Journal of Industrial Microbiology & Biotechnology (1997) 18, 30–36

Stress co-tolerance and trehalose content in baking strains of *Saccharomyces cerevisiae*

JG Lewis[1], RP Learmonth[2], PV Attfield[3] and K Watson[1]

[1]*Department of Molecular and Cellular Biology, University of New England, Armidale, NSW 2351;* [2]*Department of Biology, University of Southern Queensland, Toowoomba, QLD 4350;* [3]*Yeast Physiology Research Group, Burns Philp Technology and Research Centre, PO Box 219, North Ryde, NSW 2113, Australia*

Fourteen wild-type baking strains of *Saccharomyces cerevisiae* were grown in batch culture to true stationary phase (exogenous carbon source exhausted) and tested for their trehalose content and their tolerance to heat (52°C for 4.5 min), ethanol (20% v/v for 30 min), H_2O_2 (0.3 M for 60 min), rapid freezing (–196°C for 20 min, cooling rate 200°C min^{-1}), slow freezing (–20°C for 24 h, cooling rate 3°C min^{-1}), salt (growth in 1.5 M NaCl agar) or acetic acid (growth in 0.4% w/v acetic acid agar) stresses. Stress tolerance among the strains was highly variable and up to 1000-fold differences existed between strains for some types of stress. Compared with previously published reports, all strains were tolerant to H_2O_2 stress. Correlation analysis of stress tolerance results demonstrated relationships between tolerance to H_2O_2 and tolerance to all stresses except ethanol. This may imply that oxidative processes are associated with a wide variety of cellular stresses and also indicate that the general robustness associated with industrial yeast may be a result of their oxidative stress tolerance. In addition, H_2O_2 tolerance might be a suitable marker for the general assessment of stress tolerance in yeast strains. Trehalose content failed to correlate with tolerance to any stress except acetic acid. This may indicate that the contribution of trehalose to tolerance to other stresses is either small or inconsistent and that trehalose may not be used as a general predictor of stress tolerance in true stationary phase yeast.

Keywords: *Saccharomyces cerevisiae*; stress tolerance; baking yeast; trehalose; oxidative stress

Introduction

Stress co-tolerance (cross-tolerance) is the phenomenon of tolerance to one type of stress tending to be associated with tolerance of another. For example, in the yeast *Saccharomyces cerevisiae*, relationships have been noted between tolerance to heat and osmotic stress [40], freezing and dehydration stress [10], and freezing and alcohol stress [30]. Experiments examining the heat-shock response have provided other examples of stress co-tolerance, where cells subjected to a mild heat-shock have not only increased thermotolerance but also increased tolerance to ethanol [45], freezing [23], H_2O_2 [7,38] and other stresses [44]. These apparent relationships between stresses may imply that there is some mechanistic similarity between them, either in the type of injuries caused by the stresses, mechanisms for protecting the cell from them, or mechanisms for repairing damage that is sustained.

One factor which has been implicated in stress co-tolerance is the cellular accumulation of the disaccharide, trehalose. High intracellular concentration of trehalose in yeast has been linked with tolerance to a range of stresses including heat, dehydration, high osmotic pressure and freezing [42,46]. This may imply that cells with high trehalose levels will be tolerant to a variety of stresses and demonstrate stress co-tolerance. However, like many single physiological traits which have been associated with stress tolerance, the relationship is not absolute and exceptions to the general trend have been noted [18,31,36,47]. In addition, many studies which have linked trehalose with stress tolerance have examined trehalose levels in only a few strains of yeast which may limit the general applicability of the results [20,21,32].

In the present study we have examined stress co-tolerance and the contribution of trehalose to tolerance of heat, ethanol, H_2O_2, rapid freezing, slow freezing, salt and acetic acid stress in 14 strains of baker's yeast, *S. cerevisiae*. Cells were grown in batch culture to stationary phase, defined as exhaustion of ethanol [25]. Correlation analysis of stress tolerance between strains showed several strong instances of stress co-tolerance, in particular the association of tolerance to H_2O_2 with many other types of stress. In contrast, correlation of stress tolerance with trehalose content of the strains was generally poor, implying that the contribution of trehalose to stress tolerance may not be as general as has been previously suggested.

Materials and methods

Yeast strains and culture conditions

Yeast used were baking strains of *Saccharomyces cerevisiae*, designated A1 to A12, A16 and A17. Genetically diverse strains of *Saccharomyces cerevisiae* used in this study were selected from the Burns Philp culture collection. They were originally isolated from various samples of dried or compressed commercial baker's yeast. All strains were prototrophic polyploids (exact ploidy not known). Molecular analysis of total DNA by Southern hybridization using a Ty1 probe was used to confirm genetic diversity (Burns Philp Technology and Research Centre). Starter cultures

Correspondence: K Watson, Department of Molecular and Cellular Biology, University of New England, Armidale, NSW 2351, Australia
Received 10 October 1995; accepted 10 September 1996

(50 ml of YEP broth containing 0.5% w/v yeast extract, 0.5% bacteriological peptone, 1% glucose, 0.3% $(NH_4)_2SO_4$ and 0.3% KH_2PO_4 in 250-ml Erlenmeyer flasks) were inoculated from a slope and incubated overnight at 25°C and 180 rpm. Cultures at this stage were in respiratory growth phase and were used to inoculate, in triplicate, 2-L Erlenmeyer flasks containing 600 ml of YEP broth as experimental cultures. Growth was monitored by assay of glucose and ethanol concentration in the culture [25]. Cultures were tested for stress tolerance and trehalose content 6–10 h after entry into stationary phase, defined as exhaustion of ethanol [25].

Stress tolerance

Cells were harvested by centrifugation ($1500 \times g$ per 2 min) and resuspended in the original volume of YEP broth without glucose (YEPNG) in order to make the stressing medium consistent for all strains. Final cell density for all the cultures was between 1×10^7 and 5×10^7 CFU ml^{-1}. Stressing protocols were as follows: (A) *Heat stress*. A 4.1-ml sample of cells was transferred to a 22-mm Pyrex test tube and heated with shaking in a 60°C water-bath to 52°C (approximately 30 s). The tube was then transferred to a 52°C shaking water-bath and incubated for 4.5 min before being cooled in ice-water to 25°C [25]. (B) *Ethanol stress*. A 1-ml sample of cells was added to 0.25 ml of ethanol in a 1.5-ml microcentrifuge tube, to give a final concentration of 20% v/v ethanol. The tube was mixed and incubated at 25°C for 30 min, after which the stress was alleviated by a ten-fold dilution in YEPNG. (C) *Hydrogen peroxide stress*. A 0.1-ml sample of cells was added to 0.9 ml of H_2O_2 in distilled water to give a final concentration of 0.3 M H_2O_2. The tube was mixed and incubated at 25°C. After 60 min the stress was halted by the addition of 10 μl of 2 mg (28 000 units) ml^{-1} catalase solution. (D) *Rapid freezing*. A 1-ml sample of cells in a 1.5-ml microcentrifuge tube was plunged into liquid nitrogen for 20 min (cooling rate approximately 200°C min^{-1}) before being thawed in a 25°C water-bath for 4 min (thawing rate approximately 190°C min^{-1}) [25]. (E) *Slow freezing*. A 1-ml sample of cells in a microfuge tube was exposed to two cycles of freezing at −20°C (cooling rate approximately 3°C min^{-1}) for 20 and 4 h respectively, and thawing at 25°C (thawing rate approximately 190°C min^{-1}). Two freezing cycles were necessary to differentiate between sensitive and resistant strains due to the high resistance of all cultures to this relatively mild stress. (F) *Salt stress*. Pour plates of 0.1-ml samples, appropriately diluted in YEPNG, were made in YEP agar (YEP broth solidified with 1% agar) containing 1.5 M NaCl [40]. (G) *Acetic acid stress*. Pour plates of 0.1-ml samples, appropriately diluted in YEPNG, were made in YEP agar containing 0.4% v/v acetic acid. Final pH of the medium was 3.9, where approximately 85% of the acid was in the undissociated form [35]. For heat, ethanol, H_2O_2, fast freezing and slow freezing stress, post-stress viability was assessed by diluting cells in YEPNG and plating them onto YEP agar plates in quadruplicate. Plates were incubated at 28°C and counted after 2–3 days. Salt plates were counted after 2 weeks and acetic acid plates after 3–4 days. Stress tolerance was taken as the percentage of colony forming units after the stress compared with an unstressed control for each culture of each strain.

Trehalose extraction and assay

Culture samples of 150 ml were centrifuged ($1500 \times g$ per 2 min), the medium decanted and the cells put on ice. Cells were subsequently washed twice with ice-cold distilled water and frozen at −70°C until assayed. Trehalose was extracted from chilled and washed cells with cold 0.5 M trichloroacetic acid and estimated by the anthrone method as previously described [24]. Samples for dry weight analysis were washed with distilled water and dried at 95°C for 24 h.

Statistical analysis

For statistical analysis, all stress tolerance data were standardised to allow comparison by expressing the viable counts of stressed cultures as a percentage of the viable counts of the corresponding unstressed control. Trehalose data were expressed as a percentage of dry cell weight. Means and standard errors were calculated for the three replicates of each strain and statistical analysis performed using the statistical software package Minitab (Release 7, Minitab Inc, State College, PA, USA). Normality of the distribution of the data was assessed by plotting data frequency histograms and normal probability plots [29,37]. Data for H_2O_2 stress tolerance, salt stress tolerance and trehalose content tended to a normal distribution and did not require transformation before analysis. Heat, ethanol, rapid freezing, slow freezing and acetic acid stress tolerance data tended to be skewed towards high measurements (ie relatively few high results having a large influence on the analysis) and required transformation to improve normality [37]. Heat, rapid freezing, slow freezing and acetic acid stress tolerance data were natural-log transformed and ethanol data were square-root transformed. Correlation coefficients were calculated for pairs of stresses and between trehalose content and each stress. Significance levels for each correlation were assigned on the basis of analysis of variance [37].

Results

Stress tolerance of the strains varied widely, especially for ethanol, rapid freezing and acetic acid stress, where up to 1000-fold differences existed between the least and most resistant strains (Table 1). Of all the stresses, rapid freezing appeared to be the most severe, with no strain having more than 1% of the population resistant. In contrast, slow freezing tolerance was high, ranging from 8.90% (strain A7) to 54.5% (A11) of the population. H_2O_2 stress also appeared very moderate, and resistance of the strains ranged from 14% (A2 and A3) to 90% (A12) of the population. Other stress protocols resulted in a wide variation between the most and least resistant strains. Heat stress tolerance of the strains ranged from 0.57% (A2) to 30.7% (A7) of the population, ethanol from 0.049% (A2) to 65.1% (A12), salt stress from 9.42% (A4) to 87.0% (A16) and acetic acid stress from 0.014% (A2) to 28.3% (A12) of the population.

No single strain was the most resistant to all stresses. A12 appeared to be the most generally stress-tolerant strain

Table 1 Stress tolerance of yeast strains

Strain	Mean % survivors after stress ± s.e.m.							Trehalose content[a]
	Heat	Ethanol	H_2O_2	Rapid freezing	Slow freezing	Salt	Acetic acid	
A1	2.62 ± 0.73	0.30 ± 0.09	68.1 ± 7.80	0.14 ± 0.01	31.9 ± 2.64	51.1 ± 2.86	1.79 ± 0.95	10.9 ± 0.71
A2	0.57 ± 0.24	0.049 ± 0.022	13.7 ± 2.50	0.0032 ± 0.0005	17.8 ± 1.33	15.7 ± 3.40	0.014 ± 0.002	3.55 ± 0.25
A3	1.73 ± 1.51	0.67 ± 0.31	14.1 ± 6.18	0.0086 ± 0.0079	13.0 ± 1.70	25.0 ± 4.49	0.11 ± 0.02	7.22 ± 0.30
A4	3.12 ± 0.77	11.9 ± 6.82	77.8 ± 2.86	0.14 ± 0.02	13.7 ± 1.37	9.42 ± 0.51	0.37 ± 0.03	4.92 ± 0.57
A5	3.21 ± 0.09	45.9 ± 3.88	59.2 ± 4.88	0.45 ± 0.10	19.3 ± 3.16	40.0 ± 3.35	7.28 ± 3.26	8.62 ± 1.64
A6	1.18 ± 0.17	5.01 ± 1.43	72.9 ± 6.06	0.13 ± 0.04	27.9 ± 4.52	26.6 ± 2.09	23.0 ± 1.70	10.3 ± 1.22
A7	30.7 ± 7.01	28.9 ± 4.31	43.9 ± 7.73	0.62 ± 0.16	8.90 ± 0.38	53.4 ± 10.3	5.16 ± 1.59	6.11 ± 0.27
A8	0.69 ± 0.12	14.7 ± 6.73	36.7 ± 0.70	0.024 ± 0.005	16.8 ± 2.81	48.2 ± 3.19	0.61 ± 0.13	7.49 ± 0.30
A9	27.6 ± 5.27	12.3 ± 10.9	87.9 ± 5.19	0.27 ± 0.01	45.9 ± 4.31	78.1 ± 0.98	0.19 ± 0.08	7.20 ± 0.17
A10	8.67 ± 0.93	2.82 ± 0.54	82.8 ± 6.56	0.063 ± 0.011	37.7 ± 2.45	52.8 ± 3.18	19.7 ± 5.81	10.5 ± 0.40
A11	14.8 ± 0.73	29.3 ± 27.0	85.0 ± 6.68	0.43 ± 0.11	54.5 ± 12.8	58.4 ± 3.48	25.1 ± 7.28	6.29 ± 0.78
A12	4.25 ± 2.11	65.1 ± 1.80	90.7 ± 3.43	0.096 ± 0.021	50.9 ± 10.1	60.4 ± 11.0	28.3 ± 2.37	8.50 ± 0.29
A16	7.02 ± 2.89	17.6 ± 8.67	83.8 ± 5.59	0.81 ± 0.03	35.9 ± 1.76	87.0 ± 3.84	14.1 ± 0.82	11.4 ± 0.50
A17	3.27 ± 0.34	17.8 ± 2.03	49.9 ± 4.37	0.33 ± 0.02	26.5 ± 2.39	17.5 ± 0.62	0.26 ± 0.07	6.29 ± 0.31

[a]Mean % dry weight ± s.e.m.

and demonstrated the maximum recorded tolerance to ethanol, H_2O_2 and acetic acid stress. This strain was also reasonably tolerant of other stresses, apart from rapid freezing. Strain A16 demonstrated maximum tolerance to rapid freezing and salt stress, while strain A11 had the highest resistance to slow freezing. Maximum heat tolerance was found for strain A7, which in contrast was also the strain most sensitive to slow freezing. Strain A2 was clearly the most stress-sensitive strain tested, and was the strain most sensitive to heat, ethanol, H_2O_2, rapid freezing and acetic acid stress. Strain A4 was most sensitive to salt stress.

Trehalose content also varied between strains, but over a much smaller range (Table 1). Levels of trehalose varied from 3.55% w/w (strain A2) to 11.4% (A16) and are comparable to those reported in the literature for baking yeast, although there is considerable variation depending on yeast strain and growth conditions [1,16].

Correlation analysis was performed on the stress-tolerance data to investigate whether tolerance of one type of stress was related to tolerance of another. Of the 21 possible correlations between each of the stresses, four positive correlations were found at the 1% level of significance and an additional seven at the 5% level (Table 2). The strongest correlations were between rapid freezing and heat tolerance ($r = 0.701$, $P = 0.005$), H_2O_2 and rapid freezing tolerance ($r = 0.673$, $P = 0.008$), H_2O_2 and slow freezing tolerance ($r = 0.721$, $P = 0.004$), and H_2O_2 and acetic acid tolerance ($r = 0.684$, $P = 0.007$). Scatter plots of the data were used to confirm that the correlations represented true relationships. Figure 1 presents examples of data pairs producing high (Figure 1a,b) and low (Figure 1c,d) correlations.

The scatter plot for heat and slow freezing showed one data point which appeared to be a clear outlier and strongly decreased an otherwise clear correlation (results not shown). The data were from strain A7 and all three replicates produced equivalent results. Removal of this point from the analysis increased the correlation to $r = 0.710$, $P = 0.007$. In relation to these two stresses, strain A7 appears to be substantially different from the other strains in this analysis.

Table 2 shows that, at the 5% level of significance, tolerance to H_2O_2 also correlated with tolerance to heat ($r = 0.553$, $P = 0.040$) and salt stress ($r = 0.554$, $P = 0.040$), leaving ethanol as the only stress with no significant association with H_2O_2 tolerance ($r = 0.431$, $P = 0.124$). Other significant correlations at the 5% level of significance were between rapid freezing and acetic acid tolerance ($r = 0.611$, $P = 0.020$), rapid freezing and ethanol tolerance ($r = 0.605$, $P = 0.022$), ethanol and acetic acid tolerance ($r = 0.533$, $P = 0.050$), salt and slow freezing tolerance ($r = 0.545$, $P = 0.044$), and salt and heat tolerance ($r = 0.619$, $P = 0.018$).

When the scatter plots were examined, all correlations found by the statistical analysis appeared reasonable, although in correlating such a large number of datasets there is a chance that some of the correlations will be the result of random scatter and not be representative of a true underlying relationship. This is especially true of those relationships with low correlation coefficients. Consequently, correlations at the 5% level of significance with r values ranging as low as 0.533 (ethanol and acetic acid stress tolerance) must be treated only as tentative relationships.

Correlation analysis of trehalose and stress tolerance data is presented in Table 3. Generally, correlations were very low and the only significant correlation was between trehalose content and acetic acid tolerance ($r = 0.654$, $P = 0.011$). Examination of scatter plots for these datasets confirmed that the relationship between trehalose and tolerance of each stress was poor (plots not shown).

Discussion

Stress co-tolerance

Tolerance to H_2O_2 stands out from the results as being important in two ways. First, all the strains tested demonstrated exceptionally high levels of tolerance towards H_2O_2 compared to previously published reports. Second, tolerance to all stresses except ethanol demonstrated a significant, positive correlation with tolerance to H_2O_2 (Table 2).

Table 2 Correlation analysis of stress tolerance data (n = 14). Data represent the correlation coefficient (r). Probability (P) was assigned on the basis of analysis of variance

	Heat	Ethanol	H_2O_2	Rapid freezing	Slow freezing	Salt
Ethanol	0.414 (P = 0.141)					
H_2O_2	0.553 (P = 0.040)	0.431 (P = 0.124)				
Rapid freezing	0.701 (P = 0.005)	0.605 (P = 0.022)	0.673 (P = 0.008)			
Slow freezing	0.271[a] (P = 0.783)	0.192 (P = 0.517)	0.721 (P = 0.004)	0.275 (P = 0.342)		
Salt	0.619 (P = 0.018)	0.350 (P = 0.220)	0.554 (P = 0.040)	0.490 (P = 0.075)	0.545 (P = 0.044)	
Acetic acid	0.394 (P = 0.164)	0.533 (P = 0.050)	0.684 (P = 0.007)	0.611 (P = 0.020)	0.435 (P = 0.120)	0.495 (P = 0.072)

[a]Excluding data from strain A7, r = 0.710, P = 0.007, n = 13.

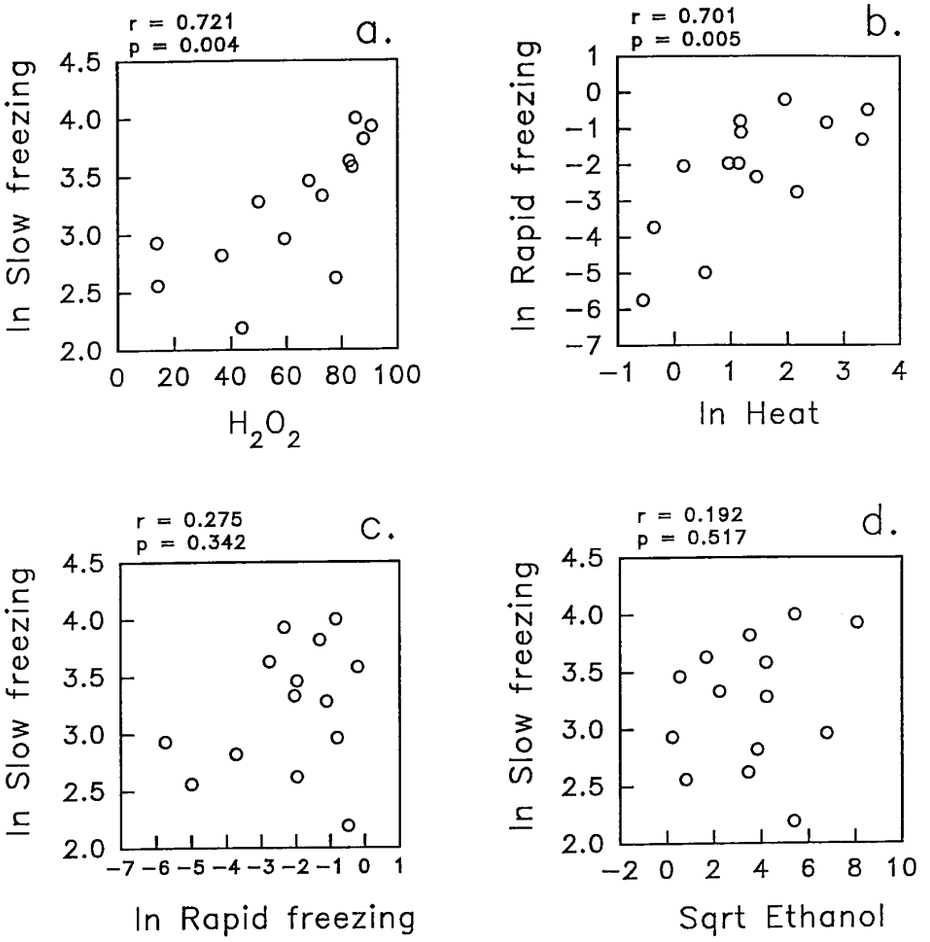

Figure 1 Scatter plots of the two highest (a, b) and two lowest (c, d) correlations of stress tolerance data. Correlation coefficients (r) and probability (P) values are indicated for each plot. Where indicated, data were transformed to improve normality as described in Materials and Methods. Data for tolerance to individual stresses are presented in Table 1.

These results suggest that oxidative processes are important in a wide variety of stress conditions.

The tolerance to H_2O_2 demonstrated by all strains used in this work was much greater than that previously reported, despite the high concentration of H_2O_2 used (300 mM for 60 min). This was far higher than those previously used in experiments with *S. cerevisiae*, which have been in the order of 0.1–10 mM [7,22] or up to 50 mM in

Table 3 Correlation analysis of stress tolerance and trehalose data (n = 14). Data represent the correlation coefficient (r). Probability (P) was assigned on the basis of analysis of variance

Stress	r
Heat	0.089 (P = 0.762)
Ethanol	–0.006 (P = 0.983)
H_2O_2	0.486 (P = 0.078)
Rapid freezing	0.338 (P = 0.237)
Slow freezing	0.430 (P = 0.125)
Salt	0.528 (P = 0.052)
Acetic acid	0.654 (P = 0.011)

our laboratory where other yeast strains were examined [38]. For example, levels of tolerance for stationary phase cells were reported by Jamieson [22] as up to 80% survivors, while in our studies cells had 80–90% survival after exposure to 60 times the highest concentration of H_2O_2 used by Jamieson. The reason for this high level of tolerance in the strains used in the present study is not clear, although it seems possible that the ability to tolerate oxidative stress may be necessary for the general stress tolerance and robustness associated with industrial yeast strains. The large number of stresses which demonstrated a relationship with tolerance to H_2O_2 stress may also indicate this. Tolerance to every stress tested, with the exception of ethanol, was correlated with tolerance to H_2O_2 and, for rapid freezing, slow freezing and acetic acid stress, these relationships were strong and statistically highly significant ($P \leq 0.01$). This result implies that injuries, or protection or repair mechanisms associated with tolerance to H_2O_2 stress are also associated with tolerance to other stresses. Either some of the cellular injuries inflicted by these stresses are mediated by oxidative processes, or mechanisms that prevent oxidative damage are also able to protect cells from a variety of other types of injury inflicted by the stresses, or there is commonality of induction of oxidative and other repair processes.

A role for oxidative processes in stress-induced injury has been previously suggested for stresses including freezing [3,39], storage at low water activity [28], acetic acid [28], dehydration [5], ethanol [8] and heat [4,7,28,38]. The mechanistic connection between oxidative processes and stress has had a number of explanations, including the suggestion that stresses may induce increased levels of free radicals within the cell [7,38]. Whatever the mechanistic explanation, there is much evidence which suggests a link between oxidative processes and cellular damage associated with a variety of types of stress. The association between H_2O_2 and other stress tolerance found in this work adds substantially to this evidence.

The other notable set of correlations is the number of stresses related to heat stress. As well as H_2O_2 tolerance, heat tolerance is related to salt tolerance (r = 0.619, P = 0.018), slow freezing (r = 0.710, P = 0.007) and rapid freezing (r = 0.701, P = 0.005). It is possible that tolerance to heat, salt and slow freezing may be connected through the oxidative processes which we have suggested may link H_2O_2 tolerance with each of these stresses. However, other links are also likely between these stresses, including the protective power of accumulated intracellular solutes, such as trehalose, which protect against these types of stress [6,11] and the possible role of heat-shock proteins (hsps) in amelioration of cellular injury induced by these stresses [44]. However, salt and slow freezing stress are also correlated with each other (r = 0.545, P = 0.044). This adds support to the first of these two explanations as the two stresses have previously been suggested to be related on the basis of mechanistic similarities and the ability for both to be relieved by the addition or accumulation of protective solutes [6,11].

The other correlation with heat is rapid freezing, and the relationship is reasonably strong (r = 0.701, P = 0.005). Superficially, there seems little to link these two stresses, as heating at 52°C and freezing at −196°C appear to be fundamentally different processes. However, heat-shock may induce tolerance not only to heat, but also to rapid freezing [23] and it was concluded that hsps were vital for this to occur. Results from our laboratory tend to support the view that expression of hsps is necessary for the induction of cryotolerance [26]. This suggests that some aspects of stress-induced injury may be the same for both heat and rapid freezing and the apparent influence of hsps may imply that the link is through protein denaturation.

Trehalose

In the literature there is strong evidence of a relationship between trehalose content and stress tolerance in *S. cerevisiae*. Trehalose has been associated with tolerance to heat [1,13–15,31], dehydration [9,17,19,32], hyperosmotic stress [12,27], freezing stress [1,9,12,18] and cold storage [12,36]. However, in the work presented here, correlation of trehalose content with stress tolerance was generally very poor.

Thermotolerance is the most widely reported association with trehalose, yet in this work the correlation of trehalose content with heat stress resistance is essentially random (r = 0.089, P = 0.762; Table 3). For example, strain A8 showed the second lowest heat tolerance (0.69% survivors) and a trehalose content of 7.5% (Table 1), while the most heat-tolerant strain, A7 (30.7% survivors), had 6.1% trehalose (Table 1). Tolerance to freezing has also been widely reported as being associated with trehalose content, but in this present work correlations were low, although somewhat higher than for heat (slow freezing, r = 0.430, P = 0.125; rapid freezing, r = 0.338, P = 0.237; Table 3). For osmotolerance, the other previously-reported association with trehalose among the stresses tested here, a higher correlation was found with a probability just outside the 5% level of significance (r = 0.528, P = 0.052; Table 3). These results imply that the influence of trehalose on stress tolerance under the experimental conditions used in this study is generally weak, and in some cases is non-existent, a finding which appears to contradict previously published work.

Most studies which have related trehalose content to stress tolerance have been carried out by manipulating trehalose levels within one or two strains of yeast [19,32] or by examining the effect of exogenous trehalose on cell tolerance [9,17]. Under these circumstances it may be possible to find clear relationships between trehalose content and stress tolerance, and some workers have suggested an almost linear relationship across different treatments and

even different strains [13,20]. However, using similar methods, a significant proportion of papers, while still suggesting a relationship between trehalose and stress tolerance, have also noted inconsistencies between trehalose level and stress tolerance [18,31,36,47]. For example, Gelinas *et al* [18] found that there was a relationship between trehalose level and freezing tolerance under some growth conditions but not others, and Slaughter and Nomura [36] found a relationship between trehalose content and yeast viability during storage, but concluded that trehalose content had no general predictive power between one set of storage conditions and another.

This lack of a clear relationship is supported by evidence from a recent detailed study which examined the trehalose content, and heat- and freezing-tolerance of 12 closely related strains of *S. cerevisiae* [2]. Results demonstrated some inconsistency between trehalose content and heat- and freezing stress tolerance, and supported the concept that trehalose content and stress tolerance are not strictly related in a linear manner. In the present study, we have employed *S. cerevisiae* strains related only by their use as commercial baking yeast and shown by molecular analysis to be genetically diverse. Thus their physiological differences are likely to be significant. This is reflected in the large variation observed in the stress tolerance of the strains (Table 1). Under these circumstances, only physiological or genetic characteristics which are both strong and consistent within the species *S. cerevisiae* are liable to be detected by the correlation analysis. In the case of trehalose, results presented here suggest that its contribution to tolerance appears to be too small or too inconsistent for it to be used as a marker of general stress tolerance in unrelated strains. This does not imply that trehalose does not substantially affect heat and freezing tolerance, but that its effects may be outweighed by the combined effects of all other factors which may contribute to tolerance.

The lack of strong correlation of trehalose with heat and freezing found in this study may also explain some of the variation in the reported influence of trehalose and hsps on acquired stress tolerance after heat-shock. Evidence has been presented to support the dominance of hsps over trehalose [33,34,47] and *vice versa* [13,20,21]. One possible explanation for these conflicting results may be that the effect is strain-dependent, and for some strains hsps are of paramount importance in stress tolerance, while trehalose may be the greatest influence for others. Furthermore, the relative importance of trehalose and other factors may vary over the distinct phases of growth in batch culture. Recent work employing isogenic strains with varied capacity for trehalose accumulation, indicates that cellular trehalose may be significant as a thermoprotectant in yeast during early respiratory phase in batch culture, but that in later stages of growth (approaching true stationary phase), the disaccharide appears to be less important and a number of other factors make the dominant contribution to stress tolerance [2]. Thus, in this present study, where cells were analysed in stationary phase, the effects of trehalose could be obscured.

Another explanation for the lack of correlation between trehalose and stress tolerance in this work may be the concept of a threshold value of trehalose for stress tolerance. This concept has been previously suggested [17] and supported by recent studies which showed that trehalose content and stress tolerance were not related in a linear manner but that a threshold level of trehalose appeared to be necessary for relatively high stress tolerance for both heat and freezing stress [2]. Those strains which accumulated less than approximately 4% w/w trehalose invariably had poor stress tolerance compared with those which accumulated 5% (w/w) or greater. However, above 5% (w/w) the correlation relationship between trehalose content and heat or freezing tolerance was apparently random [2]. In results presented here virtually all strains accumulated 5% (w/w) trehalose or greater and the only strain which accumulated less than 4% (w/w) trehalose was very stress-sensitive. Strain A2 accumulated 3.6% (w/w) trehalose and was the most sensitive strain to heat, ethanol, H_2O_2, rapid freezing and acetic acid stress and had low levels of resistance to slow freezing and salt stress. These results are consistent with the concept that the threshold level of trehalose required for stress tolerance is around 5%, and above this level no direct benefit accrues to the cell by accumulating more trehalose.

The only significant correlation of stress resistance with trehalose content was that with acetic acid ($r = 0.654$, $P = 0.011$; Table 3) which was unexpected and to our knowledge has not been previously suggested. It is possible that accumulated trehalose acts as an energy source for the cell during the early stages of exposure to acetic acid where proton extrusion is necessary to restore internal pH balance and requires rapid energy availability [43]. A high intracellular trehalose content may act as an energy buffer, allowing cells time to respond to the drop in intracellular pH and adapt to the stress before irreversible cellular injury is induced. This explanation may be supported by the observation that when internal acidification of cells is induced by exposure to acetate, trehalase is activated [41].

In summary, results presented here suggest that H_2O_2 tolerance may have application as a marker of stress tolerance in baking strains of *S. cerevisiae*, with the exception of salt stress. This may indicate an underlying widespread role of oxidative processes in many types of environmental stress. In contrast, trehalose content of cells had poor predictive power for tolerance to stresses other than acetic acid, implying that its influence on tolerance to other stresses is weak or inconsistent in true stationary phase cells.

Acknowledgements

This work was supported by an Australian Postgraduate Research Award – Industry (JGL) and University of New England internal research grants. We are grateful to Dr Ian Davies for assistance with statistical analysis. We also thank the Management of Burns Philp Research and Development Pty Ltd for their kind permission to publish this work.

References

1 Attfield PV, A Raman and CJ Northcott. 1992. Construction of *Saccharomyces cerevisiae* strains that accumulate relatively low concentrations of trehalose, and their application in testing the contribution

of the disaccharide to stress tolerance. FEMS Microbiol Lett 94: 271–276.
2 Attfield PV, S Kletsas and BW Hazell. 1994. Concomitant appearance of intrinsic thermotolerance and storage of trehalose in *Saccharomyces cerevisiae* during early respiratory phase of batch culture is *CIF1*-dependent. Microbiology 140: 2625–2632.
3 Baird-Parker A and E Davenport. 1965. The effect of recovery medium on the isolation of *Staphylococcus aureus* after heat-treatment and after storage of frozen or dried cells. J Appl Bacteriol 28: 390–397.
4 Begonia GB and ML Salin. 1991. Elevation of superoxide dismutase in *Halobacterium halobium* by heat shock. J Bacteriol 173: 5582–5584.
5 Beudeker RF, HW Van Dam, JB Van Der Plaat and K Vellenga. 1990. Developments in baker's yeast production. In: Yeast: Biotechnology and Biocatalysis (Verachtert H and R De Mot, eds), pp 103–146, Marcel Dekker, New York.
6 Brown AD. 1990. Microbial Water Stress Physiology. Wiley, Chichester.
7 Collinson LP and IW Dawes 1992. Inducibility of the response of yeast cells to peroxide stress. J Gen Microbiol 138: 329–335.
8 Costa V, E Reis, A Quintanilha and P Moradas-Ferreira. 1993. Acquisition of ethanol tolerance in *Saccharomyces cerevisiae*: the key role of mitochondrial superoxide dismutase. Arch Biochem Biophys 300: 608–614.
9 Coutinho C, E Bernardes, D Felix and AD Panek. 1988. Trehalose as a cryoprotectant for preservation of yeast strains. J Biotechnol 7: 23–32.
10 Crowe JH, JF Carpenter, LM Crowe and TJ Anchordoguy. 1990. Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. Cryobiology 27: 219–231.
11 Crowe JH, FA Hoekstra and LM Crowe. 1992. Anhydrobiosis. Ann Rev Physiol 54: 579–599.
12 D'Amore T, R Crumplen and GG Stewart. 1991. The involvement of trehalose in yeast stress tolerance. J Ind Microbiol 7: 191–196.
13 De Virgilio C, U Simmen, T Hottiger, T Boller and A Wiemken. 1990. Heat shock induces enzymes of trehalose metabolism, trehalose accumulation, and thermotolerance in *Schizosaccharomyces pombe*, even in the presence of cycloheximide. FEBS Lett 273: 107–110.
14 De Virgilio C, T Hottiger, J Dominguez, T Boller and A Wiemken. 1994. The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. Eur J Biochem 219: 179–186.
15 Eleutherio ECA, PS Araujo and AD Panek. 1993. Protective role of trehalose during heat stress in *Saccharomyces cerevisiae*. Cryobiology 30: 591–596.
16 El Mokadem MT, MI Mahmoud and ZH Kairalla. 1986. The effect of growth medium on the levels of trehalose and glycogen content of some yeasts. Egypt J Microbiol 21: 103–109.
17 Gadd GM, K Chalmers and RH Reed. 1987. The role of trehalose in dehydration resistance of *Saccharomyces cerevisiae*. FEMS Microbiol Lett 48: 249–254.
18 Gelinas P, G Fiset, A LeDuy and J Goulet. 1989. Effect of growth conditions and trehalose content on cryotolerance of bakers' yeast in frozen doughs. Appl Environ Microbiol 55: 2453–2459.
19 Hottiger T, T Boller and A Wiemken. 1987. Rapid changes of heat and desiccation tolerance with changes of trehalose content in *Saccharomyces cerevisiae* cells subjected to temperature shifts. FEBS Lett 220: 113–115.
20 Hottiger T, T Boller and A Wiemken. 1989. Correlation of trehalose content and heat resistance in yeast mutants altered in the RAS/adenylate cyclase pathway: is trehalose a thermoprotectant? FEBS Lett 255: 431–434.
21 Hottiger T, C De Virgilio, W Bell, T Boller and A Wiemken. 1992. The 70-kilodalton heat-shock proteins of the SSA subfamily negatively modulate heat-shock-induced accumulation of trehalose and promote recovery from heat stress in the yeast, *Saccharomyces cerevisiae*. Eur J Biochem 210: 125–132.
22 Jamieson DJ. 1992. *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. J Bacteriol 174: 6678–6681.
23 Komatsu Y, SC Kaul, H Iwahashi and K Obuchi. 1990. Do heat shock proteins provide protection against freezing? FEMS Microbiol Lett 72: 159–162.
24 Lewis JG, RP Learmonth and K Watson. 1993. The role of growth phase and ethanol in freeze-thaw stress resistance of *Saccharomyces cerevisiae*. Appl Environ Microbiol 59: 1065–1071.
25 Lewis JG, CJ Northcott, RP Learmonth, PV Attfield and K Watson. 1993. The need for consistent nomenclature and assessment of growth phases in diauxic cultures of *Saccharomyces cerevisiae*. J Gen Microbiol 139: 835–839.
26 Lewis JG, RP Learmonth and K Watson. 1995. Induction of heat, freezing and salt tolerance by heat and salt shock in *Saccharomyces cerevisiae*. Microbiology 141: 687–694.
27 Mackenzie KF, KK Singh and AD Brown. 1988. Water stress plating hypersensitivity of yeasts: protective role of trehalose in *Saccharomyces cerevisiae*. J Gen Microbiol 134: 1661–1666.
28 Martin SE, RS Flowers and ZJ Ordal. 1976. Catalase: its effect on microbial enumeration. Appl Environ Microbiol 32: 731–734.
29 Miller RB. 1988. Minitab Handbook for Business and Economics. PWS-Kent, Boston.
30 Nagodawithana TW and NB Trivedi. 1990. Yeast selection for baking. In: Yeast Strain Selection (Panchal CJ, ed), pp 139–184, Marcel Dekker, New York.
31 Panek AC, JJM Vania, MF Paschoalin and D Panek. 1990. Regulation of trehalose metabolism in *Saccharomyces cerevisiae* mutants during temperature shifts. Biochimie 72: 77–79.
32 Pearce DA, AH Rose and IP Wright. 1989. Trehalose and trehalase in active dried yeast. Yeast 5: S453–S457.
33 Sanchez Y and SL Lindquist. 1990. HSP104 required for induced thermotolerance. Science 248: 1112–1115.
34 Sanchez Y, J Taulien, KA Borkovich and S Lindquist. 1992. Hsp104 is required for tolerance to many forms of stress. EMBO J 11: 2357–2364.
35 Silliker JH, RP Elliot, AC Baird-Parker, FL Bryan, JHB Christian, DS Clark, JC Olson and TA Roberts. 1980. Microbial Ecology of Foods, Vol 1. Factors Affecting Life and Death of Microorganisms. Academic Press, New York.
36 Slaughter JC and T Nomura. 1992. Intracellular glycogen and trehalose contents as predictors of yeast viability. Enzyme Microb Technol 14: 64–67.
37 Sokal RR and FJ Rohlf. 1981. Biometry. WH Freeman, New York.
38 Steels EL, RP Learmonth and K Watson. 1994. Stress tolerance and membrane lipid unsaturation in *Saccharomyces cerevisiae* grown aerobically or anaerobically. Microbiology 140: 569–576.
39 Swartz HM. 1971. Effect of oxygen on freezing damage. II. Physical-chemical effects. Cryobiology 8: 255–264.
40 Trollmo C, L Andre, A Blomberg and L Adler. 1988. Physiological overlap between osmotolerance and thermotolerance in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 56: 321–326.
41 Valle E, L Bergillos, S Gascon, F Parra and S Ramos. 1986. Trehalose activation in yeasts is mediated by internal acidification. Eur J Biochem 154: 247–251.
42 Van Laere A. 1989. Trehalose, reserve and/or stress metabolite? FEMS Microbiol Rev 63: 201–210.
43 Warth AD. 1988. Effect of benzoic acid on growth yield of yeasts differing in their resistance to preservatives. Appl Environ Microbiol 54: 2091–2095.
44 Watson K. 1990. Microbial stress proteins. Adv Microb Physiol 31: 183–223.
45 Watson K and R Cavicchioli. 1983. Acquisition of ethanol tolerance in yeast cells by heat shock. Biotechnol Lett 5: 683–688.
46 Wiemken A. 1990. Trehalose in yeast, stress protectant rather than reserve carbohydrate. Antonie van Leeuwenhoek 58: 209–217.
47 Winkler K, I Kienle, M Burgert, JC Wagner and H Holzer. 1991. Metabolic regulation of the trehalose content of vegetative yeast. FEBS Lett 291: 269–272.