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Roles of Sugar Alcohols in Osmotic Stress Adaptation. Replacement of Glycerol by Mannitol and Sorbitol in Yeast[1]

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For many organisms there is a correlation between increases of metabolites and osmotic stress tolerance, but the mechanisms that cause this protection are not clear. To understand the role of polyols, genes for bacterial mannitol-1-P dehydrogenase and apple sorbitol-6-P dehydrogenase were introduced into a *Saccharomyces cerevisiae* mutant deficient in glycerol synthesis. Sorbitol and mannitol provided some protection, but less than that generated by a similar concentration of glycerol generated by glycerol-3-P dehydrogenase (GPD1). Reduced protection by polyols suggested that glycerol had specific functions for which mannitol and sorbitol could not substitute, and that the absolute amount of the accumulating osmoticum might not be crucial. The retention of glycerol and mannitol/sorbitol, respectively, was a major difference. During salt stress, cells retained more of the six-carbon polyols than glycerol. We suggest that the loss of >98% of the glycerol synthesized could provide a safety valve that dissipates reducing power, while a similar high intracellular concentration of retained polyols would be less protective. To understand the role of glycerol in salt tolerance, salt-tolerant suppressor mutants were isolated from the glycerol-deficient strain. One mutant, sr13, partially suppressed the salt-sensitive phenotype of the glycerol-deficient line, probably due to a doubling of [K^+] accumulating during stress. We compare these results to the "osmotic adjustment" concept typically applied to accumulating metabolites in plants. The accumulation of polyols may have dual functions: facilitating osmotic adjustment and supporting redox control.

Several mechanisms are involved in the adaptation to water stress by *Saccharomyces cerevisiae*, which experiences both osmotic stress and ion toxicity when exposed to NaCl. To respond to a high external osmotic environment, the cells accumulate glycerol, seemingly to compensate for differences between the extracellular and intracellular water potential (Brown, 1990). High osmolarity is perceived as a signal by two membrane osmosensors. The signal is transferred via a MAP-kinase cascade (HOG) and, among many other effects, enhances the expression of the glycerol biosynthetic pathway (Maeda et al., 1994). Glycerol-3-P dehydrogenase (GPD), which is encoded by two GPD genes, catalyzes the first reaction from dihydroxyacetone-P to glycerol-3-P, which is then converted to glycerol by glycerol-3-phosphatase (GPP), which is encoded by the GPP genes (Albertyn et al., 1994; Norbeck et al., 1996; Ansell et al., 1997). Increased glycerol production leads to an increase in intracellular glycerol concentration, likely due to altered membrane permeability to glycerol under osmotic stress (Brown, 1990; Albertyn et al., 1994).

In contrast, the salt-tolerant *Zygosaccharomyces rouxii* seems to achieve glycerol accumulation by increased retention or uptake rather than by increased production during osmotic stress (Brown, 1990). The energy cost required for maintaining high glycerol concentrations seems to limit further increases in salt tolerance in this species. Fps1p (*fdp1* suppressor), a glycerol transporter that shows homology to water channel proteins, has been isolated. Expression of FPS was not regulated by the HOG signaling pathway that regulates glycerol biosynthesis (Luyten et al., 1995). It has recently been shown that Fps1p exerts control over glycerol accumulation and release in yeast, that the protein seems to be important as an exporter of glycerol, and that its action is controlled by a separate signaling pathway (Tamas et al., 1999). It would be interesting to determine whether other polyols, such as mannitol and sorbitol, could replace glycerol and show less leakage during osmotic stress.

To reduce Na^+ toxicity, yeast cells need to maintain ion homeostasis under salt stress, especially for Na^+ and K^+. A low Na^+ to K^+ ratio is essential for salt tolerance. A low Na^+ concentration in the cytoplasm is usually achieved by an increased efflux via the Na^+-ATPase and the Na^+-H^+ antiporter proteins under salt stress (Haro et al., 1991; Garciadeblas et al., 1993; Wieland et al., 1995; Prior et al., 1996). Mutants that failed to maintain low Na^+ concentrations in cells were salt sensitive despite normal glycerol accumulation (Yagi and Tada, 1988; Ushio et al., 1992; Garciadeblas et al., 1993; Welihinda et al., 1994). K^+ is taken up via high- and low-affinity K^+ transporters (Ko and Gaber, 1991). The high-affinity K^+ transporter shows a higher K^+ to Na^+ discrimination than the low-affinity transporter. Under salt stress, high-affinity K^+ uptake allows cells to accumulate more K^+ than Na^+ and thus maintain a low Na^+ to K^+ ratio (Haro et al., 1993).

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Overexpression of halotolerant genes such as HAL1 and HAL3 results in a decreased Na^+ to K^+ ratio, and thus increased salt tolerance (Gaxiola et al., 1992; Ferrando et al., 1995). Increased Na^+ to K^+ discrimination by a wheat high-affinity K^+ transporter enhanced salt tolerance of yeast strains deficient in K^+ uptake (Rubio et al., 1995). In addition, the vacuole may play an important role in salt tolerance, because mutants defective in vacuole morphology and vacuolar protein targeting are salt-sensitive (Latterich and Watson, 1991), and a mutant in subunit C of the vacuolar ATPase shows increased sensitivity to Na^+ and Li^+ (Haro et al., 1993). The essential function of the vacuole may be associated with both compartmentation of ions and osmoregulation.

The details of the signal transduction pathway regulating ion homeostasis remain unknown. Studies have revealed that calcineurin (a protein phosphatase 2B) and protein phosphatase PPZ may be involved (Nakamura et al., 1993; Mendoza et al., 1994; Posas et al., 1995). The activity of calcineurin requires Ca^{2+} and calmodulin. Null mutants of calcineurin were shown to be salt sensitive because they failed to fully induce ENA1 (encoding the major Na^+-ATPase) gene expression and failed to switch from low- to high-affinity K^+ transport under salt stress (Mendoza et al., 1994). In contrast, deletions of the protein phosphatases PPZ1 and PPZ2 increased salt tolerance and enhanced expression of ENA1 (Posas et al., 1995), suggesting an essential role of phosphatases in the regulation of yeast ion homeostasis.

Accumulation of glycerol is essential for salt tolerance, since mutants that cannot accumulate glycerol are salt-sensitive (Albertyn et al., 1994). Glycerol can function either as an osmolyte, contributing to the maintenance of water balance, or as an osmoprotectant, allowing the operation of many cellular processes during osmotic stress. To test the concept that "osmotic adjustment" by glycerol is a determinant for salt tolerance in yeast, we used a mutant unable to produce glycerol and substituted the presumptive function of glycerol with genes leading to either mannitol or sorbitol production. Neither mannitol or sorbitol could replace glycerol completely. Mutants from the glycerol-deficient strain that partially suppressed salt sensitivity did so by accumulating significantly more K^+ under salt stress. Taken together, the results seem to support complex roles for accumulating metabolites, including osmotic adjustment and metabolic functions.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions

The yeast (*Saccharomyces cerevisiae*) strains used in this study were isogenic to W303-1A(MATα and MATa leu2-3, 112 ura3-1 try1-1 his3-11, 15 ade2-1 can1-100 SUC2 GAL mal0). The gpd1Δ gpd2Δ strain was constructed by deletion/replacement of two GPD genes, as described previously (Albertyn et al., 1994; Ansell et al., 1997). Salt-resistant suppressor mutants were isolated from ethyl methanesulfonate (EMS)-treated gpd1Δ gpd2Δ cells, which grew at 0.7 M NaCl in yeast peptone dextrose (YPD) plates. EMS mutagenesis of gpd1Δ gpd2Δ cells was carried out as described previously (Kaiser et al., 1994). The percentage of cells surviving after EMS treatment was 40%. Fifteen salt-resistant suppressor lines were isolated from approximately 10^5 cells. One such suppressor mutant, sr13, was used here. gpd/cnb1 and sr13/cnb1 diploids were constructed according to the method of Kaiser et al. (1994).

Haploid cnb1Δ strain (MATa leu2-3, 112 ura3-1 try1-1 his3-11, 15 ade2-1 can1-100 SUC2 GAL mal0 cnb1Δ::LEU2-3) (Mendoza et al., 1994) was mated with haploid gpd1Δ gpd2Δ strain (MATα leu2-3, 112 ura3-1 try1-1 his3-11, 15 ade2-1 can1-100 SUC2 GAL mal0 gpd1Δ::TRP1 gpd2Δ::URA3) and haploid sr13 (MATα leu2-3, 112 ura3-1 try1-1 his3-11, 15 ade2-1 can1-100 SUC2 GAL mal0 gpd1Δ::TRP1 gpd2Δ::URA3 sr13). Diploids were selected on synthetic medium without Leu, Trp, or uracil. Cells were grown in either YPD medium (1% [w/v] yeast extract, 2% [w/v] peptone, 2% [w/v] dextrose) or synthetic yeast nitrogen base (YNB) medium prepared as described previously (Kaiser et al., 1994). NaCl was added to the medium as indicated.

Gene Constructions and Transformation

The yeast expression vector pXKL2 (a gift from K. Luyten, Katholicke Universiteit, Leuven-Haverlee, Belgium) was derived from YEplac181 (Gietz and Sugino, 1988) by inserting a 1.8-kb *Hin*dIII fragment of pMA91 at the *Hin*dIII site. It contains the constitutive strong promoter and terminator of PGK, the yeast phosphoglyceride kinase gene (Mellor et al., 1983). PCR fragments of a bacterial mannitol-1-P dehydrogenase gene (MTLD; Novotny et al., 1984) and an apple gene encoding sorbitol-6-P dehydrogenase (S6PDH) (Kanayama et al., 1992) were cloned into the *Bgl*II site of pXKL2, resulting in pXKL-MTLD and pXKL-S6PDH, as shown in Figure 1. The gene constructs were introduced into gpd1Δ gpd2Δ using the lithium acetate method.

Determination of Sugar Alcohols and Ions

Sugar alcohols were determined by HPLC as described previously (Tarczynski et al., 1992) with some modifica-

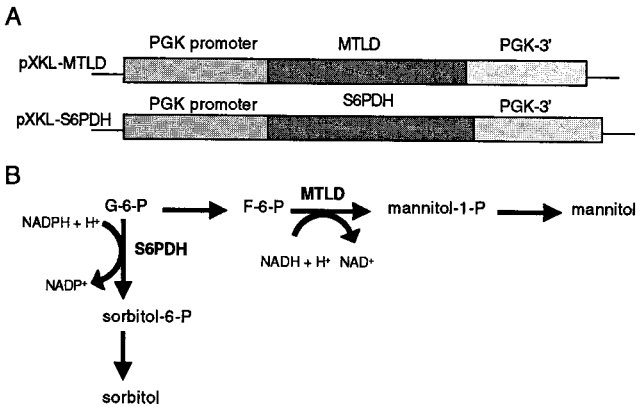

Figure 1. Gene constructs (A) and polyol biosynthetic pathway (B). PGK represents the promoter and 3′ noncoding region of the yeast gene encoding phosphoglyceride kinase. The connection of polyol biosynthesis to carbon flux pathways in yeast is indicated.

Table I. *Intracellular polyol content in yeast transformants*

Yeast cells were grown in synthetic selective media in the absence of NaCl or in the presence of 0.6 M NaCl. Cells not in NaCl stress were collected for polyol measurement when the OD_{600} reached 1.0. Cells in NaCl stress were collected after 3 h of culture in the presence of 0.6 M NaCl. Values are means ± SE of three independent transformants. gpd, gpd1Δgpd2Δ strain transformed with pXKL2 vector; GPD1, gpd1Δgpd2Δ strain transformed with the GPD1 gene (Albertyn et al. 1994); MTLD, gpd1Δgpd2Δ strain transformed with pXKL-MTLD; S6PDH, gpd1Δgpd2Δ strain transformed with pXKL-S6PDH. ND, Not detected. The detection limit of HPLC for polyol was 0.1 nmol/50 μL injection.

Strain	Glycerol		Mannitol		Sorbitol	
	−NaCl	+NaCl	−NaCl	+NaCl	−NaCl	+NaCl
	mmol g^{-1} dry wt					
gdh	2.3 ± 0.4	44.4 ± 13.5	ND	ND	3.9 ± 0.4	20.4 ± 1.5
GPD1	75.3 ± 8.1	400.7 ± 33.7	ND	ND	0.3 ± 0.1	2.0 ± 0.2
MTLD	1.0 ± 0.6	14.3 ± 1.1	48.7 ± 6.2	213.4 ± 31.0	7.9 ± 0.5	12.0 ± 1.4
S6PDH	0.7 ± 0.4	15.9 ± 1.7	ND	ND	116.6 ± 1.9	375.8 ± 21.3

tions. Yeast cells were collected and rinsed with the same growth medium without Glc to remove the remainder of the medium without disturbing the pellets. The pellet was extracted with 500 μL of extraction solvent (chloroform: ethanol:water = 3:5:1). After 10 min of vortexing, 500 μL of water was added. The extract was centrifuged at 12,000 rpm (model MC12C centrifuge, Sorvall) for 5 min. Extract supernatant (400 μL) was added to a small ion-exchange column filled with Amberlite IRA-68-OH (Rohm and Haas, Philadelphia) and Dowex-H^+ (Dow Chemicals, Midland, MI) to remove salt from the samples. The column was washed with four column-volumes of ethanol:water (1:1), and all elutes were vacuum dried. Dried samples were re-suspended in 500 μL of water and filtered through a Sep-Pak (Waters) column or a nylon filter. The analysis of sugar alcohols was performed using a HPLC system (Dionex, Sunnyvale, CA) as previously described (Tarczynski et al., 1992).

The concentration of cations was also determined with the HPLC system. Cells were collected, washed with an iso-osmotic sorbitol solution three times, and extracted with extraction solvent (chloroform:ethanol:water = 3:5:1). The supernatant of the extracts was filtered through a nylon filter (0.2 μm, Gelman, Ann Arbor, MI), and 100 μL was injected into the HPLC. The separation and analysis of cations was carried out on an IonPac CS14 column with a IonPac CG14 guard column (both from Dionex). The elutant was 10 mM methanesulfonic acid at a flow rate of 1.0 mL/min. Cations were detected by conductivity using a cation self-regenerating suppressor.

RESULTS

Replacement of Glycerol by Mannitol and Sorbitol in gpd1Δ gpd2Δ

To determine whether sorbitol and mannitol could substitute for glycerol in stress adaptation, a bacterial gene encoding MTLD and an apple gene encoding S6PDH were introduced into the glycerol-deficient mutant gpd1Δ gpd2Δ. In this strain, two GPD genes had been deleted, which led to the accumulation of only trace amounts of glycerol under salt stress, resulting in high sensitivity to salt stress and poor growth under anaerobic conditions (Ansell et al., 1997). The genes encoding MTLD and S6PDH, respectively, were cloned into a multicopy expression vector under the control of a strong, constitutive phosphoglycerate kinase (PGK) promoter (Fig. 1). Transformants that received the GPD1 gene were used as positive controls. Polyol amounts in these transformants are shown in Table I.

Yeast cells grown under salt stress accumulated 3- to 5-fold-higher amounts of polyols than cells grown without Na^+, suggesting that membrane permeability to polyols may be reduced under salt-stress conditions or that metabolic flux may be changed to favor polyol biosynthesis. Under salt stress, the amount of sorbitol in the S6PDH transformants was 375 μmol/g dry weight, which is close to 400 μmol/g dry weight glycerol in the GPD1 transformants, while mannitol in the MTLD transformants was approximately one-half of the glycerol found in the GPD1

Table II. *Leakage of polyols during culture in synthetic medium*

Yeast cells were cultured in synthetic medium in the absence of NaCl or in the presence of 0.6 M NaCl and collected at an OD_{600} of approximately the amount of polyol in the medium and in cells was determined. Values (%) are the amount of polyol in the cell divided by the total amount of polyol produced. Strains were labeled as in Table I.

Strain	Polyol Content					
	−NaCl			+0.6 M NaCl		
	Cells	Medium		Cells	Medium	
	μmol/L culture medium		%	*μmol/L culture medium*		%
Glycerol in GPD1	99 ± 4	13,274 ± 287	0.7	309 ± 25	19,497 ± 880	1.6
Sorbitol in S6PDH	99 ± 16	328 ± 16	23.1	166 ± 9	186 ± 3	47.3
Mannitol in MTLD	77 ± 4	218 ± 27	26.0	184 ± 30	156 ± 23	54.2

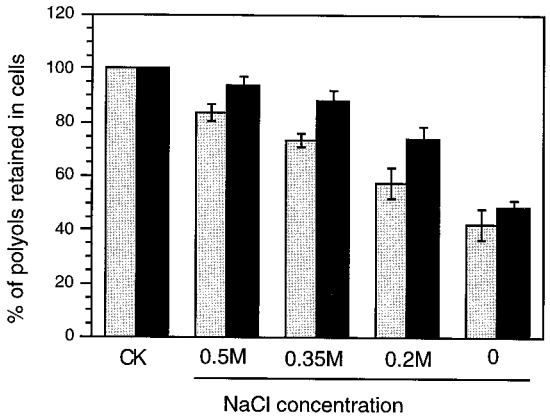

Figure 2. Acute leakage of polyols during hypo-osmotic shock. GPD1 and S6PDH transformants were grown in YNB selective medium for 2 d. Equal amounts of yeast cell pellet were resuspended in solution containing different NaCl concentrations and centrifuged immediately. Glycerol in the GPD1 transformant (shaded bars) and sorbitol in the S6PDH transformant (black bars) were measured by HPLC. CK, Control cells. The percentage of polyol retained in cells is equal to the amount of polyol recovered in the pellet after washing, divided by the amount of polyol in the pellet without washing.

transformants. Mannitol was not found in wild-type yeast cells, while low amounts of sorbitol were detected. Interestingly, the gpd1Δ gpd2Δ strain accumulated 10-fold more sorbitol than the GPD1 transformants, indicating that glycerol biosynthesis down-regulates endogenous sorbitol biosynthesis. S6PDH and MTLD transformants accumulated less glycerol than the gpd1Δ gpd2Δ strain (Table I). During culture in salt medium, GPD1 transformants leaked more than 98% of the glycerol produced into the medium, whereas less than 50% of the sorbitol and mannitol leaked out in the S6PDH and MTLD transformants. The retention of polyols by the cell increased significantly under salt stress (Table II). When cells were washed with hypo-osmotic solutions, the loss of glycerol was greater than the loss of sorbitol (Fig. 2), indicating that membrane permeability for glycerol was much higher than that for sorbitol and mannitol under salt stress.

Salt Tolerance of S6PDH and MTLD Transformants

Compared with the gpd1Δ gpd2Δ strain, MTLD and S6PDH transformants showed some improvement in salt tolerance at 0.5 M NaCl (Fig. 3), but this was not comparable to the protection provided by glycerol in the GPD1 transformants, which continued to grow in 0.8 M NaCl (Fig. 3). The I_{50} is the salt concentration that inhibits growth by 50% in liquid culture. Compared with gpd1Δ gpd2Δ, the I_{50} for S6PDH and MTLD transformants increased from 0.4 to 0.5 M NaCl, whereas the I_{50} for the GPD1 transformants was 0.9 M NaCl, about 2-fold higher than that of the gpd1Δ gpd2Δ strain (data not shown). These results indicate that mannitol and sorbitol do provide protection against salt stress, but that this protection is much smaller than the protection provided by an equal concentration of glycerol in GPD1 transformants.

Isolation of a Salt-Tolerant Suppressor Mutant

To better understand the role of glycerol in salt-stress adaptation, the gpd1Δ gpd2Δ strain was mutagenized with EMS and screened for salt-tolerant suppressor mutants. Fifteen colonies (termed "sr") that could grow in YPD medium containing 0.7 M NaCl were found, while the gpd1Δ gpd2Δ cells ceased to grow in NaCl concentrations

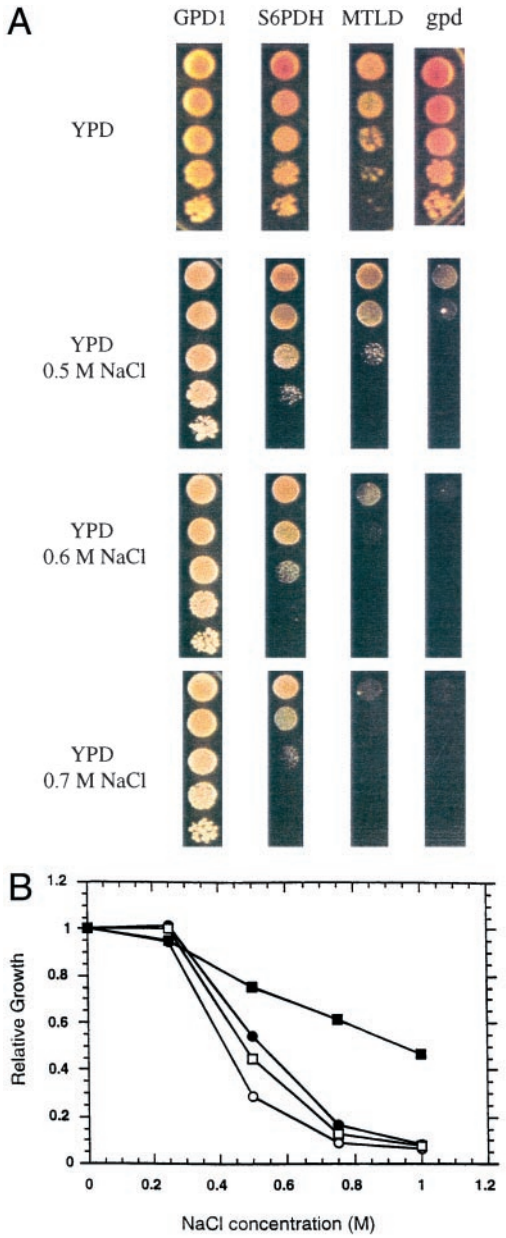

Figure 3. Growth of polyol-producing transformants under salt stress. A, Growth on solid medium. Cells were grown in YNB synthetic, selective medium supplemented with 2% (w/v) Glc for 2 d. Cells (OD_{600} approximately 1.3) from the stationary growth phase were spotted in a 1:10 dilution series onto YPD with NaCl, and grown at 30°C for 3 d. B, Relative growth of cells in suspension at different concentrations of NaCl. ■, GPD1; ●, S6PDH; □, MTLD; ○, gpd. A single experiment is shown; repeat experiments showed identical kinetics of growth.

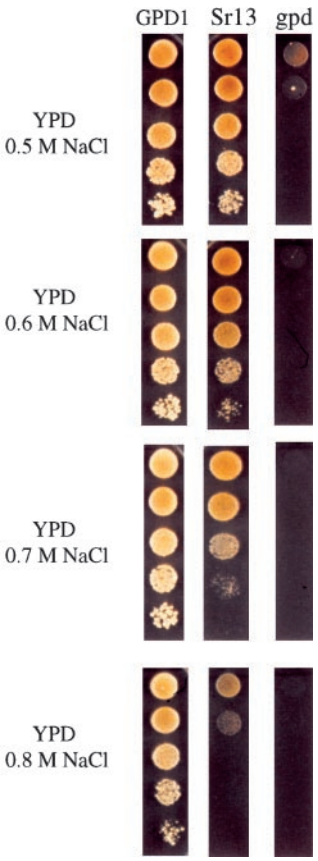

Figure 4. Growth of the sr13 mutant under salt stress. Cells were grown in YNB medium. Cells grown to the end of the logarithmic growth phase were spotted in a 1:10 dilution series onto YPD medium containing different NaCl concentrations, and grown at 30°C for 3 d. Sr13 is a salt-resistant suppressor mutant. GPD1 and gpd are as in Table I.

higher than 0.4 M. Sr13 showed the highest salt tolerance and was therefore analyzed further (Fig. 4). To determine if sr13 accumulated other polyols, which would replace the function of glycerol in salt adaptation, sugar extracts from sr13 and gpd1Δ gpd2Δ strain were analyzed by HPLC. The amounts of Glc, Fru, and Suc were identical in both strains (data not shown). Glycerol amounts in sr13 were approximately doubled compared with gpd1Δ gpd2Δ under salt stress, but were only 25% of the amount found in the GPD1 transformant (Fig. 5, Table I). The amount of sorbitol in sr13 increased about 4-fold, whereas trehalose decreased about one-third compared with gpd1Δ gpd2Δ under salt stress (Fig. 5).

To further determine changes that distinguished sr13 from the original strain, the amounts of Na^+ and K^+ were measured. Sr13 accumulated more than twice the amount of K^+ under salt stress as the wild type, while Na^+ levels remained comparable to the amounts found in wild type (Fig. 6). Without salt stress, the amount of K^+ in sr13 was similar to that found in wild-type cells, indicating that the accumulation of K^+ in sr13 was salt induced. In a sr13/cnb1 diploid (defective in calcineurin), the K^+ level was similar to wild type rather than to sr13, indicating that the

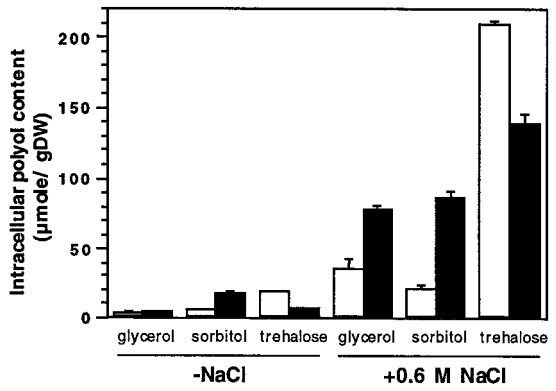

Figure 5. Polyol content in sr13 (black bars) and gpd1Δ gpd2Δ (white bars) strains. Cells were grown in synthetic medium in the absence of NaCl for 24 h or in the presence of 0.6 M NaCl for 36 h. Intracellular polyol content was measured as described. Strains were labeled as in Figure 4. Values are means ± SE of three to six measurements from three independent experiments. DW, Dry weight.

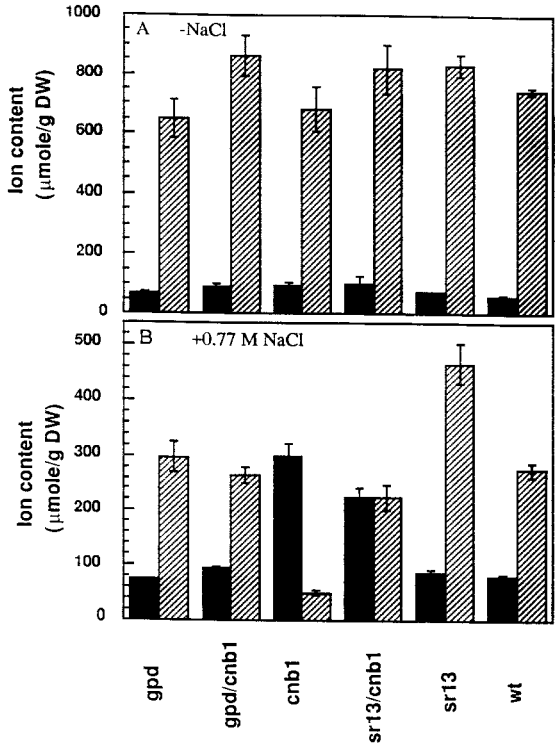

Figure 6. Intracellular content of Na^+ (black bars) and K^+ (hatched bars). Cells were grown in YPD medium in the absence (A) or presence of NaCl (B). gpd, gpd1Δ gpd2Δ; cnb1, a deletion mutant of the calcineurin regulatory subunit (Mendoza et al., 1994); wt, wild-type strain W3031-A; gpd/cnb1 and sr13/cnb1 are diploid strains. Ion concentrations under NaCl stress were measured after a 36-h incubation in 0.77 M (4.5%) NaCl. DW, Dry weight.

accumulation of K^+ was recessive. Na^+ levels in the sr13/cnb1 diploid, however, did not revert to wild-type levels for reasons that remain unknown (but may have been due to the interaction between CNB and SR13). In control experiments, the amounts of both Na^+ and K^+ in the gpd/cnb1 diploid were indistinguishable from the amounts found in the wild type (Fig. 6).

DISCUSSION

The Compatible Solute Concept

The accumulation of compatible solutes plays an essential role in osmotic stress adaptation (Brown, 1990; McCue and Hanson, 1990). In yeast, polyols are the main compatible solute and glycerol is the predominant osmolyte, and a correlation between the accumulation of glycerol and osmotolerance has been established. Although the essential role of glycerol in the adaptation to osmotic stress has been demonstrated by analysis of mutants deficient in glycerol production (Albertyn et al., 1994), the mechanisms by which glycerol can confer such tolerance are not clear. An obvious physiological mechanism is that glycerol is involved in osmotic adjustment by facilitating water flux across the plasma membrane. To determine whether glycerol was sufficient for the acquisition of osmotolerance and whether glycerol could be replaced by other osmolytes, we introduced the coding regions of enzymes leading to mannitol and sorbitol production into a glycerol-deficient mutant, and our results indicated that accumulation of either sorbitol or mannitol was not sufficient to replace glycerol completely.

Although some protective effects were observed with accumulation of mannitol and sorbitol, these effects were much less than the protective effect provided by glycerol. By reintroducing one of the deleted yeast GPD genes into the strain, the accumulation of glycerol in the glycerol-deficient mutant resulted in a significant increase in tolerance and the I_{50} increased to 0.9 M NaCl, compared with 0.4 M in the gpd1Δ gpd2Δ strain and 0.55 M in the sorbitol or mannitol accumulators. If osmotic adjustment by glycerol were the only requirement for successful adaptation, an equal concentration of sorbitol or mannitol would be expected to confer the same tolerance. This interpretation was also supported by comparing the gpd strain and the mutant sr13, which accumulated K^+. Strain sr13 was more tolerant than gpd.

When the intracellular polyols (glycerol and sorbitol) and trehalose were compared (Fig. 5), trehalose, which accumulated more in gpd, did not appear to add significantly to tolerance. However, trehalose amounts are correlated with stress tolerance, and the inability to synthesize trehalose is correlated with salt sensitivity in *S. cerevisiae* and other organisms (e.g. Hounsa et al., 1998; Marin et al., 1998). Among the possible roles for trehalose in microorganisms and in higher plants are sugar sensing and osmotic stress protection (e.g. Goddijn and Smeekens, 1998; Zentella et al., 1999), but the precise role and the mechanism of action for trehalose remain enigmatic.

Additional Functions of Polyols

The results suggest that the concept underlying the term "osmotic adjustment" may not be the essential or the only determinant for adaptation to high salinity. The consequence of this view is that glycerol has specific protective functions for which mannitol and sorbitol cannot substitute. Blomberg and Adler (1992) reported that conditioning of yeast cells at low NaCl concentrations leads to tolerance to a higher NaCl concentration. When glycerol made by the conditioned cells was flushed out, the cells still retained the acquired osmotolerance for some time. Also, glycerol accumulation in cycloheximide-treated cells could not protect against osmotic stress, indicating that the continuous maintenance of other functions was necessary and possibly enhanced or protected by glycerol. Alternatively, the less-protective effects by mannitol and sorbitol could be caused by different intracellular distribution of polyols. The compartmentation of polyols in yeast cells is not clear, but glycerol seems to be evenly distributed in the cells (Blomberg and Adler, 1992).

Another explanation is that the synthesis of different polyols might have different metabolic effects. For example, glycerol formation is required for the removal of excess NADH during anaerobic growth (Ansell et al., 1997). Based on the biochemical pathways, mannitol and sorbitol biosynthesis should have the same effect with respect to regeneration of NAD^+, and phosphate would be equally recovered in these pathways. However, there are two differences: (a) sorbitol and mannitol contain twice the number of carbon atoms, and (b) most of the glycerol synthesized leaks from the cells (Table II) or is exported through the glycerol transporter Fps1p (Tamas et al., 1999). Comparing the energy expenditure for equal intracellular amounts of the polyols, and considering how much is exported, glycerol synthesis consumed approximately 15-fold more carbon than either mannitol or sorbitol. As a hypothesis, the accumulation and leakage/export of glycerol may have two effects: osmotic adjustment and lowering of [NADH], with the latter having beneficial effects on mitochondrial respiration, energy charge, and, possibly, reduced radical oxygen production in the cells.

Leakage or export of glycerol into the medium and/or energy production may be limiting factors for further increases of salt tolerance in *S. cerevisiae*. Less than 2% of the glycerol produced was retained by the cells during salt stress, increasing the energy cost of achieving glycerol accumulation (Table II). In contrast, the extremely salt-tolerant *Z. rouxii* accumulated glycerol through increased retention or uptake rather than increased production, which would reduce the energy cost (Brown, 1990). If the hypothesis that glycerol has a role in redox control and/or energy charge is correct, then *Z. rouxii* should exert a more stringent control over mitochondrial respiration than *S. cerevisiae*. Sorbitol and mannitol showed a much higher percentage of retention than glycerol, indicating that membrane permeability to sorbitol and mannitol was lower compared with glycerol. Less leakage of sorbitol during salt stress could be an advantage for the replacement of glycerol by sorbitol. During salt stress, the polyol contents

are increased 4- to 5-fold. Since the activity of the PGK promoter is actually reduced during stress (S. Hohmann, unpublished data), the increase in polyols was most likely not due to an increased amount of the enzymes; it was probably due to metabolic changes that stimulated polyol biosynthesis and/or to decreased membrane permeability during salt stress.

Suppression of Glycerol Deficiency

To search for other mechanisms that might replace the function of glycerol during salt adaptation, mutants were isolated that suppressed salt sensitivity of the gpd1Δ gpd2Δ strain. Several mutants were isolated that accumulated more K^+ under salt stress, indicating that K^+ may partially replace the glycerol function up to approximately 0.75 M NaCl. One suppressor mutant strain showed no difference in $Na^+$and K^+ content and the polyol levels were comparable to the gpd1Δ gpd2Δ strain under salt stress. However, this mutant still showed resistance to 0.75 M NaCl (Shen, 1997), implying that other mechanisms may lead to salt tolerance. In addition to glycerol accumulation and ion homeostasis, mutants with a salt-sensitive phenotype that are defective in other cellular components and impaired in, for example, the biosynthesis of plasma membrane ATPase, vacuole, or cell walls, have been identified (McCusher et al., 1987; Latterich and Watson, 1991; Shimizu et al., 1994; Nelson et al., 1998).

K^+ plays an important role in salt tolerance in both yeast and plants. In yeast, Na^+ becomes toxic at a Na^+ to K^+ ratio greater than 0.5 (Gaxiola et al., 1992). Increased salt tolerance was correlated with enhanced K^+ accumulation (Gaxiola et al., 1992, 1996; Ferrando et al., 1995; Rubio et al., 1995). Similarly, tobacco cells adapted to NaCl showed an increased capacity for K^+ uptake. Inhibition of K^+ uptake by NaCl was not observed in these long-term salt-adapted cells (Watad et al., 1991). Arabidopsis SOS1 mutants were hypersensitive to salt stress due to a defect in high-affinity K^+ uptake, indicating an important role of K^+ for salt tolerance in plants (Wu et al., 1996).

We isolated a salt-tolerant suppressor mutant, sr13, which can partially suppress the salt sensitivity of gpd1Δ gpd2Δ. Compared with the amounts of glycerol, sorbitol, and trehalose that yeast normally synthesize, the mutant showed some differences. The sr13 mutant accumulated double the amount of K^+ and glycerol, 4-fold more sorbitol, and less trehalose compared with gpd1Δ gpd2Δ under salt stress. However, the concentration of glycerol in sr13 amounted to only 25% of that found in GPD1 transformants. S6PDH transformants accumulated 4-fold more sorbitol than sr13, but were more sensitive to NaCl compared with sr13, suggesting that increased sorbitol in sr13 was not the cause for salt tolerance. Thus, increased salt tolerance in sr13 seems to be caused by increased glycerol or higher K^+ amounts.

Recently, a salt-tolerant mutant has been isolated from wild-type yeast. The mutant cells have a lower Na^+ to K^+ ratio than wild type, again supporting the essential role of K^+ in salt tolerance (Gaxiola et al., 1996). Additional experiments (Shen, 1997, and data not included) indicated that the mutation sr13 is either downstream of calcineurin or in a separate signaling pathway. Calcineurin is a protein phosphatase of the 2B type that is involved in the regulation of K^+ uptake and Na^+ extrusion in yeast (Nakamura et al., 1993; Mendoza et al., 1994). The diploid strain sr13/cbn1 (defective in calcineurin) showed wild-type K^+ amounts under salt stress, suggesting that K^+ accumulation in sr13 is recessive. In contrast to the mutants HAL1, HAL3, and calcineurin, all of which result in salt sensitivity and less K^+ accumulation when the genes are disrupted, sr13 enhanced salt tolerance and K^+ accumulation when disrupted.

Yeast provides an excellent cellular model that has added to our understanding of osmotic stress tolerance and aided in the analysis of plant tolerance mechanisms (Frommer and Ninnemann, 1995; Rubio et al., 1995; Nelson et al., 1998; Zentella et al., 1999). The results presented here could indicate that "osmotic adjustment," the term widely used for explaining the accumulation of a variety of metabolites in higher plants and microorganisms, may be just one aspect of the beneficial function of accumulating metabolites. The export of glycerol affecting energy charge and/or redox control in yeast would not be unprecedented, because indicators exist for radical oxygen species as major contributors to damage under osmotic stress conditions in yeast and in photosynthesis-competent organisms (Allen et al., 1997; Godon et al., 1998; Smirnoff, 1998).

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