APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 3818–3824 Vol. 63, No. 10
0099-2240/97/$04.00+0

The Freeze-Thaw Stress Response of the Yeast *Saccharomyces cerevisiae* Is Growth Phase Specific and Is Controlled by Nutritional State via the *RAS*-Cyclic AMP Signal Transduction Pathway

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Received 26 March 1997/Accepted 21 July 1997

The ability of cells to survive freezing and thawing is expected to depend on the physiological conditions experienced prior to freezing. We examined factors affecting yeast cell survival during freeze-thaw stress, including those associated with growth phase, requirement for mitochondrial functions, and prior stress treatment(s), and the role played by relevant signal transduction pathways. The yeast *Saccharomyces cerevisiae* was frozen at −20°C for 2 h (cooling rate, less than 4°C min^{-1}) and thawed on ice for 40 min. Supercooling occurred without reducing cell survival and was followed by freezing. Loss of viability was proportional to the freezing duration, indicating that freezing is the main determinant of freeze-thaw damage. Regardless of the carbon source used, the wild-type strain and an isogenic petite mutant ([*rho*^{0}]) showed the same pattern of freeze-thaw tolerance throughout growth, i.e., high resistance during lag phase and low resistance during log phase, indicating that the response to freeze-thaw stress is growth phase specific and not controlled by glucose repression. In addition, respiratory ability and functional mitochondria are necessary to confer full resistance to freeze-thaw stress. Both nitrogen and carbon source starvation led to freeze-thaw tolerance. The use of strains affected in the *RAS*-cyclic AMP (*RAS*-cAMP) pathway or supplementation of an *rca1* mutant (defective in the cAMP phosphodiesterase gene) with cAMP showed that the freeze-thaw response of yeast is under the control of the *RAS*-cAMP pathway. Yeast did not adapt to freeze-thaw stress following repeated freeze-thaw treatment with or without a recovery period between freeze-thaw cycles, nor could it adapt following pretreatment by cold shock. However, freeze-thaw tolerance of yeast cells was induced during fermentative and respiratory growth by pretreatment with H_2O_2, cycloheximide, mild heat shock, or NaCl, indicating that cross protection between freeze-thaw stress and a limited number of other types of stress exists.

Freezing is used for many industrial, medical, food technological, and scientific purposes, such as strain preservation, organ preservation, and cryosurgery. The yeast *Saccharomyces cerevisiae* is an appropriate eukaryotic organism with which to study the physiological parameters that affect a cell's ability to survive freeze-thaw injury, since it has been extensively characterized both biochemically and genetically. Moreover, there is a range of available mutations affecting cellular responses to various types of stress that may be incurred during freeze-thaw injury which can be exploited to understand the nature of freeze-thaw injury and how to avoid it. Intracellular ice formation during freezing has been described for many cellular systems, and the rate at which ice is formed determines the type of freezing damage suffered by cells (25, 30). Cells can be injured during freezing by physical factors such as ice crystal formation and dehydration. At high freezing rates, intracellular freezing occurs, leading to cell damage mainly by ice crystal formation. However, at low freezing rates, extracellular ice formation predominates, leading to intracellular dehydration (25). The freezing rate is determined by the characteristics of a cell, such as shape, structure, surface area-to-volume ratio, and membrane permeability. Therefore, each cell has its own specific freezing rate for fast or slow freezing, and for yeast, freezing rates below 7°C min^{-1} are known to result in slow freezing (25).

Cells can also suffer biochemical damage, including, for example, oxidative stress by reactive oxygen species formed during the thawing process (13). Yeast cells show different degrees of tolerance to stresses depending on their growth state (23, 34), indicating that the physiological state of cells decides their stress resistance. Yeast cells in the G_0 state induced by starvation are more refractory to stress conditions such as heat shock (34). Changes as cells enter the G_0 phase are mediated in part by the *RAS* signal transduction pathway, which controls the level of cellular cyclic AMP (cAMP) (3). The *RAS*-cAMP signal transduction pathway modulates expression of genes regulated by stress response elements (29). Pretreatment of cells with a mild stress induces higher stress tolerance, and in some cases this confers cross protection to other, different types of stress (23). Hence, it has been assumed that different stress conditions act through a variety of effects in cells, such as generation of abnormal or denatured proteins, internal acidification, alterations in the cytoskeleton, or modulation of second-messenger levels, to produce signals that can converge and stimulate more general stress-responding systems (6, 8, 9).

Like for other stresses, yeast cells may cope with freeze-thaw stress by synthesis of stress proteins (19) or metabolites such as trehalose and glycerol (15, 21), which are produced in large amounts by cells in abnormal situations. Trehalose stabilizes the intracellular water structure and cell membranes under

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stress conditions (15). Disruption of the *ATH1* gene, encoding acidic trehalase, confers high freeze-thaw tolerance to yeast (18), and freeze-thaw-tolerant yeast strains have higher levels of trehalose (14), indicating that trehalose is a possible protectant for freeze-thaw stress. Cell membrane flexibility is thought to be an important factor in the freeze-thaw stress resistance of a cell, since the membrane is a major target of freeze-thaw damage. It is known that membrane flexibility can be modified by the binding of saccharides acting as cryoprotectants (15) or by alteration of the phospholipid and neutral lipid compositions (27).

In the present study, physiological factors that affect yeast cell survival during freeze-thaw stress have been characterized, including the growth phase, starvation for C or N source, and requirement for mitochondrial functions. The ability of cells to adapt to survive freeze-thaw stress following other stress treatments, including osmotic stress, heat shock, oxidative stress, and metabolic stress following cycloheximide treatment, has been examined, as has the requirement for signal transduction pathways that may be relevant to cells maintaining freeze-thaw resistance.

MATERIALS AND METHODS

Yeast strains and culture conditions. *S. cerevisiae* CY4 (*MAT***a** *ura3-52 leu2-3 leu2-112 trp1-1 ade2-1 his3-11 can1-100*) and an isogenic petite strain, CY4p (12), were used for all experiments except for analysis of the *RAS*-cAMP pathway. Strains JC482 (*MAT*α *ura3-52 leu2 his4*) (5), JC302-26B (*MAT*α *ura3-52 leu2 his4 ras2*::*LEU2*), JC303-79 (*MAT*α *ura3-52 leu2 his4 ras2*::*LEU2 sra1-13*) (4), and LRA85 (*MAT*α *his4 leu2 ura3-52 cdc35-11*) were from K. Tatchell. Strain OL520-1 (*MAT*α *his3 leu2 ura3 trp1 rca1 cdc25-5*) was from M. Jacquet. Strains GG18 (*MAT***a** *leu2 ura3 his3 trp1 ade8 cta1-2 CTT1-18/7x-LEU2-lacZ*) and GG18hog1 (*MAT***a** *leu2 ura3 his3 trp1 ade8 cta1-2 CTT1-18/7x-LEU2-lacZ hog1*::*TRP1*) were from H. Ruis. Strains W303-1A (*MAT***a** *suc2 ade2 can1 his3 leu2 trp1 ura3*) and W303-1Ahog1 (*MAT***a** *suc2 ade2 can1 his3 leu2 trp1 ura3 hog1*::*TRP1*) were from C. Gustin.

Strains CY4 and CY4p were grown at 30°C with shaking at 180 revolutions/min in minimal medium. Cells were inoculated to an optical density of 0.2 at 600 nm. Samples for fermentative growth were taken after 6 to 7 h (A_{600} = 1), and samples for respiratory growth were taken after 48 h (A_{600} = 3.8 to 4.3), except as otherwise specified. Cells in the fermentative phase were producing ethanol, and those in the respiratory phase were consuming ethanol. S medium, containing 0.17% (wt/vol) yeast nitrogen base (Difco), 0.5% (wt/vol) ammonium sulfate (Oxoid), and appropriate auxotrophic requirements, was used as a base. This medium was supplemented with carbon sources: 2% (wt/vol) glucose for SD medium or 3% (vol/vol) glycerol and 1% (vol/vol) ethanol for SGE medium. For nitrogen (N) starvation, STMD medium, containing 2% (wt/vol) glucose, 0.17% (wt/vol) yeast nitrogen base (Difco) without amino acids and ammonium sulfate, and limited amounts of auxotrophic requirements (1 mg/liter for tryptophan and 5 mg/liter for all other cases), was used (1). For carbon (C) starvation conditions, S medium was used. Strain LRA85 and OL520-1 were grown at 23°C with shaking at 180 revolutions/min in YEPD, containing 2% (wt/vol) glucose, 2% (wt/vol) Bacto Peptone, and 1% (wt/vol) yeast extract. All other strains were grown at 30°C with shaking at 180 revolutions/min in YEPD. YEPD medium was solidified by adding 2% (wt/vol) agar.

Ethanol determination. Samples of spent media were taken at different points during the yeast growth cycle, and the ethanol concentration was determined by using a Perkin Elmer Autosystem gas chromatograph. The column used was a BP31 capillary column (Scientific Glass Engineering), and the carrier gas was H_2. The column temperature was 180°C, the injector temperature was 180°C, and the detector temperature was 200°C.

Freezing and thawing conditions. Cells were harvested by centrifugation, washed in 0.1 M sodium phosphate buffer (pH 7.0), and suspended to an A_{600} of 3 in the same buffer. Aliquots (0.3 ml) of cells were transferred into thin-walled 1.5-ml polycarbonate tubes (for PCRs; Greiner Labortechnik) and frozen at −20°C for 2 h. Cooling and warming rates during the freeze-thaw process were measured with a microprocessor-based thermometer inserted into the tubes. Samples were thawed at 0°C for 40 min, and survival was determined by diluting cells into YEPD at room temperature and plating on YEPD plates at appropriate dilutions to determine cell viability. Cells were grown at 30°C for 2 days before colony counting. A different freezing protocol in which samples were thawed at room temperature was used without significantly altering the cell survival.

Stress pretreatment conditions. Oxidative pretreatment of cultures growing at 30°C was applied by treatment with H_2O_2 at final concentrations of 0.1, 0.2, and 0.4 mM for 15 min. Cycloheximide pretreatment was at concentrations of 5, 10, and 50 μg/ml for 15 min. In the case of heat pretreatment, aliquots of cells growing at 30°C were transferred to 37°C or 42°C, and samples were taken after 15, 30, and 60 min. For cold shock pretreatments, aliquots of cells growing at 30°C were transferred to 4, 10, and 16.5°C, and samples were taken after 30, 60, 90, and 120 min. Salt pretreatment was carried out by adding NaCl to a concentration of 0.3 or 0.7 M to cultures at 30°C, and samples were taken after 1, 2, and 3 h. In the cases of H_2O_2, cycloheximide, and heat pretreatments, samples from the fermentative growth phase were pretreated in either culture medium or 0.1 M sodium phosphate buffer (pH 7.0). Since the cells retained similar viabilities, samples from the respiratory growth phase were pretreated only in phosphate buffer. Following all treatments, cells were washed and suspended to an A_{600} of 3 in phosphate buffer and frozen and thawed as described above.

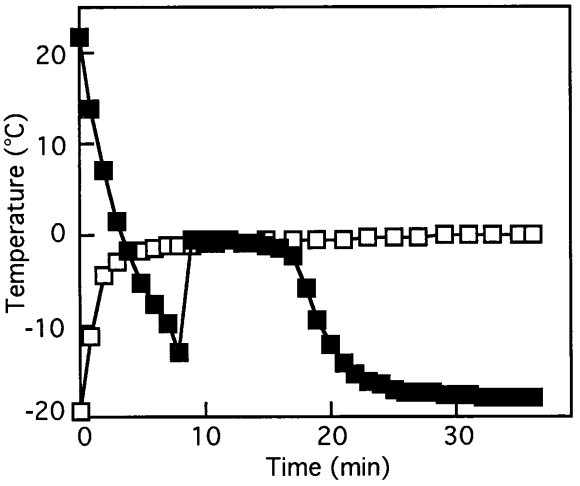

FIG. 1. Temperature changes during freezing and thawing. The temperature of cells suspended (A_{600} = 3) in 0.1 M phosphate buffer (pH 7) was measured during freezing at −20°C (■) until the sample temperature reached −20°C and during thawing at 0°C (□). The data shown are from a representative experiment.

RESULTS

Freeze-thaw stress causes a loss of viability in yeast. Before examining the effects of different physiological parameters on freeze-thaw injury or the effects of mutations on cell survival, it was necessary to identify whether yeast cells were susceptible to freeze-thaw damage and to identify a suitable protocol for freezing and thawing. Cultures were frozen in 1.5-ml reaction tubes (1-cm diameter) that were placed in a freezer, and they reached −20°C within 30 min (Fig. 1). Samples were supercooled before the release of latent heat, which took place at around −13°C and was followed by ice nucleation. After freezing for 2 h, samples were transferred to 0°C and thawed for 40 min, with the temperature changes shown in Fig. 1.

Under these freeze-thaw conditions, yeast cells lost viability as an exponential function of freezing duration (Fig. 2). Cells thawed at room temperature and at 0°C did not differ in viability (data not shown). In some tubes the supercooling state was prolonged at −20°C, and this did not affect the viability of early-exponential-growth-phase cells (A_{600} = 1), which are the most sensitive to freeze-thaw damage (Fig. 2). These results indicated that the main damage to cells comes from freezing rather than thawing and that cell survival can be used as a measure of freezing damage. They also provided a method with which to examine the effects of cellular growth phase on the ability of the cells to survive.

Survival of cells following freeze-thaw damage depends on the physiological growth state. The survival of CY4 following freeze-thaw damage was determined throughout the growth phases on defined minimal medium (SD) with glucose as a carbon source. Stationary-phase cells were inoculated into SD medium, and freeze-thaw tolerance was tested as cells exited

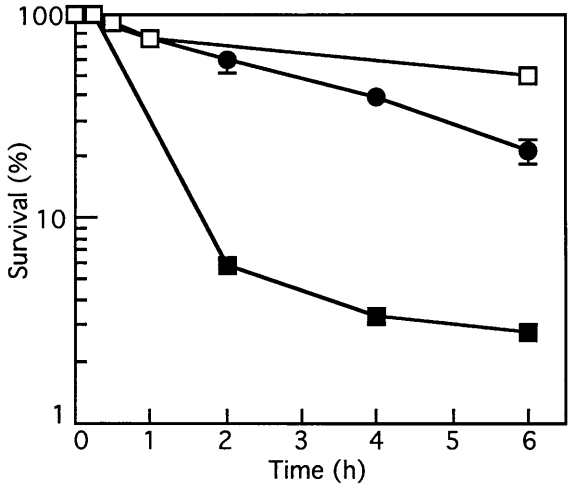

FIG. 2. Effect of freezing duration and supercooling on cell survival. Strain CY4 (wild type) grown to early exponential phase (A_{600} = 1) or to late exponential phase (A_{600} = 3.6) on minimal (SD) medium was exposed to −20°C. Survival of frozen (■) or supercooled (□) samples from early exponential phase and of frozen samples from late exponential phase (●) was determined for 6 h. Percent survival is expressed relative to the culture viability immediately prior to freezing. The data shown are means from triplicate measurements from a representative experiment. Error bars represent the standard errors of the means.

the lag phase and through exponential, diauxic-shift, and stationary-phase growth (Fig. 3). The growth phase was monitored both by changes in A_{600} and by the ethanol concentration in the medium. Cells gradually lost resistance as they progressed through the lag phase into the exponential growth phase, and the lowest freeze-thaw resistance was observed during the early exponential growth phase. Cells in the diauxic-shift phase were most resistant to freeze-thaw damage (approximately 85% survival). Resistance declined slowly during the respiratory phase while ethanol was being utilized and then fell further during starvation (data not shown). These observations indicate that cells are most resistant during lag phase. The resistance of cells was lower during the fermentative growth phase than during the respiratory growth phase, implicating mitochondrial functions.

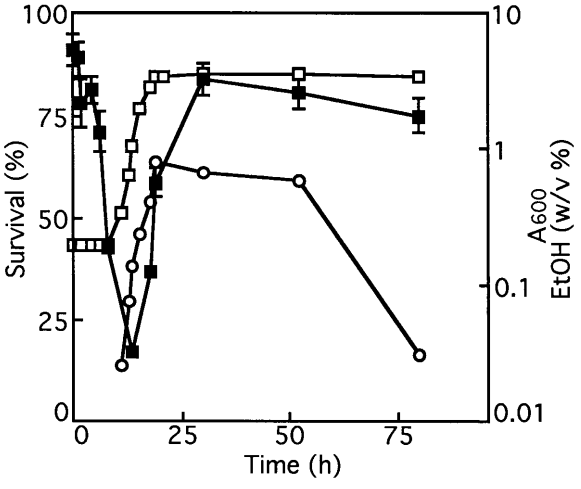

FIG. 3. Cells show different freeze-thaw tolerances depending on growth phase. Strain CY4 (wild type) was grown on minimal (SD) medium, and freeze-thaw tolerance was determined by measuring viability after exposure to −20°C (■). The growth phase was determined by measuring A_{600} (□) and ethanol (EtOH) concentration in the culture medium (○). Percent survival is expressed relative to the culture viability immediately prior to freezing. The data shown are means from triplicate measurements from a representative experiment. Error bars represent the standard errors of the means.

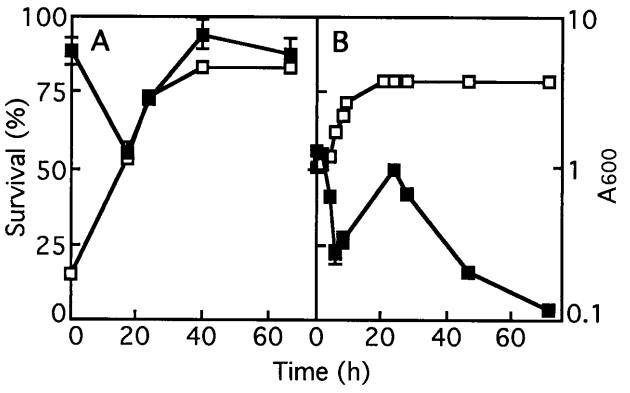

FIG. 4. Effect of respiration and mitochondrial function on freeze-thaw tolerance. The freeze-thaw tolerance of strain CY4 (wild type) grown on minimal (SGE) medium (A) and the isogenic petite strain grown on minimal (SD) medium (B) was determined at each growth phase. The growth phase was determined by measuring A_{600} (□). Percent survival (■) is expressed relative to the culture viability immediately prior to freezing. The data shown are means from triplicate measurements from a representative experiment. Error bars represent the standard errors of the means.

Respiratory growth effects on freeze-thaw stress. To examine the effect of respiratory versus fermentative growth, cells were grown on two nonfermentable carbon sources, glycerol and ethanol. The overall resistance of cells grown on nonfermentable carbon sources was higher than that of cells grown on glucose, but the same pattern of resistance was still observed; resistance decreased as the cells exited lag phase and increased as cells grew through exponential phase into stationary phase (Fig. 4A). In a respiratory-deficient petite strain lacking mitochondrial function, the overall freeze-thaw tolerance pattern of resistance was the same as that of the wild type, but the tolerance was lower (Fig. 4B). The petite strain was most freeze-thaw tolerant as it finished exponential growth. During stationary phase resistance decreased, but the untreated cells also lost viability (data not shown). These results indicate that mitochondrial function is involved in freeze-thaw stress resistance.

Carbon and nitrogen starvation induce freeze-thaw tolerance. Since increased freeze-thaw tolerance was observed during the depletion of nutrients in media, we investigated whether different forms of starvation could induce freeze-thaw tolerance of yeast cells. Cells of exponentially growing strain CY4 (A_{600} = 2) were transferred to S and STMD media for carbon source and nitrogen source starvation, respectively. In both cases, cells responded to starvation with increased freeze-thaw tolerance (Fig. 5). The response was more rapid in the case of carbon starvation, with about twofold-increased freeze-thaw tolerance after 3 h, while cells transferred to STMD medium showed a decreased freeze-thaw tolerance during the first 2 h but showed increased tolerance after 5 h. These results indicate that starvation can confer freeze-thaw stress resistance, and they raise the question of which cellular signal transduction pathways may be involved.

The *RAS*-adenylate cyclase signal transduction pathway affects resistance to freeze-thaw stress. The *RAS*-cAMP signal transduction pathway responds to nutrient depletion with changes in the intracellular cAMP level (29). We examined the involvement of the *RAS* signal transduction pathway in the freeze-thaw stress response, since starvation triggered in-

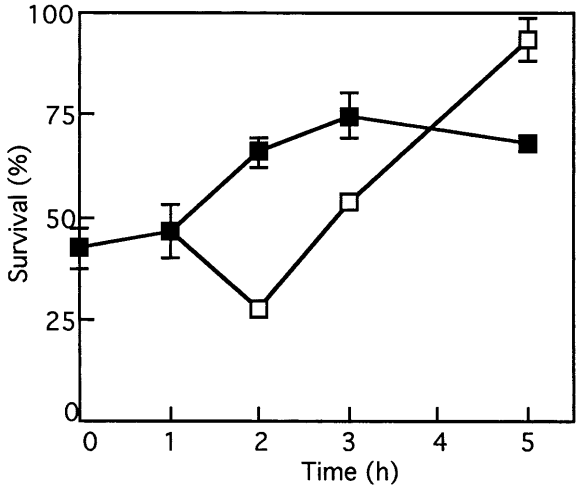

FIG. 5. Starvation induces freeze-thaw tolerance. Cells of strain CY4 (wild type) were grown to exponential phase (A_{600} = 2) on minimal (SD) medium and transferred to C starvation (S) medium (■) or N starvation (STMD) medium (□). Freeze-thaw tolerance was determined every 2 h under each condition. Percent survival is expressed relative to the culture viability immediately prior to freezing. The data shown are means from triplicate measurements from a representative experiment. Error bars represent the standard errors of the means.

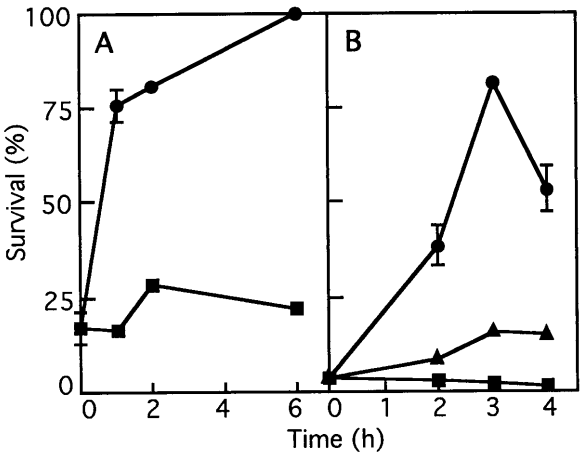

FIG. 7. cAMP is a mediator of freeze-thaw stress resistance. (A) Strain LRA85 (*cdc35* mutant) grown to exponential phase (A_{600} = 2) on YEPD medium at 22°C (■) was shifted to 37°C (●). (B) Strain OL520-1 (*cdc25* mutant) grown to exponential phase (A_{600} = 1) on YEPD medium at 22°C (■) was shifted to 37°C with 4 mM cAMP (▲) or without cAMP (●). Freeze-thaw tolerance was determined during culture under each condition. Percent survival is expressed relative to the culture viability immediately prior to freezing. The data shown are means from triplicate measurements from a representative experiment. Error bars represent the standard errors of the means.

creased freeze-thaw tolerance. A *ras2* disruption mutant (JC302-26B) was more freeze-thaw tolerant than the isogenic wild-type strain (Fig. 6). In a strain containing both a *ras2* disruption and an *sra1-13* point mutation (*sra1-13* is an allele of the *BCY1* gene that suppresses *ras2* [4]), freeze-thaw tolerance was reduced, confirming that the *RAS* signal transduction pathway is required for freeze-thaw stress resistance. The role of cAMP in maintaining freeze-thaw tolerance was confirmed by using temperature-sensitive mutants LRA85 (*cdc35-11*) (defective in adenylate cyclase) and OL520-1 (*cdc25-5 rca1*) (defective in the regulator of adenylate cyclase and able to take up exogenous cAMP). Both strains showed a rapid increase of freeze-thaw tolerance after the shift from the permissive (23°C) to the restrictive (37°C) temperature (Fig. 7). Since this effect was nullified by adding cAMP to strain OL520-1 (Fig. 7B), the ability to survive freeze-thaw stress is under the control of the *RAS*-cAMP signal transduction pathway with cAMP as a mediator.

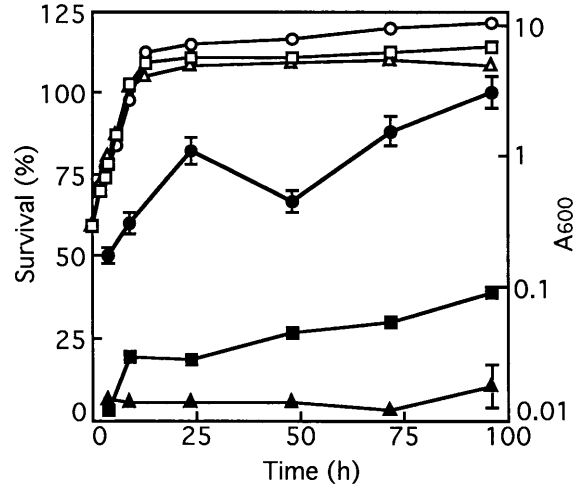

FIG. 6. Freeze-thaw stress resistance is under the negative control of the *RAS* signal transduction pathway. Strains JC482 (wild type) (■), JC302-26B (*ras2* mutant) (●), and JC303-79 (*sra1 ras2* mutant) (▲) were grown on YEPD medium, and freeze-thaw tolerance was determined at each growth phase. The growth phase was determined by measuring A_{600} (open symbols). Percent survival (closed symbols) is expressed relative to the culture viability immediately prior to freezing. The data shown are means from triplicate measurements from a representative experiment. Error bars represent the standard errors of the means.

Can cells adapt to freezing stress? Yeast can adapt to other types of stress, including those caused by heat (8) or oxidants (7, 16), so we tested the adaptability of yeast cells from the fermentative and respiratory growth phases by refreezing with or without recovery periods. When cells were refrozen immediately after a first freeze-thaw, survival was in each case higher than that for the first freeze-thaw cycle, with higher survival ratios shown by cells in the respiratory growth phase (data not shown). It appeared that surviving cells were more resistant to repeated freeze-thaw stress, but it was unclear whether this was true adaptation or the result of selecting resistant cells from the original population. During this treatment, the synthesis or modification of stress protectants was unlikely to occur rapidly; hence, the cells were given a recovery period between the first and second freeze-thaw cycles. Cells were treated with a first freeze-thaw and then allowed to recover in minimal (SD) medium at 30°C. During recovery, the cells began to lose resistance to freeze-thaw injury, as they began to grow after an initial lag phase (data not shown). This was similar to the behavior of cells recovering from a lag phase (Fig. 3). These results indicated that cells cannot adapt to freeze-thaw stress by repeated freezing and thawing.

Induction of freeze-thaw tolerance by other types of stress. To determine whether there was cross protection caused by adaptation to other forms of stress, fermentative- and respiratory-phase cells were pretreated with different levels of H_2O_2 (0.1 to 0.4 mM), cycloheximide (5 to 50 μg/ml), heat shock (shifts from 30 to 37 or 42°C for up to 60 min), NaCl (0.3 and 0.7 M), and cold shock (4, 10, and 16.5°C) for different periods.

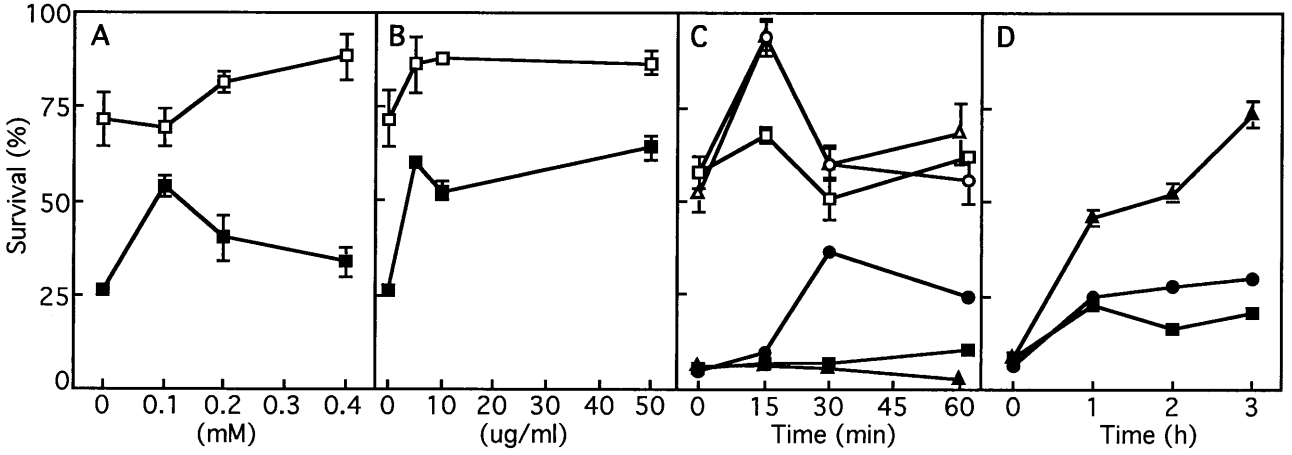

FIG. 8. Induction of freeze-thaw tolerance by other stresses. Cells of strain CY4 from the fermentative growth phase (A_{600} = 1) (closed symbols) and respiratory growth phase (open symbols) were pretreated before freeze-thaw tolerance determination with H_2O_2 (A), cycloheximide (B), heat shock (C) (squares, control; circles, 37°C; triangles, 42°C), or NaCl (D) (squares, control; circles, 0.3 M; triangles, 0.7 M). Percent survival is expressed relative to the culture viability immediately prior to freezing. The data shown are means from triplicate measurements from a representative experiment. Error bars represent the standard errors of the means.

Of these pretreatments, H_2O_2, cycloheximide, heat shock, and NaCl caused changes in freeze-thaw tolerance (Fig. 8).

Pretreatment with H_2O_2 or cycloheximide led to increased resistance for both fermentative- and respiratory-phase cells (Fig. 8A and B). Fermentative-phase cells showed optimal induction at 0.1 mM H_2O_2, whereas respiratory-phase cells required 0.4 mM. For cycloheximide-induced stress resistance, 5 μg/ml was as effective as higher concentrations.

Heat shock from 30 to 37°C led to increased resistance of cells, but the period that cells were exposed to the temperature was important; the increased resistance required 30 min for fermentative-phase cells and 15 min for respiratory-phase cells (Fig. 8C). For cells in the respiratory phase, heat shock led to increased freeze-thaw resistance regardless of the temperature used, but curiously, for fermentative-phase cells only 37°C treatment was effective and 42°C did not confer any increase.

NaCl pretreatment at 0.3 M was used since 0.3 M NaCl induces the expression of genes responsive to osmotic conditions via the *HOG* pathway (31), and the high concentration of 0.7 M was used since other osmotic-signal-responding systems, such as protein phosphatase calcineurin, operate at high salt concentrations (24). The most dramatic effect was seen for fermentative-phase cells pretreated with 0.7 M NaCl. Under these conditions, the cells pretreated for 3 h became about 75% resistant to a freeze-thaw cycle (Fig. 8D). However, pretreatment with 0.3 M NaCl had no significant effect. The involvement of the *HOG* pathway was examined by subjecting two sets of *hog1* mutant and isogenic wild-type strains to freezing and thawing. The mutant and wild-type strains showed similar levels of survival of, and cross protection by, 0.3 M NaCl pretreatment (data not shown), indicating that the *HOG* pathway does not play a significant role in the resistance of cells to freeze-thaw damage.

Pretreatments with low temperature that induced appropriate adaptive responses in prokaryotes and cold shock protein synthesis in yeast (17, 20) did not affect freeze-thaw tolerance (data not shown).

DISCUSSION

Yeast cells are capable of adapting to many types of stress; for example, there are inducible responses to heat shock (8), treatment with reactive oxygen species such as hydrogen peroxide (7) and free radical-generating agents (10), or osmotic stress (31). Moreover, when yeast cells respond to one particular form of stress, they often acquire cross-resistance to another type of stress; for example, heat shock treatment confers resistance to various types of oxidative stress (7, 10). Our results show that yeast cells do not appear to have an inducible response to freeze-thaw stress, since repeated freezing and thawing did not induce tolerance even when cells were allowed to recover in fresh growth medium at an optimal growth temperature. However, heat shock, oxidative stress induced by H_2O_2, metabolic stress caused by cycloheximide, or osmotic stress induced by 0.7 M NaCl did offer cross protection conferring freeze-thaw tolerance.

H_2O_2 pretreatment sufficient to induce full resistance to oxidative stress (7) also led to freeze-thaw tolerance. This could be taken to indicate that some of the damage caused by freezing and thawing is due to reactive oxygen species and that the induction of defenses against oxidants may form part of the resistance to freeze-thaw stress. Other explanations are possible, although previous work with transgenic alfalfa showed that a high-level expression of superoxide dismutase conferred freeze-thaw stress resistance (26). Moreover, in several systems, oxidative damage and changes in activities of antioxidant enzymes were reported to occur during thawing (13). Taken together, these results indicate that cells are probably exposed to oxidative stress during the freeze-thaw process.

Protein synthesis induced by heat shock is required for the acquisition of resistance to rapid freezing, as shown by previous studies using cycloheximide as an inhibitor (19, 22). However, cycloheximide treatment, which inhibits protein synthesis (28), may cause metabolic stress and stimulate general stress response pathways. Radioactive labeling showed that protein synthesis did not occur during the cycloheximide pretreatment (data not shown), and hence cycloheximide may cause metabolic stress through a mechanism that involves protein modification rather than synthesis.

NaCl at 0.3 M induces the expression of genes responsive to osmotic conditions via the *HOG1* signal transduction pathway (31). At higher salt concentrations other signal systems, such as protein phosphatase calcineurin, are more important (24). Interestingly, 0.7 M NaCl was required to observe an effect on cellular resistance to freeze-thaw stress, indicating that an os-

mostress defense system other than the *HOG1* pathway is involved in freeze-thaw tolerance. This may indicate that a significant change in the concentration of intracellular salt concentration occurs during slow freezing.

Cold shock pretreatment did not induce freeze-thaw tolerance of yeast. A temperature downshift induces a cold shock response in bacteria, and cold-shock-induced freeze-thaw tolerance in bacteria is dependent on the synthesis of cold shock proteins (17). Yeast is known to respond to low temperature, specifically 10°C, by turning on *TIP1*, *TIR1*, and *TIR2*, which encode cold shock proteins (17, 20). Disruption of *TIP1* showed that this protein is not related to the freeze-thaw stress response (20); the roles of other cold shock proteins have not yet been clarified. From our results, this cold shock response is not related to freeze-thaw stress resistance in yeast.

While it is clear that yeast cells cannot adapt to freeze-thaw stress following a cycle of freezing and thawing, the ability of cells to survive damage depends strongly on the physiological state of the cells prior to freezing. The marked dependence of freeze-thaw tolerance on the phase of growth in a batch culture reflects the fundamental physiological changes in cells that occur during these phases. When starved cells are placed in a good nutritional environment, many changes occur; these include the degradation of storage carbohydrates, including trehalose and glycogen. On the other hand, when cells enter stationary phase, they accumulate glycogen and trehalose, develop thick cell walls, and become thermotolerant and more resistant to H_2O_2, superoxide-generating agents, and mutagens (23, 33). The observation that starved cells lost freeze-thaw stress resistance during the return to vegetative growth and gained it as they returned to stationary phase is consistent with the acquisition of resistance to other forms of stress. Our data showed that resistance to freeze-thaw damage increased as cells entered lag phase regardless of whether they were previously growing in a fermentative or respiratory mode. This indicates that the cells are responding to some starvation signaling system common to these different nutritional states.

The results with mutants defective in components of the *RAS*-cAMP signal transduction pathway clearly showed that this system is needed to provide resistance to freeze-thaw damage. This finding is consistent with the postulated role of the *RAS*-cAMP signaling pathway as a mediator of a pleiotropic response to nutrient starvation (3). Sudden depletion of carbon or nitrogen resulted in increased freeze-thaw tolerance, whereas prolonged starvation in stationary phase resulted in a gradual loss of tolerance. These results are consistent with the known mode of operation of *RAS*-cAMP control (2, 32).

There has been much speculation about the nature of cellular damage during freezing and thawing. Mazur (25) has highlighted that more than one factor can cause damage, and these include whether ice forms intracellularly (at high freezing rates) or extracellularly (at low freezing rates) and the effect on solute concentrations inside cells as ice forms. Since freezing rates can extensively affect viability loss in cells, it seems likely that much of the damage to cells occurs during freezing rather than thawing. We observed that loss of viability of the cells in a population was an exponential function of the duration of freezing at least over the first 6 h. This implies that there is an ongoing process affecting cellular integrity that continues well beyond the time taken for the supporting medium to freeze and reach the external temperature. This also shows that damage is caused largely during freezing, since exponential-phase cells underwent extensive viability loss for several hours after the extracellular medium was frozen. Moreover, ice nucleation is a prerequisite for this viability decrease, since under conditions in which supercooling of the medium to −20°C occurred, the cells showed high survival, in agreement with results found for plant cells (11). The ongoing viability loss is therefore due not to the subzero temperature but to the external freezing, which subsequently leads to dehydration or ice formation inside the cell. It has previously been shown that external freezing precedes internal freezing (30).

Here we have shown that the ability of yeast cells to survive freezing and thawing injury is dependent on their physiological state prior to freezing, and we have identified that the *RAS*-cAMP signal transduction pathway is important in determining the extent to which cells survive this type of damage. Our data also indicate that the cellular response to freezing can be modulated by a range of other stress signals. This indicates that freeze-thaw stress may result from a combination of several subsets of other forms of damage, including oxidative and osmotic stress. This work has laid a foundation for examination of these possibilities and indicates that analyses of yeast mutants specifically affected in signal transduction pathways or stress response systems can provide answers to these questions.

ACKNOWLEDGMENTS

We thank the Australian Research Council and the Cooperative Research Center (CRC) for Food Industry Innovation for financial support. J.-I. Park was supported by the Overseas Postgraduate Research Scholarship and the CRC for Food Industry Innovation.

We thank K. Tatchell, M. Jacquet, H. Ruis, and C. Gustin for providing us with strains and Brian Hazell for guidance in the ethanol determination.

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