in a bead mill (Vibrogen-Zellmüle, Edmund Bühler, Tübingen-Weilheym, Federal Republic of Germany) with 1 g of glass beads (0.5 mm in diameter). The homogenate was collected, and the homogenization vessel was rinsed with 0.5 ml of homogenization buffer. This washing was pooled with the homogenate. The crude extract was centrifuged at 18,000 × *g* for 15 min. The supernatant was desalted by passage through a PD-10 column (Pharmacia Fine Chemicals, Uppsala, Sweden) and then assayed for enzyme activity. The final protein concentration of the extract was around 10 mg/ml. Protein concentration was determined by the method of Sedmak and Grossberg (20), using gamma globulin (no. G-5009) (Sigma Chemical Co., St. Louis, Mo.) as the standard.

Assays of enzyme activity. The assays were performed in a recording Shimadzu UV-240 double-beam spectrophotometer (Shimadzu Co., Kyoto, Japan) at 25°C in 1-ml cuvettes. NADH depletion or production was monitored spectrophotometrically at 340 nm. To calculate the enzyme activities, the reaction rate between 30 and 90 s and an absorption coefficient for NADH of 6.2×10^3 M^{-1} cm^{-1} were used (1 U = 1 μmol of NADH or NAD consumed per min).

GPDH (EC 1.1.1.8) was assayed in a buffer containing 20 mM imidazole-HCl (pH 7.0), 1 mM dithioerythritol, 1 mM $MgCl_2$, 0.67 mM dihydroxyacetonephosphate (DHAP), and 0.09 mM NADH. The reaction was started by the addition of DHAP and was linear for at least 2 min. No background NADH oxidase activity was detected. At least five protein concentrations in the range of 0.05 to 0.5 mg/ml in the cuvette were recorded per sample. Specific activities of GPDH were dependent on protein concentration in the cuvette (8): the higher the protein concentration, the higher the specific activity. Consequently, from the graph presenting the inverse of specific activity against the inverse of the protein concentration, the line fitted by least-square regression gave the maximum specific activity as the specific activity extrapolated from an infinite protein concentration. This mathematical transformation of the recorded specific activities of a sample into the maximum specific activity of a sample did not change the relative order between different samples, i.e., the fitted lines never crossed each other in quadrant 1. The maximum specific activity of GPDH will, throughout this report, be referred to as the specific activity.

Alcohol dehydrogenase (ADH) (EC 1.1.1.1) was assayed in a buffer containing 100 mM glycine–40 mM hydrazine sulfate (pH 8.5), 1 mM NAD^+, and 100 mM ethanol. Aldehyde dehydrogenase $[NAD(P)]^+$ (ALDH) (EC 1.2.1.5) was assayed in 100 mM Tris–HCl buffer (pH 8.0), 1 mM dithiothreitol, 100 mM KCl, 1 mM NAD^+, and 0.065 mM acetaldehyde. For both alcohol dehydrogenase and aldehyde dehydrogenase, the specific enzyme activity was independent of protein concentration in the cuvette.

Washing procedure. Samples (30 ml) of the culture were centrifuged at 3,000 × *g* for 5 min, and the pellet was dissolved in 30 ml of cold (4°C) salt solution (0 to 0.7 M NaCl). The procedure was repeated, and the obtained cell suspension was used for further analyses.

RESULTS

Water stress plating hypersensitivity. In an exponentially growing culture of *S. cerevisiae*, only a minor subpopulation is viable (capable of forming colonies) on plates containing high concentrations of solute, i.e., salt or sugar (3, 11). This subpopulation constituted about 0.02% of the total population, when plated on medium containing 1.4 M NaCl (Table 1). Dramatic and rapid changes in the size of the viable subpopulation occurred during conditioning of the cells at low salinities (Fig. 1). For the cells conditioned in 0.35 M NaCl, the size of the osmotolerant subpopulation was quickly raised (within 20 min) but never exceeded 20% of the total population. After 1 h of conditioning in 0.7 M NaCl, however, 100% of the population was viable on the 1.4 M NaCl plates.

Inhibition of protein synthesis. Cycloheximide addition (100 μg/ml) to the conditioning medium reduced the rate of protein synthesis to a maximum of 7% (2×10^3 dpm/mg [dry weight] per min) of the rate obtained for cells conditioned without the inhibitor. During conditioning in 0.7 M NaCl medium, the initial osmotic dehydration per se hampered the rate of protein synthesis to 15% (34×10^3 dpm/mg [dry

TABLE 1. Osmotolerant fraction of the total population of *S. cerevisiae* that was viable on 1.4 M NaCl plates

NaCl concn (M) in conditioning medium[a]	Treatment	Osmotolerant fraction[b]
0 (nonconditioned cells)	None	0.02
0.35	None	16
	Cycloheximide (100 μg/ml)	0.05
0.7	None	100
	Cycloheximide (100 μg/ml)	0.2
	Washed in 0.7 M NaCl	96
	Washed in 0.35 M NaCl	96
	Washed in 0 M NaCl	50

[a] Cells conditioned for 60 min in medium with indicated concentration of NaCl.

[b] Quantified as the percent of the total population (CFU on 0 M NaCl plates) that formed colonies on 1.4 M NaCl plates. Data represent mean values of three independent experiments, except for washed cells (single experiment).

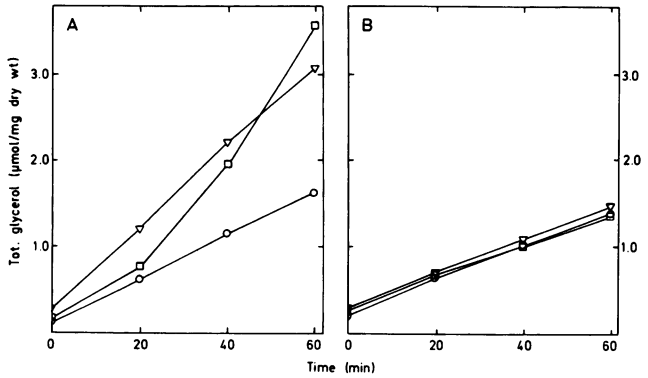

FIG. 2. Total (Tot.) production of glycerol during conditioning for *S. cerevisiae* without cycloheximide (A) or with the addition of cycloheximide (100 μg/ml) (B). Symbols: ○, control in 0 M NaCl medium; ▽, conditioning in 0.35 M NaCl medium; □, conditioning in 0.7 M NaCl medium. Data represent mean values of three independent experiments.

weight] per min) of the rate during growth in the basal medium (235 × 10^3 dpm/mg [dry weight] per min). Conditioning for 20 min reestablished the rate to about 45% (104 × 10^3 dpm/mg [dry weight] per min) of the control. When the cytoplasmic protein synthesis was blocked during conditioning by cycloheximide treatment, only minor increases in viability on 1.4 M NaCl plates were observed (Table 1).

Glycerol production. The apparent specific glycerol production rate in basal medium (0 M NaCl) was 1.4 μmol of glycerol per mg (dry weight) per h (Fig. 2), in good agreement with a previous report (3). When the NaCl concentration of the conditioning medium was increased to 0.35 M, the specific rate of glycerol production was enhanced to 3.1 μmol of glycerol per mg (dry weight) per h. Both in the basal medium and in the 0.35 M NaCl conditioning medium, the rate of glycerol production remained constant over the entire conditioning period (60 min). For cells conditioned in 0.7 M NaCl, the rate of glycerol production equaled that of the control for the first 20 min; thereafter, there was a steady increase in the production rate for the subsequent 40 min. The specific glycerol production rate for the last 20 min of the conditioning period was around 4.5 μmol of glycerol per mg (dry weight) per h, which was about threefold higher than the production rate for the control.

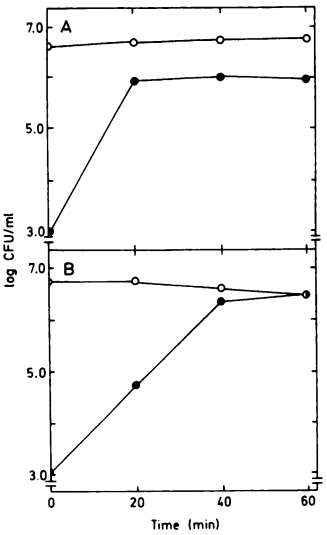

FIG. 1. Viability of *S. cerevisiae* on YEPD plates supplemented with 0 or 1.4 M NaCl during conditioning in 0.35 M NaCl medium (A) or 0.7 M NaCl medium (B). Symbols: ○, 0 M NaCl; ●, 1.4 M NaCl. Data represent mean values of three independent experiments.

After inhibition of the cytoplasmic protein synthesis by addition of cycloheximide (100 μg/ml), no increase in the specific glycerol production rate was observed with increased concentrations of NaCl (Fig. 2B), and the rate observed remained roughly the same as that for the control with no salt and no cycloheximide addition. Surprisingly, there was no reduction in the glycerol production rate at 0.7 M NaCl when cycloheximide was added; despite the dehydrated state of the cells, the rate of glycerol production remained identical to that of the control (Fig. 2B).

Specific activity of GPDH. When the rate of glycerol formation was increased as a response to increased concentrations of NaCl in the medium (Fig. 2A), the specific activity of GPDH (EC 1.1.1.8) was enhanced (Fig. 3). After the cells were conditioned for 60 min in 0.7 M NaCl, the specific activity was about sixfold higher than that for nonconditioned cells. Upon cycloheximide treatment, neither the rate of glycerol production nor the specific activity of GPDH increased (Fig. 3), thus supporting induction of GPDH as a prerequisite for increased rate of glycerol formation.

GPDH, which is the first enzyme in the pathway from glycolysis to glycerol, may be a bottleneck in glycerol production. Theoretically, all the enzymes involved in a metabolic pathway can be allocated controlling power on the flux by estimates of the flux control coefficient for each enzyme (23). The inset (Fig. 3) shows a plot of the logarithm of GPDH activity versus the logarithm of glycerol production rate, from which an apparent flux control coefficient of 0.6 can be estimated (the slope). As the summation of all the flux control coefficients for a specific pathway will be 1.0, GPDH will thus have a major impact on the flux to glycerol. Comparison with the flux control coefficients of other claimed bottlenecks, such as phosphofructokinase and hexokinase, which have values of 0.4 and 0.3, respectively (23), verifies the use of the term as far as GPDH is concerned.

Acetate production. In the production of glycerol, the DHAP provided by glycolysis is not the only essential substrate; cytoplasmic NADH is required in equimolar

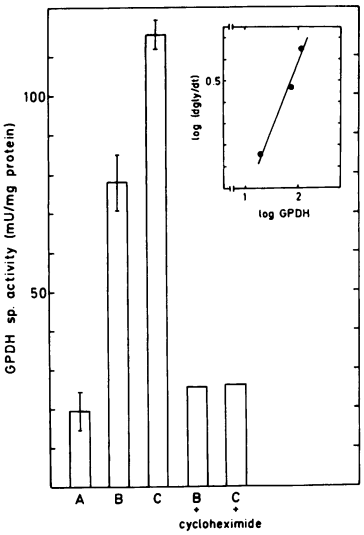

FIG. 3. Specific (sp.) activity of GPDH of *S. cerevisiae* before and after the period of conditioning. Bars: A, nonconditioned cells; B, cells conditioned in 0.35 M NaCl medium; C, cells conditioned in 0.7 M NaCl medium. When indicated, cycloheximide (100 μg/ml) was added during the conditioning. Data represent mean values of three independent experiments (A, B, and C) or single experiments (cycloheximide treatments). Error bars indicate standard deviations. The inset shows an estimate of the flux control coefficient. The dependence of the glycerol production rate (*d*gly/*dt*) on the specific activity of GPDH is plotted in a double logarithmic plot, and the slope of the curve equals the flux control coefficient.

amounts (14). The enhanced need for NADH during the conditioning in 0.7 M NaCl was partly accounted for by an increased rate of acetate production (Fig. 4), the salt-induced rate being 1.4 μmol/mg (dry weight) per h. For every mole of acetate produced, 2 mol of NADH will be available for further reduction compared with that in ethanol fermentation, which is NADH neutral (5, 14). The enhanced rate of acetate production during conditioning in 0.7 M NaCl was reflected in changed activities of the two enzymes competing for acetaldehyde as substrate; the metabolic flow to acetate was favored by a reduction in ADH activity and an increment in ALDH activity. For nonconditioned cells, the specific activities (in milliunits per milligram of protein; mean ± standard deviation of three independent experiments) were 595 ± 75 for ADH and 13.5 ± 3 for ALDH, while for cells

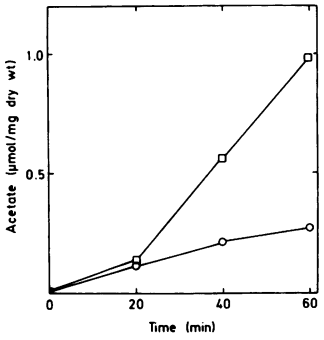

FIG. 4. Acetate production during conditioning of *S. cerevisiae*. Symbols: ○, control cells and 0 M NaCl medium; □, cells conditioned in 0.7 M NaCl medium. Data represent mean values of three independent experiments.

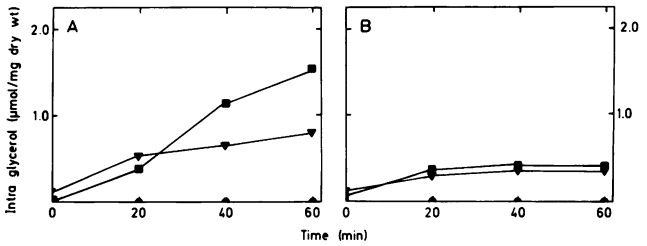

FIG. 5. Glycerol accumulated (Intra glycerol) during conditioning for *S. cerevisiae* without cycloheximide (A) or with the addition of cycloheximide (100 μg/ml) (B). Symbols: ●, control and 0 M NaCl medium; ▼, conditioning in 0.35 M NaCl medium; ■, conditioning in 0.7 M NaCl medium. Data represent mean values of three independent experiments.

conditioned in 0.7 M NaCl, the specific activities were 208 ± 74 for ADH and 28.7 ± 4 for ALDH.

Glycerol accumulation. Glycerol accumulation was demonstrated for various salt concentrations of the conditioning medium (Fig. 5). Even though production occurs in the case of basal medium (see above), no glycerol accumulated intracellularly. Since the glycerol production rate increased as the salt concentration increased, the accumulation could reflect an enhanced production rate, if the production rate exceeded the rate of glycerol excretion or leakage. On the contrary, cells exposed to 0.7 M NaCl produced the same amount of glycerol during the first 20 min as the control but retained a high proportion (around 50%) (Fig. 2A and 5A). Furthermore, cycloheximide-treated cells with no salt-induced increase in glycerol production rate retained glycerol during the conditioning (Fig. 2B and 5B).

Glycerol content of washed cells. The accumulated glycerol of the salt-conditioned cells could be removed by washing, and the glycerol levels retained after the washing procedure were proportional to the salt concentration of the washing solution (Fig. 6). When cells were washed in distilled water, no residual intracellular glycerol could be detected.

Cell viability for washed cells. Table 1 shows how the osmotolerance of conditioned cells was affected by complete or partial removal of glycerol by washing. Partial glycerol depletion by washing with 0.35 M NaCl yielded approximately the same level of intracellular glycerol as that for

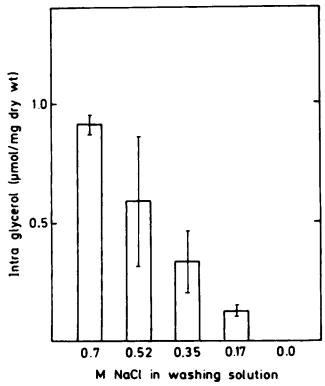

FIG. 6. Residual intracellular glycerol (Intra glycerol) in *S. cerevisiae* conditioned in 0.7 M NaCl medium and then washed in washing solutions with different concentrations of NaCl. No detectable glycerol remained after washing in 0 M NaCl solution. Data represent mean values of two independent experiments, and error bars indicate standard deviations.

cells conditioned in medium supplemented with cycloheximide (Fig. 5B and 6). No reduction in the osmotically resistant population was observed for these washed cells. On complete removal of glycerol by washing in distilled water, a twofold reduction in viability was observed for cells conditioned in 0.7 M NaCl medium.

DISCUSSION

The osmosensitivity phenotype of exponentially growing cells of *S. cerevisiae* can be altered into an osmotolerance phenotype by short-time conditioning to an intermediate osmotic stress. During this short period of conditioning, the cells enter a state of acquired osmotolerance. Phenomenologically, this rapid reprogramming of the cells at a sublethal stress to permit survival and growth under a more severe stress has traits in common with acquired thermotolerance (12). In an exponentially growing culture, only 0.02% of the cells exhibited a capacity to form colonies on 1.4 M NaCl plates. Conditioning in 0.7 M NaCl renders the whole population osmotolerant. For 0.35 M NaCl, however, the osmotolerant subpopulation never exceeded 20% of the total. To fully understand the mechanisms behind this differential dynamics within the population, more knowledge has to be obtained about the osmosensitive Achilles' heel(s) of the cell.

If the cytoplasmic protein synthesis was inhibited by cycloheximide addition during conditioning, no significant rise in the size of the osmotolerant subpopulation was observed. Synthesis of new proteins or enhanced levels of preexisting proteins may therefore contribute to the acquired osmotolerance phenotype.

Since glycerol has been shown to be the main compatible solute in *S. cerevisiae* during growth under osmotic stress (4, 18), its role in acquired osmotolerance was of interest. During conditioning in 0.7 M NaCl, the rate of glycerol production increased about threefold. In a previous report, the salt-induced increased rate of glycerol formation of the osmotolerant yeast *Debaryomyces hansenii* never exceeded 1 μmol of glycerol per mg (dry weight) per h during adaptation to saline medium (calculated from reference 2). The salt-induced value of *D. hansenii* is comparable with that of cells of *S. cerevisiae* grown in basal medium (1.4 μmol/mg [dry weight] per h). Thus, the glycerol-producing capacity per se does not seem to be a determinant for osmotolerance.

More importantly, as far as the osmotolerance of the cells is concerned, the intracellular levels of glycerol rose to roughly 0.8 and 1.5 μmol/mg (dry weight) in 0.35 and 0.7 M NaCl, respectively. These levels are comparable with reported values for accumulated glycerol at the same salinities in the osmotolerant species *D. hansenii* (2). For cells conditioned in 0.7 M NaCl, about 50% of the total glycerol produced was retained within the cells throughout the whole conditioning period, while in 0.35 M NaCl, not more than 25% remained intracellularly (Fig. 2A and 5A). The increased accumulation during conditioning was not just a result of the increased production rate, since cycloheximide-treated cells with no increase in production rate retained glycerol during the conditioning. This points to some other mechanism than just increased glycerol production rate being responsible for the accumulation of glycerol. Our hypothesis is that the initial osmotic dehydration perturbs the permeability of the plasma membrane, which results in glycerol accumulation in the cells. This permeability change was shown to be glycerol specific, since no intracellular accumulation of ethanol or acetate could be detected (results not shown).

The accumulated glycerol appeared to be osmotically active and the level appeared to be osmotically controlled, since the glycerol that remained after washing was proportional to the concentration of salt in the washing solution. The cells were totally depleted of glycerol by washing with distilled water, which supplied us with a tool for determining the contribution of glycerol to the osmotolerance phenotype. Partial replenishing of glycerol did not affect osmotolerance, while complete depletion conferred a minor reduction in osmotolerance. In addition, the glycerol accumulated during the conditioning of cycloheximide-treated cells did not protect the major part of the population from osmotic shock. Thus, glycerol, being clearly involved in osmoregulation, did not have a major impact on the osmotolerance of the cells.

The specific activity of GPDH, which links glycerol formation to glycolysis, has been shown to increase in cells growing exponentially under osmotic stress (8). The increase in the specific glycerol production rate during conditioning in 0.7 M NaCl, as well as the basal levels obtained after the addition of cycloheximide, may be taken to indicate induction of enzyme(s) involved in glycerol production. In view of the relatively high apparent flux control coefficient of GPDH in the glycerol production, one can picture the enzyme as a bottleneck in this production. However, to approach a complete understanding of the factors governing the rate, one has to take into consideration all enzymes and metabolite pools that are directly or indirectly coupled to glycerol production. Since GPDH seemed to limit the rate of glycerol production, cells with a high specific GPDH activity would have the capacity for a high rate of glycerol formation and an enhanced osmoregulatory potential.

In conjunction with induction of GPDH, the activities of two other enzymes involved in NADH-NAD recycling were shown to be regulated. ADH activity was repressed while ALDH activity was induced, both changes cooperating to yield an enhanced production of NADH and finally, of glycerol. This is supported by increased production of glycerol by an ADH I-deficient mutant of *S. cerevisiae* (10).

To summarize, de novo protein synthesis partly contributed in establishing the state of acquired osmotolerance of the cells. Because of the indicated rate-limiting function of GPDH in glycerol production, this enzyme is a good candidate for a protein instigating induced osmotolerance. However, a high level of GPDH is not the single prerequisite for *S. cerevisiae* to transform into an osmotolerance phenotype, since ethanol-grown cells, having a high specific activity of GPDH, have been shown to exhibit a low osmotolerance (3). Other proteins might have to be coordinately regulated with GPDH, for example, ADH and ALDH, for this enzyme to express its full power as a determinant in the osmotolerance phenotype of the cells.

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