APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1993, p. 1065–1071
0099-2240/93/041065-07$02.00/0

Vol. 59, No. 4

Role of Growth Phase and Ethanol in Freeze-Thaw Stress Resistance of *Saccharomyces cerevisiae*

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Received 13 October 1992/Accepted 2 February 1993

The freeze-thaw tolerance of *Saccharomyces cerevisiae* was examined throughout growth in aerobic batch culture. Minimum tolerance to rapid freezing (immersion in liquid nitrogen; cooling rate, approximately 200°C min^{-1}) was associated with respirofermentative (exponential) growth on glucose. However, maximum tolerance occurred not during the stationary phase but during active respiratory growth on ethanol accumulated during respirofermentative growth on glucose. The peak in tolerance occurred several hours after entry into the respiratory growth phase and did not correspond to a transient accumulation of trehalose which occurred at the point of glucose exhaustion. Substitution of ethanol with other carbon sources which permit high levels of respiration (acetate and galactose) also induced high freeze-thaw tolerance, and the peak did not occur in cells shifted directly from fermentative growth to starvation conditions or in two respiratorily incompetent mutants. These results imply a direct link with respiration, rather than exhaustion of glucose. The role of ethanol as a cryoprotectant per se was also investigated, and under conditions of rapid freezing (cooling rate, approximately 200°C min^{-1}), ethanol demonstrated a significant cryoprotective effect. Under the same freezing conditions, glycerol had little effect at high concentrations and acted as a cryosensitizer at low concentrations. Conversely, under slow-freezing conditions (step freezing at −20, −70, and then −196°C; initial cooling rate, approximately 3°C min^{-1}), glycerol acted as a cryoprotectant while ethanol lost this ability. Ethanol may thus have two effects on the cryotolerance of baker's yeast, as a respirable carbon source and as a cryoprotectant under rapid-freezing conditions.

Freezing and thawing is a substantial and often lethal stress to yeast. The ability to cope with this stress depends on many factors, including growth phase and rate, nutritional status, rate of freezing, and the presence of cryoprotective compounds in the freezing menstruum (26). In the baking industry, freeze-thaw tolerance is a necessary trait for yeast used in frozen doughs, as postthaw leavening activity is essential prior to baking. Normally, 4 to 5% yeast is used in frozen doughs, compared with 3% for standard dough, representing a significant increase in cost (22). While tolerance to freezing may be increased by addition of various cryoprotectants (15, 16), strains with intrinsic freeze-thaw tolerance would be preferable, and much work is in progress to produce such strains (32).

It is generally assumed that nongrowing cells are more stress tolerant than their actively growing counterparts. Actively growing yeast cells have been demonstrated to be more sensitive to stresses, including heat (33, 42), chemical mutagens (33), and freezing (29), than are nongrowing cells. However, inconsistencies in the use of terms to describe the growth phases of *Saccharomyces cerevisiae* after respirofermentative (exponential) growth are common (20) and make it difficult to assess whether maximum stress tolerance is in the stationary phase or some other postrespirofermentative growth phase. There is also evidence that the type of cellular metabolism, fermentative or respiratory, influences tolerance of heat and oxidative stress and that respiratory metabolism induces intrinsically greater stress tolerance (12). In this study, we examined the freeze-thaw tolerance of *S. cerevisiae* with regard to growth phase and metabolism in aerobic batch culture to help define the growth conditions that induce maximum freeze-thaw tolerance. We present results that demonstrate that maximum tolerance to freezing and thawing occurs not in the stationary phase but rather during respiratory growth on ethanol or other respirable carbon sources. In addition, the role of ethanol as a cryoprotectant was examined. Very little work has been done to investigate the use of ethanol as a cryoprotectant, owing to its perceived toxicity and several early accounts of a poor cryoprotective effect (15). However, *S. cerevisiae* is particularly tolerant to ethanol, owing to its highly fermentative nature, so it may be amenable to cryoprotection by this compound. Under conditions of rapid freezing, ethanol demonstrated a significant cryoprotective effect.

MATERIALS AND METHODS

Yeast strains. *S. cerevisiae* K7 (ATCC 26422), a sake strain; SG195 (ATCC 38554), a wild-type strain isolated from canned cherries; and A9, a wild-type baking strain, were maintained on agar slopes containing 0.5% yeast extract (Oxoid), 0.5% bacteriological peptone (Oxoid), 1% glucose, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, and 1.5% agar (all wt/vol) (YEP agar). Slopes were stored at 4°C.

Culture conditions. Starter cultures (50 ml of YEP broth in a 250-ml Erlenmeyer flask) were inoculated from a slope and incubated overnight at 25°C and 180 rpm. Starters at this stage were in the respiratory growth phase and were subsequently used to inoculate 2-liter Erlenmeyer flasks containing 600 ml of YEP broth as experimental cultures. Duplicate cultures were inoculated simultaneously from the same starter to an optical density (OD) at 640 nm of 0.10 and incubated under the same conditions as the starter cultures. The position of the culture in the growth curve was monitored by OD at 640 nm and changes in glucose and ethanol

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concentrations. Glucose was estimated with indicator strips (Diabur-Test 5000; Boehringer GmbH, Mannheim, Germany), and ethanol concentration was estimated by gas chromatography with flame ionization detection.

For experiments specifically examining the effect of cell metabolism on cryotolerance, cells were grown in minimal medium consisting of 0.67% (wt/vol) yeast nitrogen base without amino acids (YNB; Difco) with addition of 1% glucose. When glucose was exhausted, cells were centrifuged, washed twice with YNB without a carbon source, and transferred to minimal medium containing either 0.5% (wt/vol) glucose, 0.5% (wt/vol) galactose, 0.5% (wt/vol) potassium acetate, 0.3% (wt/vol) ethanol, or no carbon source. Freeze-thaw tolerance was monitored for the next 10 h.

All experiments were repeated at least three times and gave essentially identical results.

Freeze-thaw tolerance. Generally, the freeze-thaw tolerance of cultures was tested as follows: 5 to 10 ml of a culture was centrifuged at 1,500 × *g* for 2 min at 25°C, and the pellet was suspended in YEP broth with no glucose (YEPNG). Volumes of 1 ml were distributed into 1.5-ml microcentrifuge tubes, immersed in liquid nitrogen for 5 min (cooling rate, approximately 200°C min^{-1}), and subsequently thawed in a 25°C water bath for 10 min (warming rate, approximately 190°C min^{-1}). Viability was assessed by diluting cells in YEPNG and plating them onto YEP agar in quadruplicate. Plates were incubated at 25°C for 24 to 48 h before counting. Stress tolerance was taken as the percentage of survivors after freezing and thawing compared with an unstressed control.

Effects of cryoprotective compounds. To assess the cryoprotective effects of endogenous and exogenous ethanol, cells were stressed in the culture medium (the ethanol concentration depended on the stage of growth of the culture [see Fig. 1B]) or centrifuged and then suspended in YEPNG with or without addition of 0.16% (wt/vol) ethanol. Tubes were frozen in liquid nitrogen as described above. To compare the cryoprotective efficacy of ethanol with that of glycerol, 0.5 ml of resuspended cells was added to the same volume of YEPNG containing double the required final concentration of the cryoprotectant and the tubes were mixed and frozen as described above. For cryoprotectant concentrations, see Fig. 3.

Rate of freezing. To investigate the interaction between the cryoprotectant and the rate of freezing, stationary-phase cells (6 h after ethanol exhaustion) were frozen in YEP broth without glucose with addition of 0.5 or 2 M ethanol, 2 or 5 M glycerol, or no cryoprotectant. Two equilibration times were used, 0 and 20 min, along with two freezing regimens, designated slow (−20°C for 2 h, shift to −70°C for 2 h, and shift to −196°C for 20 h; initial cooling rate, approximately 3°C min^{-1}) and fast (−196°C for 24 h, cooling rate, approximately 200°C min^{-1}). Cooling rates were measured with a fine-gauge copper-constantan thermocouple inserted into the culture during freezing.

Trehalose estimation. Trehalose was extracted from chilled and washed cells with 0.5 M trichloroacetic acid as previously described (21). Trehalose in the extract was estimated by the method outlined by Stewart (38) and the anthrone reagent described by Spiro (37).

RESULTS

Maximum intrinsic freeze-thaw tolerance. The intrinsic freeze-thaw tolerance of strain A9 during growth in aerobic batch culture changed dramatically as the culture passed through various growth stages (Fig. 1). Minimum tolerance was associated with respirofermentative growth on glucose, but tolerance increased rapidly as glucose became depleted. Maximum freeze-thaw tolerance occurred several hours after exhaustion of glucose, shortly after entry into respiratory growth on ethanol (Fig. 1B and C). Freeze-thaw tolerance at this point was higher than that of stationary-phase cells of the same culture; this indicates that maximum freeze-thaw tolerance is not always associated with a nongrowth period. This finding was confirmed with *S. cerevisiae* K7 and SG195, which both demonstrated higher freeze-thaw tolerance immediately after entry into the respiratory phase (78 and 80% survivors, respectively) than in the stationary phase (51 and 49% survivors).

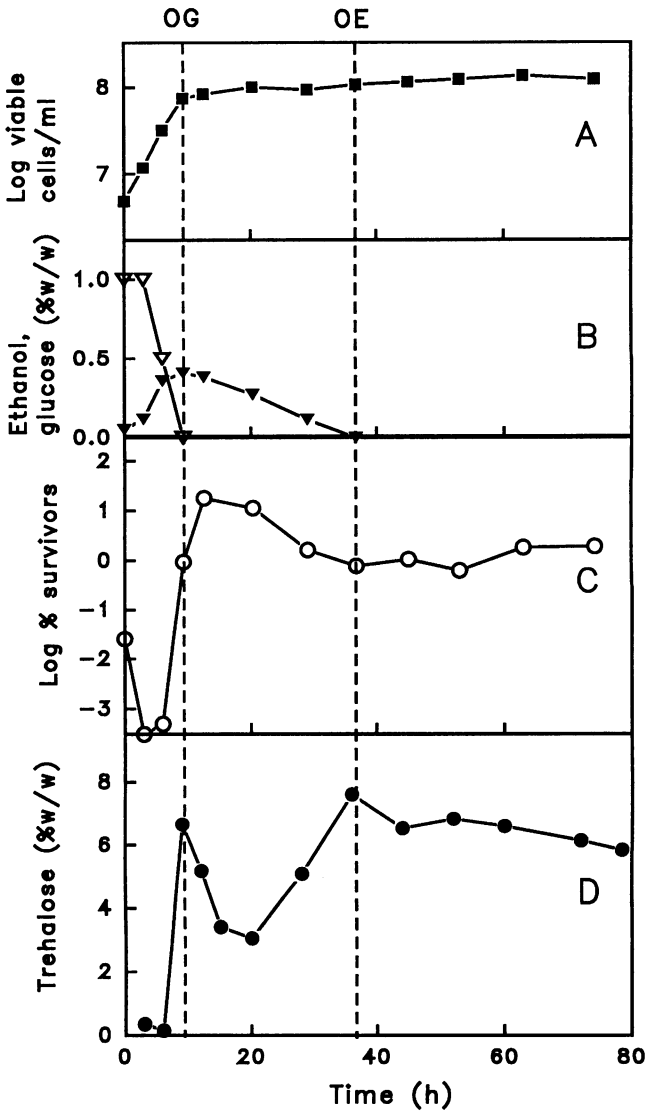

FIG. 1. Changes in freeze-thaw tolerance and trehalose during growth of *S. cerevisiae* in aerobic batch culture. (A) Log_{10} viable count ml^{-1}. (B) Glucose (▽) and ethanol (▼) concentrations in the medium. (C) Log_{10} percentage of survivors after freeze-thaw stress compared with an unstressed control. (D) Intracellular trehalose concentration. 0G, zero glucose in the culture medium; 0E, zero ethanol. The results shown are averages of duplicate independent experiments.

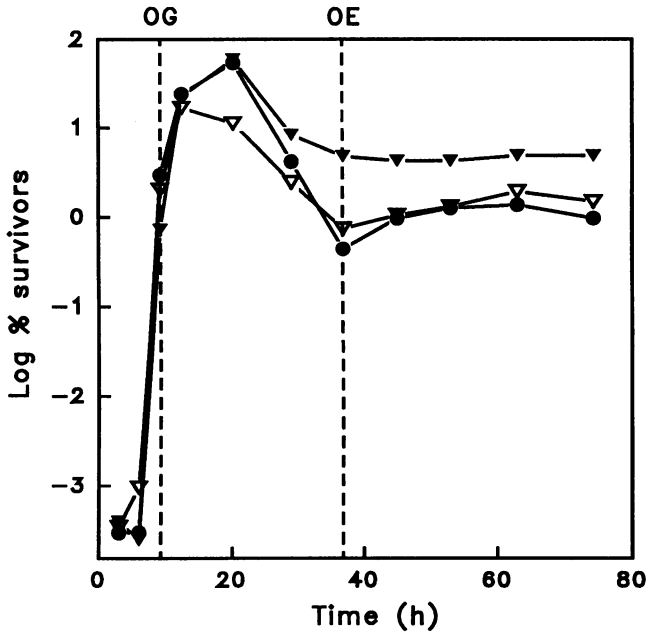

FIG. 2. Cryoprotective effects of endogenous and exogenous ethanol during growth in batch culture. Cells were frozen directly in culture medium, and the ethanol concentration was dependent on the position on the growth curve (●). Cells were centrifuged and suspended in YEPNG with (▼) or without (▽) 0.16% (wt/vol) ethanol prior to freezing. 0G, zero glucose in the culture medium; 0E, zero ethanol. The results shown are expressed as percentages of unstressed controls and are averages of duplicate independent experiments.

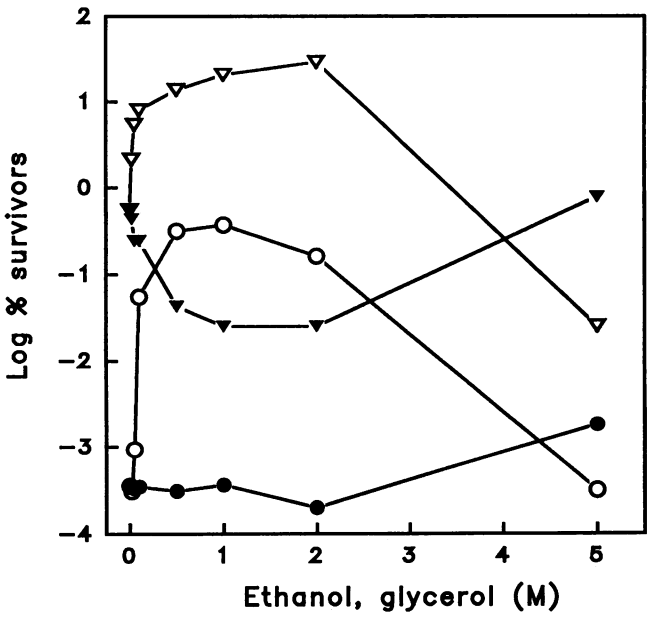

FIG. 3. Cryoprotection of respirofermentative (○, ●)- and respiratory (▽, ▼)-phase cells by ethanol (○, ▽) and glycerol (●, ▼) under conditions of fast freezing (cooling rate, approximately 200°C min^{-1}). Cells were centrifuged, washed, and suspended in YEPNG with addition of the appropriate concentration of a cryoprotectant. The results shown are expressed as percentages of an unstressed control and are averages of triplicate independent experiments.

Effect of trehalose. The role of trehalose as a powerful cryoprotectant in yeast and other cell systems has been well documented (6, 41). Trehalose was accumulated transiently in the diauxic lag phase (Fig. 1D), but its level declined immediately thereafter and did not correspond to the observed peak in freeze-thaw tolerance. As well, maximum trehalose accumulation occurred upon entry into the stationary phase, where freeze-thaw tolerance was high but not maximal.

Cryoprotective effect of ethanol. As maximum freeze-thaw tolerance was found to be associated with significant levels of ethanol in the culture medium, its possible cryoprotective effect was examined (Fig. 2). The presence of endogenous or exogenous ethanol in the freezing menstruum led to an increase in the maximum freeze-thaw tolerance of the culture, and the peak of maximum tolerance was shifted slightly. However, in all cases, maximum freeze-thaw tolerance still occurred during the respiratory phase rather than the stationary phase. Throughout the respiratory and stationary phases, ethanol acted as a strong cryoprotectant, increasing cryotolerance between 1.4 and 7.6 times. This was evident in the nonresuspended culture while ethanol was present (i.e., before ethanol exhaustion) and in the culture resuspended with addition of ethanol (Fig. 2). In a separate experiment, we compared the cryoprotectant efficacy of ethanol with that of glycerol by using cells in the respirofermentative phase (4.5 h, 0.15% [wt/vol] ethanol accumulated in the culture) and in the respiratory phase (28 h, 0.20% [wt/vol] ethanol remaining in the culture) at several concentrations (Fig. 3). Ethanol demonstrated a strong cryoprotective effect in both respirofermentative- and respiratory-phase cells at a low concentration, whereas at the same concentration glycerol showed a marked cryosensitizing effect. Only at the highest concentration did glycerol begin to demonstrate a significant cryoprotective effect, although even at this concentration it did not equal the cryoprotection afforded by ethanol at low concentrations.

As glycerol has previously been widely demonstrated to be a powerful cryoprotectant for many types of cells, including those of *S. cerevisiae* (15, 16), we speculated that our freezing methodology may favor the action of ethanol over that of glycerol. Normally, the best cryoprotective action is the result of a brief equilibration time (15 to 30 min [1]) and moderately slow freezing rates (27), whereas our method involved extremely rapid freezing immediately after addition of the cryoprotectant. Under slow-freezing conditions (3°C min^{-1}), glycerol surpassed ethanol as a cryoprotectant and ethanol acted as a cryosensitizer (Table 1), particularly at the higher concentration. However, during fast freezing (200°C min^{-1}), ethanol clearly demonstrated superiority over glycerol, resulting in cell survival levels between 9 and 23 times that of control cells. Glycerol, on the other hand, had little effect at 5 M and produced a marked decrease in cell survival at 2 M. Equilibration time generally had little effect.

Effect of carbon source. As maximum freeze-thaw tolerance was observed during respiratory growth on ethanol, the effect of active respiration was investigated. Freeze-thaw tolerance during growth on carbon sources supporting various levels of fermentation and respiration was measured to assess whether the peak in freeze-thaw tolerance was the result of active respiration or related to exhaustion of glucose (Fig. 4). The cultures growing purely by respiration of acetate or ethanol (growth inferred from increasing OD) demonstrated increased cryotolerance compared with non-

TABLE 1. Cryoprotective effects of ethanol and glycerol under slow[a]- and fast[b]-freezing regimens and two equilibration times[c]

Cryoprotectant (concn [M])	Mean[d] % survivors ± SE			
	Slow, 0 min	Slow, 20 min	Fast, 0 min	Fast, 20 min
None (control)	72.6 ± 5.07	69.7 ± 6.81	3.10 ± 0.46	6.34 ± 0.18
Ethanol (0.5)	27.6 ± 7.33	36.8 ± 11.4	54.6 ± 4.91	57.1 ± 6.35
Ethanol (2.0)	3.15 ± 1.28	5.68 ± 1.62	72.4 ± 6.00	70.9 ± 4.76
Glycerol (2.0)	88.0 ± 7.91	71.1 ± 8.66	0.76 ± 0.19	1.11 ± 0.25
Glycerol (5.0)	83.6 ± 12.8	72.8 ± 8.14	7.63 ± 3.78	3.25 ± 1.63

[a] Cooling rate, approximately 3°C min^{-1}.
[b] Cooling rate, approximately 200°C min^{-1}.
[c] Equilibration times, 0 and 20 min.
[d] The results shown are means of triplicate experiments.

growing cells (no added carbon source, no increase in OD). Glucose, which supports a high level of fermentation and strongly represses respiration (19), immediately induced a marked decrease in cryotolerance. However, glucose was rapidly exhausted and cryotolerance again increased as the culture began to respire on the accumulated ethanol. Growth on galactose, which is fermented but also supports a high level of respiration (19), was also rapid but still induced increased cryotolerance compared with nongrowing cells. The growth rate of the cultures may be inferred from their increase in OD (Fig. 5). A low growth rate has previously been associated with high stress tolerance, and in these experiments this was the case for ethanol, acetate, and starved cells. Conversely, high rates of growth have been associated with low stress tolerance, and while this was true during rapid growth of cells on glucose, galactose at no time induced the same marked cryosensitivity as glucose, despite equally rapid growth after an initial lag period (Fig. 5).

The effect of respiratory growth was also considered by the use of two spontaneously respiratorily incompetent mutants derived from strain A9, designated A91 and A92. The mutants were characterized by slow growth on glucose and inability to grow on ethanol or glycerol. Unlike that of their parent strain, the freeze-thaw tolerance of both mutants in aerobic batch culture failed to increase past the point of glucose exhaustion (Fig. 6). Freeze-thaw tolerance was comparable to that of the parent strain in the stationary phase (Fig. 1C) and was considerably less than that of A9 during respiratory growth.

DISCUSSION

Two major roles for ethanol in the cellular response of *S. cerevisiae* to freeze-thaw stress are implied by these results. The first is that ethanol, under some circumstances, may act as a powerful cryoprotectant for preservation of yeast cells

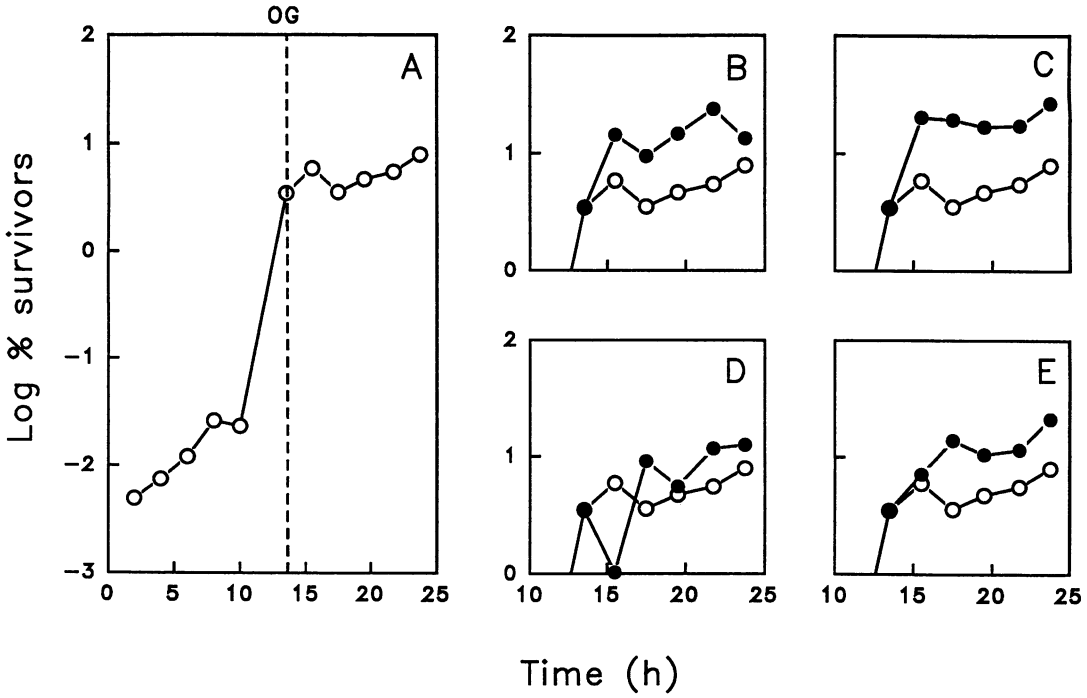

FIG. 4. Effect of carbon source on cryotolerance. Cells were grown on YNB minimal medium with 1% glucose until glucose was exhausted (0G) and then centrifuged, washed in YNB, and suspended in YNB with no carbon source (A to E, ○), or with a carbon source (B to E, ●); B, 0.3% [wt/vol] ethanol; C, 0.5% [wt/vol] potassium acetate; D, 0.5% [wt/vol] glucose; E, 0.5% [wt/vol] galactose). Cryotolerance results are expressed as percentages of unstressed controls and are averages of duplicate independent experiments.

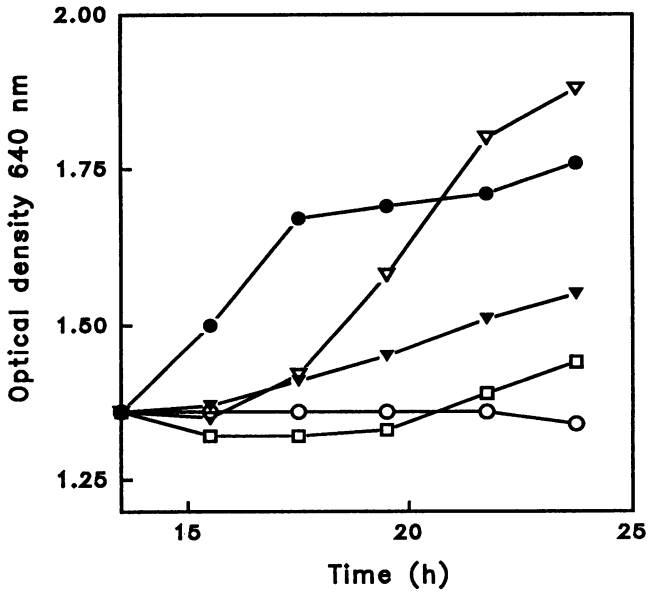

FIG. 5. Growth of *S. cerevisiae* after resuspension with various carbon sources. For experimental details, see the legend to Fig. 4. Symbols: ○, no carbon source; ▼, 0.3% (wt/wt) ethanol; □, 0.5% (wt/wt) potassium acetate; ●, 0.5% (wt/wt) glucose; ▽, 0.5% (wt/wt) galactose.

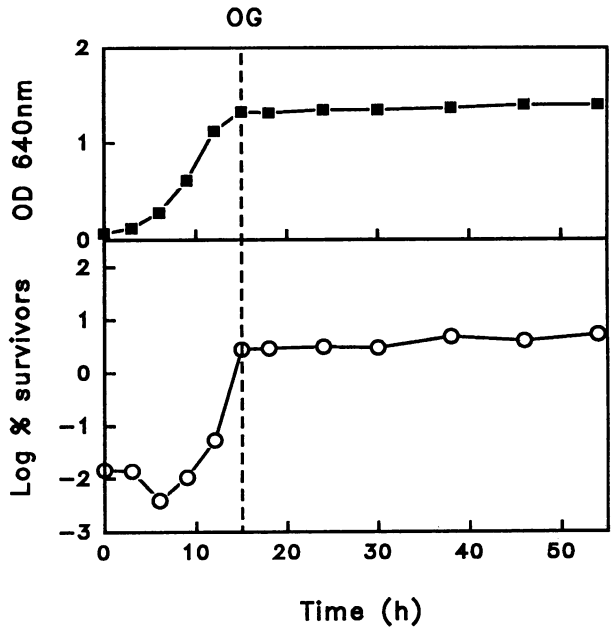

FIG. 6. Cryotolerance of respiratorily incompetent mutant A91 in aerobic batch culture. The results obtained with mutant A92 were essentially identical. 0G, zero glucose. The results shown are averages of duplicate independent experiments.

from extreme freeze-thaw stress. The second is that, acting as a respirable carbon source, ethanol may be responsible for the physiological state that induces maximum tolerance to freeze-thaw stress.

While polyhydric alcohols, such as glycerol, glycols, and sugar alcohols, have been commonly used as cryoprotectants, little use has been made of monohydric alcohols, owing to their apparent toxicity. Ideally, cryoprotectants should be nontoxic at the concentration necessary to achieve a cryoprotective effect, both prior to freezing and after thawing, as well as at increased concentrations caused by dehydration and phase separation during freezing. Monohydric alcohols increase in toxicity with acyl chain length (13), and consequently only methanol and ethanol have been demonstrated to have any cryoprotective ability (7, 15, 16). Of the two, methanol is the only one to have a practical application, i.e., preservation of parasites (14). However, ethanol has been demonstrated to offer slight protection against haemolysis in erythrocytes (23) and to maintain yeast gassing power after freezing (39). In the latter experiment, however, cell viability was not measured.

Under our experimental conditions, ethanol led to low cell viability after slow freezing and high viability after fast freezing (Fig. 3 and 4 and Table 1). Glycerol produced the opposite effect, demonstrating cryoprotection during slow freezing but none under conditions of fast freezing. While the toxicity of ethanol under conditions of slow freezing is readily explained in terms of the extremely high concentration of the compound accumulated as the water freezes, it is not clear how ethanol is able to protect cells under conditions of rapid freezing. Under these conditions, the extracellular solute concentration increases more rapidly than the intracellular concentration, owing to the inability of water to diffuse from the cells rapidly enough. This leads to intracellular ice crystal formation and results in very low cell viability upon thawing (28). Addition of ethanol may mitigate the effects of ice crystal formation, possibly by inhibiting intracellular ice crystal formation completely (leading to a vitrified state within the cells) or by inhibiting ice crystal propagation. A more thorough investigation of the cryoprotective properties of ethanol and other monohydric alcohols under controlled freezing conditions may be warranted to reassess their cryoprotective properties and applications.

The cryoprotective effect of ethanol in the growth medium, however, does not explain the peak in freeze-thaw tolerance during respiratory growth. It has long been accepted that, for most stresses, tolerance is linked to the growth rate of yeast cells. Most of the work has been done with cells in batch culture, and many workers have compared rapidly proliferating cells with their nonproliferating counterparts. Virtually all reports have indicated that stationary-phase cells are far more stress resistant than those in the exponential growth phase. However, the extremely large increases observed in the cellular stress tolerance of cultures progressing from exponential respirofermentative growth on glucose to respiratory growth on ethanol has tended to obscure smaller variations in stress tolerance after this point. Combined with the common usage of the term stationary phase to refer to any time after exhaustion of the initial fermentable carbon source (20), this has led to a general assumption that stationary-phase cells are invariably more stress resistant than growing cells. The sensitivity of growing cells to freeze-thaw stress and the relative resistance of their nongrowing counterparts has been previously documented for bacteria (25, 26) and yeasts (5, 29, 31, 34, 36). However, the results presented here demonstrate that growing cells may be more freeze-thaw tolerant than stationary-phase cells (Fig. 1), provided that respiratory metabolism is not fully repressed (Fig. 4). The effect is not limited to slow growth on nonfermentable carbon sources but extends to

rapid growth on fermentable carbon sources that support high levels of respiration (Fig. 4 and 5). Additionally, respiratorily incompetent cells cannot attain the same cryotolerance as their respiratorily competent counterparts (Fig. 1 and 6). Two other reports have also noted that growing cells are more tolerant to stress than are nongrowing cells. While Terasima and Yasukawa (40) found plateau-phase mammalian cells to be more freeze-thaw tolerant than rapidly growing cells, Frim et al. (8) reported the opposite. Similarly, Mitchel and Morrison (30) found that stationary-phase yeast cells were more resistant to UV radiation than were respirofermentative cells, but the opposite has also been reported (33). Parry et al. (33) suggested that while cells may be more sensitive to UV stress while growing, necessary repair mechanisms may also be more active, leading to an overall greater resistance of the population in the respirofermentative phase than in the stationary phase. Frim et al. (8) also considered this hypothesis, and it is possible that freeze-thaw tolerance is a similar case, in which an essential repair or protection system, or combination of systems, is better expressed during the respiratory phase than during the stationary phase.

Many intrinsic physiological characteristics have been associated with cryotolerance in *S. cerevisiae*, including membrane composition (4, 10, 18), heat shock proteins (17), glycerol (15, 16), and trehalose (6, 9, 11, 41). However, it seems unlikely that any of these characteristics individually could explain the complex behavior observed during changes in the growth phase (Fig. 1). There is abundant evidence that trehalose is responsible for much of the freeze-thaw tolerance observed in cells after the respirofermentative phase, and in our experiments, levels of trehalose and cryotolerance both remained high compared with those of respirofermentative-phase cells (Fig. 1D). However, the lack of correlation between trehalose and maximum freeze-thaw tolerance here, and elsewhere (9), implies that other factors are also important, especially during respiratory growth. Membrane lipid composition varies during growth in batch culture (43), but the fairly minor changes reported seem unlikely to influence freeze-thaw tolerance as strongly as demonstrated here. The glycerol concentration in batch culture also varies but, except under conditions of osmotic stress, never reaches significant concentrations (24) and seems unlikely to affect cryotolerance.

Growth phase (3, 35) and carbon source (2, 35) both affect the expression of proteins, including heat shock proteins. Hsp104, in particular, is preferentially expressed during respiratory growth and has been correlated with tolerance to a wide variety of stresses in *S. cerevisiae* (35). It is possible that expression of stress proteins, in particular Hsp104, is partly responsible for the freeze-thaw tolerance observed during respiratory growth. Thus, in combination with high levels of trehalose, maximum cryotolerance may occur during respiratory growth rather than during the stationary phase. This hypothesis may also partly explain the results obtained by Gélinas et al. (9), who found that cryotolerance was influenced by the trehalose concentration in cells but its influence was modified by the rate of aeration and, hence, cellular respiration.

ACKNOWLEDGMENTS

We are grateful to Paul Attfield for useful discussion.

This work was supported by an Australian Postgraduate Research Award—Industry (J.G.L.) and University of New England internal research grants.

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