Fluidization of Membrane Lipids Enhances Tolerance of Freezing and Salt Stress by *Saccharomyces cerevisiae*

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Unsaturated fatty acids play an essential role in the biophysical characteristics of cell membranes and determine the proper function of membrane-attached proteins. Thus, the ability of cells to alter the degree of unsaturation in their membranes is an important factor in cellular acclimatization to environmental conditions. Many eukaryotic organisms can synthesize dienoic fatty acids, but *S. cerevisiae* can introduce only a single double bond at the Δ^9 position. We expressed two sunflower (*Helianthus annuus*) oleate Δ^{12} desaturases, *FAD2-1* and *FAD2-3*, in yeast cells of the wild-type W303-1A strain (*trp1*) and analyzed their effects on growth and stress tolerance. Production of the heterologous desaturases increased the content of dienoic fatty acids, especially 18:2$\Delta^{9,12}$, the unsaturation index and the fluidity of the yeast membrane. The total fatty acid content remained constant and the level of mono-unsaturated fatty acids decreased. Growth at 15°C was reduced in the *FAD2* strains, probably due to tryptophan auxotrophy since *trp1* (*TRP1*) transformants that produced the sunflower desaturases grew as the control strain. Our results suggest that changes in the fluidity of the lipid bilayer affect tryptophan uptake and/or the correct targeting of tryptophan transporters. The expression of the sunflower desaturases, in either Trp$^+$ or Trp$^-$ strains, increased NaCl tolerance. Production of dienoic fatty acids increased tolerance to freezing of wild-type cells pre-incubated at 30ºC or 15ºC. Thus, membrane fluidity is an essential determinant of stress resistance in *S. cerevisiae* and engineering of membrane lipids has the potential to be a useful tool of increasing freeze tolerance in industrial strains.

Freezing tolerance is an essential trait influencing viability and leavening capacity of baker's yeast in frozen dough (4, 42). The so-called frozen-dough technology has been widely accepted by consumers and bakers due to several advantages, which include supplying oven-fresh bakery products and improving labour conditions. However, no appropriate industrial strain with high freeze tolerance is available, and it is unlikely that classical breeding programs could provide significant improvements of this trait. Freezing is a complex and multifaceted stress, in which different stressors and stress responses appear to play important roles. Cells exposed to subzero temperatures are injured by the formation of ice crystals and crystal growth during frozen storage (33). At low freezing rate, the cells become exposed to hyperosmotic solutions and equilibrate by movement of water across the membranes (65). Finally, during the thawing process, cells can suffer biochemical damage by oxidative stress (16). It is not surprising; therefore, that freezing tolerance is likely to involve different mechanisms working in concert.

Biological membranes are the first barrier that separates cells from their environment, and are a primary target for damage during environmental stress. Sudden changes in environmental conditions cause alterations in the organization and dynamic structure of membrane lipids (62), and alter the function of many cellular activities. For example, a reduced incubation temperature increases the molecular order of membrane lipids, *i.e.*, rigidification (29), and alters the activity of membrane-associated enzymes and transporters (5). Other stresses, *e.g.*, heat shock, freezing and osmotic stress, induce dramatic changes in the organization and dynamic properties of membrane lipids (12, 27). However, to date, research in this field has focused on the connections between the physical state of the membrane and cold tolerance.

Many organisms have developed mechanisms to maintain the appropriate fluidity of the membrane lipids regardless of ambient temperature. These mechanisms include changes in

the proportions of types of lipid and alterations in the lipid:protein ratio (26). The most widely recognized change in cell membranes at low temperature is the unsaturation of lipid acyl chains (44, 54). Phospholipids with unsaturated fatty acids have a lower melting point and more flexibility than do phospholipids with saturated acyl chains (35). Such adaptation involves the induction of fatty acid desaturases (55, 63), which incorporate unsaturated bonds at defined positions in fatty acids that are linked to membrane glycerolipids.

In *Saccharomyces cerevisiae*, exposure to low temperature increases the expression of *OLE1* (36), which encodes the only desaturase known in this yeast (52, 53). Ole1p, a Δ^9 fatty acid desaturase, converts palmitic (16:0) and stearic (18:0) fatty acids into their corresponding mono-unsaturated fatty acids, palmitoleic acid (16:1Δ^9) and oleic acid (18:1Δ^9), respectively. Consistent with the increased expression of *OLE1*, there is an increase in the degree of unsaturation of total fatty acids when yeast cells are shifted from 30ºC to 10ºC (36). Nevertheless, *OLE1* does not appear to be essential in acclimation to low temperature, since its overexpression does not confer growth advantages at 10ºC (20). Overexpression of *OLE1* is toxic and reduces growth at 30ºC (52). Thus, the functional role of *OLE1* is unclear and the mechanism by which *S. cerevisiae* alters membrane fluidity in response to different types of stress remains unknown.

Unlike *S. cerevisiae*, many organisms synthesize polyunsaturated fatty acids *de novo* through the activity of specific desaturase enzymes. Genes encoding a range of different fatty acid desaturases (*FAD*), have been cloned from microorganisms, animals and plants, and expressed in heterologous hosts (28, 37, 38), including yeast (14, 19, 40, 43). *FAD*-expressing yeast cells contained high levels of di-unsaturated fatty acids. However, the physiological significance of this change in fatty acid composition has only rarely been studied in detail. *S. cerevisiae* cells overexpressing the *Arabidopsis FAD2* gene were more resistant to ethanol than were control cells (19). Production of a Δ^{12} desaturase from

Caenorhabditis elegans in yeast increased the growth rate at low temperature and enhanced resistance to ethanol and H_2O_2 (40).

Cultivated sunflower (*Helianthus annuus* L.) seed is rich in unsaturated fatty acids $18{:}1\Delta^{9}$ and $18{:}2\Delta^{9,12}$ (linoleic acid). The enzyme responsible for synthesizing linoleic acid is the
5 microsomal oleate desaturase (1-acyl-2-oleoyl-sn-glycero-3-phosphocholine Δ^{12} desaturase). Three different cDNA sequences, *FAD2-1*, *FAD2-2* and *FAD2-3*, encoding the sunflower microsomal oleate desaturases (FAD2) have been isolated and characterized (31). FAD2 activity in developing sunflower seeds is regulated by temperature and oxygen availability (32). FAD2 activity levels were similar at 10ºC and 20ºC, and decreased at 30ºC, which is
10 consistent with a hypothesis that FAD2 activity is decreased by high temperature. This phenomenon has been mainly attributed to the relatively low thermal stability of the major and seed-specific desaturase isoform FAD2-1 (45).

Our objective in this study was to alter the unsaturation index of the *S. cerevisiae* membrane through the heterologous expression of the sunflower desaturase genes, *FAD2-1*
15 and *FAD2-3*. We hypothesized that gene-engineered fluidization of the membrane lipids might play a protective role upon freezing. The results may have practical applications in increasing yeast resistance to freezing.

MATERIALS AND METHODS

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Strains, culture media and general methods. *S. cerevisiae* W303-1A (*MAT*a *leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL mal SUC2*) wild-type strain (57) was used throughout this work. *E. coli* strain DH10B was used as the host for plasmid construction. Yeast cells were cultured at 30ºC in YPD (1% yeast extract, 2% peptone, 2% glucose) or SD
25 (0.2% yeast nitrogen base without amino acids [DIFCO, BD Diagnostics, Sparks, MD], 0.5%

$(NH_4)_2SO_4$, 2% glucose) supplemented with the appropriate auxotrophic requirements (49). In some experiments the concentration of tryptophan was increased by supplementing the medium with 200 μg/ml of this amino acid. *E. coli* was grown in Luria Bertani (LB) medium (1% peptone, 0.5% yeast extract, 0.5% NaCl) supplemented with ampicillin (50 mg/l).
5 Yeasts were transformed by the lithium acetate method (18). *E. coli* was transformed by using an Eppendorf electroporator 2510 (Eppendorf AG, Hamburg, Germany).

Stress sensitivity tests. Cells were grown at 30ºC to mid-exponential phase, collected by centrifugation (3,000 × *g*, 2 min, 4ºC), transferred to fresh medium and incubated at various temperatures, after which growth was monitored. Doubling times (g) were calculated from
10 the formula $g = \ln 2/\mu$, where μ is the specific growth rate constant of the culture. μ was calculated from the slope of the line obtained after plotting ln *X* versus *t*, where *X* is the cell density of the culture, measured as DO_{600}, at multiple time points (*t*) during logarithmic growth. For plate phenotype experiments cultures were diluted to $OD_{600} = 0.3$ and 10-fold serial dilutions spotted (3 μl) onto SD- or YPD-agar solid media containing sorbitol or NaCl
15 at different concentrations. Unless otherwise indicated, colony growth was inspected after 2-4 days of incubation at 30ºC.

For freeze tolerance assays, cells were grown in SD medium at 30ºC or 15ºC, harvested ($OD_{600} = 3$), resuspended in YPD ($OD_{600} = 10$), and 10 μl aliquots shifted to -20ºC. At various times, samples were thawed at 30ºC for 10 min, diluted, and cells plated onto solid
20 YPD. After 2 days at 30ºC, colonies were counted. Viability is expressed as the percentage of viable cells relative to unfrozen control samples.

Plasmids. Plasmids for expressing *FAD2* desaturases were pVTHaFAD2-1 and pVTHaFAD2-3 (45), which contain the sunflower (*H. annuus*) genes *FAD2-1* (GenBank accession number, AF251842) and *FAD2-3* (GenBank accession number AF251844),

respectively, flanked by the *S. cerevisiae ADH1* promoter in the *URA3*-based multicopy vector pVT102-U (60).

Northern blot. Total RNA was prepared as previously described (49). Equal amounts of RNA (10 μg) were separated in 1% (w/v) agarose gels, containing formaldehyde (2.5% v/v),
5 transferred to a nylon membrane and hybridized with a ^{32}P-labeled probe of the *FAD2-1* and *FAD2-3* genes. Probes were obtained by restriction with BamHI of plasmids pVTHaFAD2-1 and pVTHaFAD2-3. A PCR-generated fragment of the *S. cerevisiae ACT1* gene (+10 to +1,066) was used as the loading control. Probes were radiolabeled with the random primer Ready-to-Go kit (Amersham Biosciences, Chalfont-St Giles, England) and [α^{32}P]dCTP
10 (Amersham Biosciences). Hybridization was carried out under standard conditions (49). Filters were exposed to a high-resolution BAS-MP 2040S imaging plate (Fuji, Kyoto, Japan) for 24 h and scanned in a Phosphorimager (Fuji, FLA-3000).

Lipid analysis. Cells were grown to stationary phase (OD_{600} ~ 3) in SD at 30ºC or 15ºC. Culture samples of 50 ml corresponding to approximately 45 mg cells, dry weight (OD_{600} = 1
15 equals 0.3 mg cells dry weight/ml), were centrifuged (3,000 × *g*, 2 min, 4ºC) and washed twice with ultrapure water (MilliQ RO 10 Plus, Millipore, Bedford, MA). Total lipid content and fatty acid composition of whole yeast cells were determined using the one-step method of Garcés and Mancha (15). Briefly, following the addition of 3.3 ml methanol-toluene-dimetoxypropane-H_2SO_4 (39:20:5:2, vol/vol/vol/vol) and 1.7 ml heptane to the yeast pellet,
20 the mixture was incubated at 80ºC for 1 h forming a single phase. After cooling, the upper phase containing the fatty acid methyl esters was separated, washed with 5 ml 6.7% Na_2SO_4, and evaporated to dryness under nitrogen. The methyl esters were dissolved in an appropriate volume of heptane and analyzed by gas-liquid chromatography using a HP-5890 (Hewlett-Packard, Palo Alto, CA) fitted with a capillary column (30 m length; 0.25 mm id;
25 0.20 μm film thickness) of fused silica (Supelco, Bellafonte, PA) and a FID detector.

Hydrogen was used as the carrier gas with a linear rate of 28 cm s^{-1} and a split ratio of 1/50. The injector and detector temperature were 220ºC and the oven temperature was 170ºC. Heptadecanoic acid was used as internal standard.

The yeast lipid composition was determined from cells harvested and washed as described
5 above. After heating at 100°C for 10 min to stop any enzymatic reaction, the fresh weight of the cell pellet was measured, and the yeast cells were resuspended with distilled water. Lipids were extracted from 1 ml aliquots with 3.75 ml of chloroform-methanol (1:2, vol/vol) according to the method of Bligh and Dyer (9) modified by Kates (24), and the lower phase was separated and evaporated to dryness with nitrogen. Fatty acid composition of the
10 different lipid classes was determined as described (45).

Membrane fluidity determination. Membrane fluidity was measured as previously described (27) by using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) as a reporter. The degree of fluorescence polarization (*P*) was calculated as described by Ansari et al. (3). In these experiments, decreases in *P* values reflect increases of the fluidity of the
15 lipid bilayer, which controls or affects the mobility of DHP on the membrane.

Statistical analysis. The significance of unsaturation index variations among strains was determined by a global ANOVA (available at: www.physics.csbsju.edu/stats/anova.html).

RESULTS

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Heterologous expression of sunflower *FAD2* desaturases enhances unsaturation of yeast lipids. W303FAD2-1 and W303FAD2-3 strains, which express the sunflower genes *FAD2-1* and *FAD2-3*, respectively, were analyzed for *FAD2* mRNA levels. Northern blot analysis showed a single hybridizing band, that was absent in samples of the control strain
25 transformed with an empty plasmid (data not shown). Differences in the expression levels of

FAD2-1 and *FAD2-3* were insignificant in cells growing either actively or in the diauxic-shift. Moreover, none of the transformant strains had abnormal-sized desaturase messenger RNAs (data not shown).

Heterologous expression of microsomal oleate desaturase-encoding genes led to the
5 production of palmitolinoleic ($16{:}2\Delta^{9,12}$) and linoleic acid ($18{:}2\Delta^{9,12}$), and the reduction of the amount of fatty acids $16{:}1\Delta^{9}$ and $18{:}1\Delta^{9}$, thus, altering the fatty acid profile and increasing the unsaturation index of yeast lipids (Table 1). This result indicates that the sunflower desaturases are active and act on endogenous yeast monounsaturated fatty acids, preferentially converting oleic acid to linoleic acid (Table 1). As in a previous study (45), the
10 dienoic acids were almost evenly distributed among the different lipid classes analyzed, including polar (phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol) and neutral lipids (diacylglycerol, triacylglycerol and sterol ester), although a higher percentage was detected in phosphatidylethanolamine and neutral lipids, two of the most prominent components of the yeast plasma membrane lipids (8). In
15 consonance with previous reports (32, 45), the incubation temperature altered the amount of unsaturated fatty acids present and the monoenoic/dienoic acid ratio of the transformed cells. Cells incubated at 15ºC contained higher levels of dienoic acids than did those cultivated at 30ºC (Table 1). This difference was statistically significant ($p < 0.01$) in cells expressing the *FAD2-1* gene, in which the percentage of 16:2 and 18:2 fatty acids increased 4- and 3-fold,
20 respectively, at 15ºC, relative to levels at 30ºC. However, the activity of FAD2 desaturases did not alter the total amount of fatty acids, except at 15ºC, where a moderate increase was observed (Table 1).

Effects of heterologous FAD2 desaturases on membrane fluidity and growth. Membrane fluidity in whole cells of wild-type and *FAD2*-overexpressing strains was
25 estimated by measuring fluorescent polarization (*P*) of the lipophilic membrane probe 1,6-

diphenyl-1,3,5-hexatriene (DPH). In both strains expressing *FAD2*, W303FAD2-1 and W303FAD2-3, *P* values at 25ºC were significantly lower, 0.116 ± 0.033 and 0.125 ± 0.020, respectively, than those of the control cells (0.160 ± 0.016). Thus, fluidity of yeast membrane lipids increases when monoenoic fatty acids are converted to dienoic fatty acids.

5 Overproduction of FAD2 desaturases decreased growth of wild-type cells cultivated in liquid SD medium at 10 or 15ºC, while increased growth at 30ºC (Table 2). The same phenotype was observed when cells were plated on solid media, both SD (data not shown) and YPD (Fig. 1A), but the effect of FAD2 on growth at low temperature was more pronounced. Indeed, FAD2 overproducing cells did not produce visible colonies on solid

10 YPD at 15ºC (Fig. 1A).

Nutrient uptake may be sensitive to changes in the fatty acid composition of the yeast membrane. Tryptophan uptake can affect the growth temperature profile of *S. cerevisiae* (1) and is impaired in cells exposed to low temperatures (1), and the strain used in this study is *trp1*. Yeast growth was stimulated by increasing tryptophan availability at low temperature

15 (Fig. 1A), although the growth reduction was still detectable under these conditions. The addition of excess tryptophan did not affect yeast cell growth at 30ºC. No growth differences at either 15 or 30ºC were observed between wild-type and *FAD2* overexpressing strains transformed with the integrative plasmid YIplac112, carrying the wild-type *TRP1* gene (Fig. 1A). This result was not due to a lack of activity of the heterologous desaturases in the Trp^+

20 background since both W303FAD2-1 and W303FAD2-3 accumulated dienoic fatty acids (Table 1) in a manner similar to that observed in the corresponding tryptophan-auxotroph strains (Table 1).

Membrane fluidity and tolerance to ion stress and freezing injury. Production of dienoic fatty acids did not alter the growth of yeast cells, either Trp^+ or Trp^-, exposed to

different concentrations of sorbitol (data not shown). However, overexpression of *FAD2* genes did increase Na^+-tolerance relative to strains harboring an empty plasmid (Fig. 1B).

Cultures of wild-type and *FAD2*-expressing cells were transferred from 30 to -20ºC and analyzed for cell viability after different periods of frozen storage. The viability of W303-1A (*trp1*) wild-type cells, in which no detectable dienoic fatty acids were produced, was less than that of Trp^- transformed cells carrying a high copy number expression cassette of *FAD2* (Fig. 2). The extent of unsaturation of lipids at 30ºC was critical for cell survival below 0ºC. Cells expressing *FAD2-1* died more rapidly when frozen than did the cells producing the FAD2-3 enzyme, which corresponds with their dienoic acid content (Table 1). Similar results also were observed for the Trp^+ strains, although the effects on freeze tolerance were less pronounced (Fig. 2).

Cell survival following freezing and frozen storage increased if the cells were grown at 15ºC instead of 30°C (Fig. 2), confirming that death during freezing can be prevented or alleviated by growth at low temperatures (23, 39). Again, the loss of viability following freezing was significantly larger for the control strain. In a representative experiment, ~50% of wild-type and ~90% of the FAD2-3 overproducing cells survived for 35 days at -20ºC (Fig. 2). The differences in freeze tolerance between FAD2-3 and FAD2-1-producing cells grown at 15ºC were less evident than those observed in cells grown at 30ºC, probably because the two *FAD2*-expressing strains produce similar amounts of dienoic fatty acids at low temperature (Table 1). These observations were further confirmed in Trp^+ strains (Fig. 2).

DISCUSSION

Heterologous expression of sunflower microsomal oleate desaturases in yeast cells has enabled us to evaluate the functional role of dienoic fatty acids. Cells of *S. cerevisiae* into which either of the *FAD2-1* or *FAD2-3* genes for Δ^{12} desaturases from *H. annuus* were introduced, could efficiently convert palmitoleic and oleic acid into palmitolinoleic and
5 linoleic acid, respectively. The extent of accumulation of dienoic acids was similar to that obtained by overexpression of other plant *FAD2* genes, which range from 10-50% (11, 13, 19, 25). More di-unsaturated fatty acids were produced at 15ºC than at 30ºC, especially in *FAD2-1* transformants. At this respect, reports of the expression of *Arabidopsis FAD2* in yeast had described contradictory results, in which the amount of 16:2 and 18:2 increased at
10 low temperatures (13), or remained constant (11). We found that the unsaturation index of lipids, in both Trp^+ and Trp^- W303FAD2-1 cells, varied from 0.74-0.79 at 30ºC to 0.94-0.97 at 15ºC, a difference that has been attributed to the low thermal stability of the FAD2-1 isoform (45). However, overexpression of sunflower *FAD2* genes did not increase the total unsaturated fatty acid content. This fact could be explained by possible feedback inhibition
15 of Ole1p, since exogenous 18:2 in the culture medium could repress transcription of *OLE1* (10).

Increasing the unsaturation index of yeast lipids fluidized the yeast membrane and altered the stress response of yeast cells. The finding that biosynthesis of $16{:}2\Delta^{9,12}$ and $18{:}2\Delta^{9,12}$ fatty acids increased tolerance to salt stress and freezing was not completely unexpected.
20 There are previous reports that suggest that membrane lipid composition is correlated with tolerance to different stresses, including heat shock (51), heavy metals (17) and exposure to the herbicide 2,4-dichlorophenoxyacetic acid (61). Survival following freezing/thawing also might depend on the physical properties of the membrane. Below 0ºC, cells are injured by the formation of ice crystals, which results in macromolecule and membrane denaturation
25 (33). The growth of ice crystals during frozen storage could further degrade the plasma

membrane (65). Thus, changing the fluidity of the lipid bilayer may help prevent or alleviate membrane damage due to freezing and contribute to cell survival.

The same protective mechanism also may operate in cells exposed to hyperosmolarity since osmotic stress reduces cell-membrane fluidity (27), which influences membrane
5 permeabilization and cell death (41). We found that fluidization of the yeast membrane produced a moderate increase in Na^+ tolerance, but had no effects on pure osmotic stress resistance. In *S. cerevisiae*, Na^+ is extruded by both the P-type ATPase Ena1p (30) and the H+-antiporter Nha1p (21). In addition, Na^+-compartmentation is mediated by vacuolar antiporters (48). All of these mechanisms of ion homeostasis depend on membrane proteins,
10 whose activity might be affected by changes in membrane fluidity. Phospholipid composition of the membrane affects membrane-associated processes such as plasma membrane ATPase activity (47), the higher proton motive force (34), and the transport of various amino acids (58).

In support of this view, increased unsaturation of yeast lipids altered the growth profile of
15 W303-1A (*trp1*) cells in response to ambient temperature. Unlike previous reports (40), dienoic fatty acid-enriched yeast cells grew slightly faster at 30ºC than did wild-type cells. Similar results were observed at 37ºC (data not shown). Their growth capacity at low temperatures also was greatly reduced, but the cold-sensitivity phenotype was alleviated by increasing tryptophan availability, and completely overcome by tryptophan prototrophy. No
20 phenotypic differences were detected in Trp^+ transformants grown at 30ºC or 37ºC (data not shown).

Tryptophan uptake has been termed the Achilles' heel of yeast physiology (2), since under a variety of stress conditions it becomes a limiting factor for cell growth. The sensitivity of tryptophan permeases to in response to changes in membrane fluidity also may determine the
25 growth temperature profile of *S. cerevisiae* (1). Trp^- yeast cells that overproduce Tat2p, a

high-affinity tryptophan transporter (46), grow better that the wild-type at 10 or 15ºC, while their growth at 37ºC is relatively reduced (1). Thus, Tat2p activity may be sensitive to the changes in fatty acid composition resulting from overexpression of sunflower *FAD2* desaturases.

5 Increased unsaturation of yeast lipids also may affect the protein sorting mediated by lipid rafts and the final destination of key membrane proteins. Association with lipid rafts, a sphingolipid- and sterol-rich membrane domain (50), plays an essential role in the correct localization of proteins such as Tat2p (59), Ole1p (56) or Pma1p (6, 7), the major plasma membrane H^+-ATPase in *S. cerevisiae*. Mutations in *PMA1* result in cold-sensitivity and Na^+ 10 resistance (22, 64), phenotypes shared by Trp^- *FAD2*-overexpressing cells. Thus, changes in the unsaturation index of lipids could affect the localization of key proteins and thereby cause pleiotropic phenotypes.

The data presented here provide direct evidence that overexpression of desaturases can be used to increase freeze tolerance in yeast cells. This trait is critical in determining an optimal 15 leavening of lean and sweet frozen dough products and increasing freeze resistance in industrial yeast is one of the most important biotechnological challenges in this field (42). Alteration of fatty acid composition and content by expression of heterologous desaturases also could be useful as a means to modify lipid microdomains and to study their function in cell signaling, polarity and sorting. Thus, the results reported in this study have both basic 20 and applied significance.

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Figure Legends

FIG. 1. *FAD2*-overexpressing yeast cells display *trp1*-dependent cold-sensitivity and enhanced NaCl tolerance. (A) Tryptophan auxotrophic (*trp1*) cells of the *S. cerevisiae* W303-1A wild-type strain (wt) were transformed with the plasmids pVTHaFAD2-1 (FAD2-1) or pVTHaFAD2-3 (FAD2-3), which contain the sunflower (*H. annuus*) genes *FAD2-1* and *FAD2-3*, respectively, and transformants were assayed for growth at 15ºC and 30ºC. Wild-type and *FAD2* overexpressing strains transformed with the integrative plasmid YIplac112, carrying the wild-type *TRP1* gene (*trp1 TRP1*), were also tested. Cells were grown in SD liquid medium at 30ºC until early-exponential phase and adjusted to OD_{600} = 0.3. Serial dilutions (1-10^{-3}) of the adjusted cultures were spotted (3 μl) onto standard YPD-agar plates (YPD) or YPD plates containing an excess (200 μg per ml) of tryptophan (High Trp). Plates were incubated for 2 (30ºC) or 7 (15ºC) days. (B) The same strains were cultivated at 30ºC on YPD plates containing NaCl at the indicated concentrations, and inspected after 5 days. Cultures were pre-grown, diluted and cells were spotted as described above. In all cases, a representative experiment is shown.

FIG. 2. Fluidization of yeast lipids enhances freeze tolerance. Cell cultures of the *trp1* and *trp1 TRP1 S. cerevisiae* strains, wild-type W303-1A (▲) and *FAD2-1* (○) and *FAD2-3* (●) overexpressing transformants were grown at 30ºC (DO_{600} = 0.4-0.6) and transferred directly to -20ºC or pre-incubated at 15ºC for 24 h, prior the shift to -20ºC. After 14, 28 and 35 days, cell samples were thawed at 30ºC for 30 min, diluted and plated onto solid YPD and the percent of survival was determined. Values represent the means of at least three independent experiments. The error associated with the points was calculated by using the formula: $(1.96 \times SD) / \sqrt{n}$, where n is the number of measurements.

TABLE 1. Fatty acid composition of total lipids in *S. cerevisiae FAD2* transformants grown at different temperatures[a]

	Strain	ºC	Fatty acid (mol %)						UI[b]	Total Fatty acids[c]
			16:0	16:1	16:2	18:0	18:1	18:2		
	W303-1A	30	20±0.5	34±1.2	ud[d]	11±0.3	36±1.4	ud	0.69*	21
		15	20±0.1	35±0.7	ud	8.9±0.2	37±0.6	ud	0.71*	22
trp1	W303FAD2-1	30	20±0.4	30±1.4	1.6±0.4	11±1.7	29±0.6	8.0±0.1	0.79#	18
		15	20±1.3	23±1.1	6.0±0.3	12±0.9	18±0.2	20±2.8	0.94§	26
	W303FAD2-3	30	22±0.8	27±0.8	5.2±0.0	12±1.1	16±0.4	19±1.0	0.90§	20
		15	20±0.2	27±0.5	5.1±0.1	11±0.1	15±0.2	22±0.6	0.97§	26
	W303-1A	30	21±0.5	31±0.5	ud	11±1.6	37±1.4	ud	0.68*	39
		15	19±0.9	39±0.4	ud	9.5±1.4	33±0.4	ud	0.72*#	49
trp1 TRP1	W303FAD2-1	30	20±0.9	30±0.9	ud	11±0.7	33±0.5	6.0±0.9	0.74#	36
		15	19±0.3	27±0.6	7.2±1.2	11±0.2	16±1.9	20±1.2	0.97§	45
	W303FAD2-3	30	22±0.9	23±2.8	5.4±1.7	12±0.8	16±3.3	21±2.7	0.92§	34
		15	19±0.5	27±2.0	6.6±1.3	11±1.9	16±3.5	20±1.5	0.96§	48

[a] Tryptophan auxotrophic (*trp1*) and prototrophic (*trp1 TRP1*) cells of the *S. cerevisiae* W303-1A wild-type strain expressing the sunflower genes *FAD2-1* and *FAD2-3*, were analyzed. Values represent the mean of at least three independent experiments with duplicate determinations of fatty acid composition.

[b] Unsaturation index was defined as: [(%16:1 + %18:1) + 2 (%16:2 + %18:2)] / 100. Values with different symbol are significantly different at the 0.01 level (ANOVA).

[c] µg per mg of cells, dry weight.

[d] undetected

TABLE 2. Growth at different temperatures of *FAD2* transformants

Strain	Doubling times (h)[a]		
	30ºC	15ºC	10ºC
W303-1A	2.2±0.1	11.5±0.2	89.3±5.3
W303FAD2-1	1.7±0.1	13.3±0.2	106.8±5.2
W303FAD2-3	1.7±0.1	12.8±0.1	107.3±11.7

[a] Tryptophan auxotrophic (*trp1*) cells of the *S. cerevisiae* W303-1A wild-type strain expressing the sunflower genes *FAD2-1* and *FAD2-3*, were analyzed. Cells were grown in liquid SD medium. Values represent the mean of at least two independent experiments.

A

trp1   *trp1* (*TRP1*)

wt FAD2-1 FAD2-3   wt FAD2-1 FAD2-3   wt FAD2-1 FAD2-3

15ºC

30ºC

YPD   High Trp   YPD

B

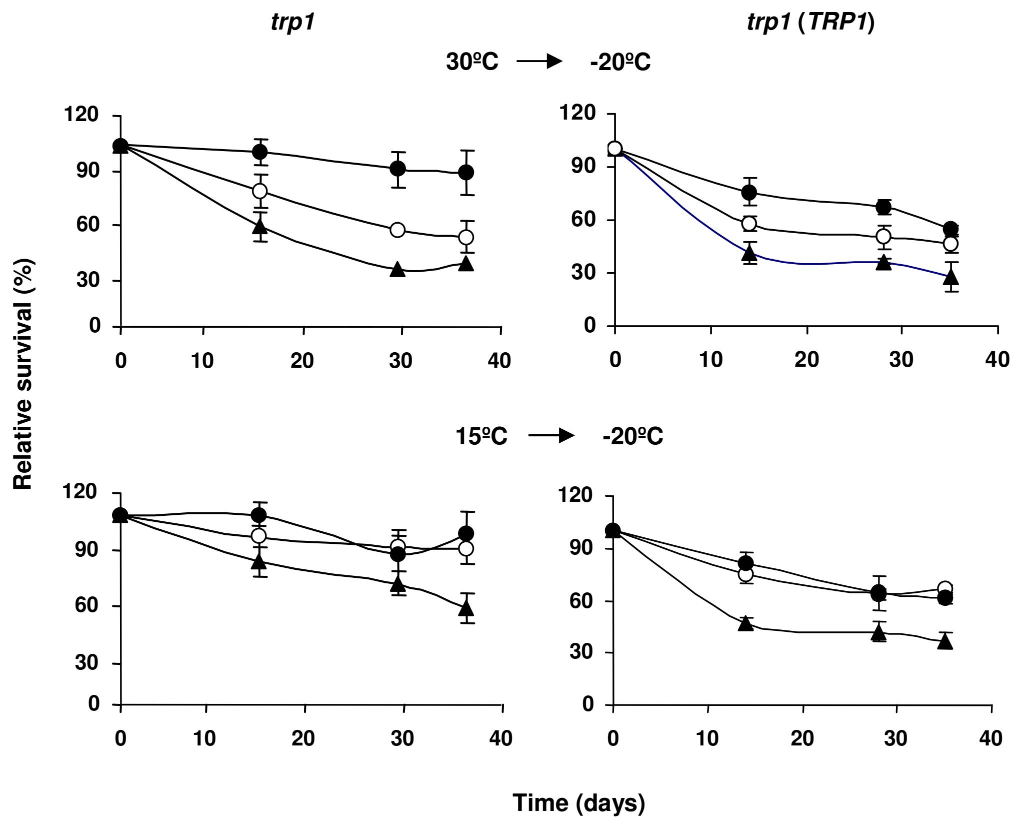
wt FAD2-1 FAD2-3   wt FAD2-1 FAD2-3   wt FAD2-1 FAD2-3

trp1

trp1 (*TRP1*)

0.5 M   1.0 M   1.5 M

NaCl



trp1
trp1 (TRP1)
30ºC → -20ºC
15ºC → -20ºC
Relative survival (%)
Time (days)
120
90
60
30
0
0
10
20
30
40