Weak organic acid treatment causes a trehalose accumulation in low-pH cultures of *Saccharomyces cerevisiae*, not displayed by the more preservative-resistant *Zygosaccharomyces bailii*

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**Abstract**

Weak organic acid food preservatives exert pronounced culture pH-dependent effects on both the heat-shock response and the thermostolerance of *Saccharomyces cerevisiae*. In low-pH cultures, they inhibit this stress response and cause strong induction of respiratory-deficient petites amongst the survivors of lethal heat treatment. In higher pH cultures, 25°C sorbic acid treatment causes a strong induction of thermostolerance without inducing the heat-shock response. In this study we show that trehalose, a major stress protectant, accumulates rapidly in *S. cerevisiae* exposed to sorbate at low pH. In pH 3.5 cultures, a 25°C sorbate treatment is as effective as a 39°C heat shock in inducing trehalose. This weak-acid-induced trehalose accumulation is enhanced in the *pfk1 S. cerevisiae* mutant, indicating that it arises through inhibition of glycolysis at the phosphofructokinase step. The more preservative-resistant food spoilage yeast *Zygosaccharomyces bailii* differs from *S. cerevisiae* in that: (1) its basal thermotolerance is not strongly affected by culture pH; (2) it does not display trehalose accumulation in response to 25°C sorbate treatment at low pH; and (3) there is no induction of respiratory-deficient petites during lethal heating with sorbate. This probably reflects *Z. bailii* being both petite-negative and better equipped for maintenance of homeostasis during weak-acid, pH or high-temperature stress. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

**Keywords**: *Saccharomyces cerevisiae*; *Zygosaccharomyces bailii*; Heat shock; Trehalose; Sorbic acid; Intracellular pH; Phosphofructokinase

1. **Introduction**

Inhibition of fungal and bacterial growth by weak organic acid food preservatives increases with medium acidification, being essentially proportional to the concentration of the undissociated acid. This uncharged form of the acid readily traverses the cell membrane and then dissociates in the higher pH environment of the cytosol, causing both a cytoplasmic acidification and intracellular accumulation of the...
membrane-impermeant acid anion. In Saccharomyces cerevisiae, intracellular pH (pH$_i$) declines more than 1 unit with 2–10 mM benzoate, this pH$_i$ depression causing an inhibition of 6-phospho-1-fructokinase (PFK1) that reduces glycolytic flux [1,2]. However, the same amounts of weak acid, though they inhibit respiration in the more preservative-resistant Zygosaccharomyces bailii, have little effect on sugar fermentation in the latter yeast [3].

Adaptation of yeasts to growth in the presence of weak-acid preservatives is an important cause of food spoilage [4–6]. An understanding of the effects of these acids on yeast cells should therefore assist the design of more effective food preservation strategies. Recently, we started to characterise, at the molecular level, the stress response elicited in S. cerevisiae cells by weak organic acid treatment. This hitherto-uncharacterised stress response enables cells to adapt so as to enable growth in the presence of millimolar levels of sorbate at pH 4.5 [7,8]. Part of the response is induction of the Hsp30 [7] and Pdr12 [8] plasma membrane proteins. Pdr12, an ATP-binding cassette transporter protein catalysing active acid eflux from the cell, is particularly important for acid resistance [8].

In an earlier study we found that sorbic and benzoic acids also have pronounced influences on the heat-shock response and thermotolerance of S. cerevisiae, effects that are strongly dependent on culture pH [9]. At low pH, these preservatives inhibit both the heat-shock protein (Hsp) and thermotolerance inductions during sublethal (39°C) heat shock. They also cause strong induction of respiratory-deficient petes among the survivors of lethal (50–52°C) heat treatment. However at pH values above 5, sorbate acts as a potent chemical inducer of thermotolerance in the absence of sublethal heat treatment, but no longer inhibits the capacity of cells to mount a heat-shock response [9]. This led us to investigate whether sorbate influences levels of the disaccharide trehalose. The cytoplasmic trehalose pool of yeast shows a striking correlation to several stress resistances, though this does not apply to all stresses or under all experimental conditions tested (reviewed in [10–13]). We show here that trehalose is induced by sorbate in low-pH 25°C S. cerevisiae cultures, but not in identically treated cultures of Z. bailii. Moreover the strong thermotolerance induction that accompanies 25°C sorbate treatment at higher pH (pH > 5) is not associated with an accumulation of trehalose and must occur by an alternative process.

2. Materials and methods

2.1. Strains and materials

All experiments were conducted using Z. bailii strain 563 (National Collection of Yeast Cultures (NCYC), Norwich, UK) and S. cerevisiae strains SUB62 (a leu2, trp1, ura3, his3, lys2); HD56 (a ura3, leu2, his3); pfk1 (a ura3, leu2, his3, pfk1::HIS3); and pfk2 (a ura3, leu2, his3, pfk2::HIS3). SUB62 was kindly provided by D. Finley. HD56 and its pfk1 and pfk2 mutant derivatives were the generous gift of J. Heinisch and are described in [14].

2.2. Yeast culture

Cultures were grown on YPD medium (2% (w/v) bactopeptone, 1% yeast extract, 2% glucose) that had been titrated to the stated pH with either HCl or NaOH prior to autoclaving. Flask cultures were shaken at 25°C to ensure aeration and used for experiments when in early exponential phase at 5 × 10^5–1 × 10^7 cells ml$^{-1}$. Potassium sorbate (Sigma) was added from a pH 7 1-M stock solution. To determine thermotolerance, small volumes of exponential phase cells were heated in glass tubes for 10 min at 52°C (attaining this temperature in 1–1.5 min), then rapidly diluted in 25°C water and plated on YPD plates. Colonies were counted after 3 days of growth at 30°C, thermotolerance being expressed relative to the survival of the original cells not exposed to 52°C.

2.3. Trehalose determinations

Cells were rapidly harvested, washed in ice-cold water and trehalose determined as in [15]. Protein determinations were performed using the Bio-Rad Protein Assay Kit and bovine serum albumin as standard. Each trehalose and protein determination was determined in triplicate, S.D. of these measurements being consistently < 15% of the mean.
3. Results

3.1. Effects of sorbate and culture pH on the basal and heat-induced trehalose levels of S. cerevisiae and Z. bailii

Rapid trehalose accumulation is one of the major consequences of sublethal heat shock of *S. cerevisiae*, this trehalose pool being equally rapidly mobilised in response to a subsequent temperature downshift [12,13]. Trehalose accumulation is known to contribute to the heat-induction of thermotolerance, especially with more severe heat-shock regimes [12,13]. The effects of culture pH on the trehalose induction by heat shock appear not to have been reported, even for the extensively studied *S. cerevisiae*. We therefore investigated trehalose in both *S. cerevisiae* and *Z. bailii* transferred to media of different pH either at 25°C, or at 39°C; both in the presence and in the absence of 9 mM sorbate (Fig. 1). 39°C is a temperature that strongly induces Hsps in both yeasts ([7,9] and data not shown). Although these trehalose measurements were made 80 min after sorbate addition, time course experiments (not shown) indicated that the trehalose levels of the non-heat-stressed cells in Fig. 1 had essentially stabilised within 10–20 min of sorbate addition and thereafter underwent little change.

Culture pH, in the absence of added sorbate, had little influence over the trehalose of *S. cerevisiae* cells. However it exerts a strong influence over heat-shock-induced trehalose accumulation, this being maximal when external pH approximated to physiological pH values (pH 6–7) and considerably lower at extremes of pH (Fig. 1A). This may reflect a greater capacity of the cells to respond to heat shock when heated at pH values close to physiological pH. It is noteworthy that the induction of thermotolerance with mild heat shock of *S. cerevisiae* is also maximal when cells are heated at, or close to, physiological pH values [9]. In contrast to these results with *S. cerevisiae*, culture pH had relatively little effect on the basal and heat-induced trehalose of *Z. bailii*, the heat-induced trehalose levels of *Z. bailii* only declining slightly as external pH was reduced (Fig. 1B). Possibly trehalose synthesis in the latter organism is less influenced by pH, reduction than is the trehalose synthesis of *S. cerevisiae* (see below). Alternatively, since external pH is known to influence pH, in *S. cerevisiae* [16], *Z. bailii* might be better equipped to maintain pH at extremes of external pH as compared to *S. cerevisiae*.

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Fig. 1. Influence of the medium pH, also the presence and absence of sorbate, on trehalose. *S. cerevisiae* strain SUB62 (A) and *Z. bailii* 563 (B) cells were grown to early exponential phase (5 × 10⁶ cells ml⁻¹) on pH 6.8 YPD medium at 25°C. They were then centrifuged and resuspended in YPD previously adjusted to pH 3.5, 4.5, 5.5, 6.5, 7.5 or 8.5. After incubation for 20 min at 25°C either without (open symbols) or with (closed symbols) 9 mM sorbic acid, cells were either incubated for a further 1 h at 25°C (○,●), or heat-shocked 1 h at 39°C (□,■), prior to determination of trehalose levels.
Fig. 1A reveals that it is culture pH, not the presence or absence of 9 mM sorbate, that is the major influence over heat-induced accumulation of trehalose in *S. cerevisiae*. This contrasts with the induction of Hsps, which is strongly inhibited by preservative treatment at low pH [9]. This difference probably reflects the trehalose accumulation and Hsp inductions of the *S. cerevisiae* heat-shock response being subject to different controls. In *Z. bailii*, the presence of sorbate caused a 25–30% increment to heat-induced trehalose at pH 5.5 and above, although sorbate inhibited the heat-induction of trehalose synthesis at low pH (Fig. 1B).

Fig. 1A reveals that sorbic acid actually induces trehalose in low-pH *S. cerevisiae* cultures in the absence of heat shock, an induction not observed with identically treated *Z. bailii* cells (Fig. 1B). In the most acidic *S. cerevisiae* cultures tested (pH 3.5), the trehalose induced by 9 mM sorbate was not further inducible by heat shock, although its level was only 30–40% of the maximal trehalose achieved by heat shock of cultures of pH 5.5–7.5 (Fig. 1A).

3.2. The trehalose accumulation triggered by sorbate treatment of low-pH *S. cerevisiae* cultures is probably due to inhibition of glycolysis at the PFK step

In low-pH *S. cerevisiae* cultures weak-acid preservatives are known to inhibit glycolysis, primarily through a lowering of pH, that in turn inhibits phosphofructokinase (PFK) [1–3]. This study has shown that there is also trehalose accumulation (Fig. 1A). Inhibition of PFK will lead to elevation of cellular glucose-6-phosphate and fructose-6-phosphate levels, the latter metabolite being a potent activator of trehalose synthase [17]. Therefore, the simplest explanation for the sorbate induction of trehalose in low-pH *S. cerevisiae* cultures is PFK inhibition. Since PFK1 contributes most of the PFK activity in glucose-grown log phase *S. cerevisiae* cells [18,19], we investigated whether the *pfk1* *S. cerevisiae* mutant would display enhanced trehalose induction with 25°C weak organic acid treatment at pH 4.5. This trehalose induction was greatly enhanced in *pfk1*, but not *pfk2* cells (Fig. 2), consistent with the lower trehalose induction in wild-type and *pfk2* cells being due to inhibition of PFK1 activity. That PFK activity becomes limiting in weak-acid-stressed *S. cerevisiae*, especially at higher temperatures, is also shown by a much greater susceptibility of *pfk1* cells, relative to *pfk2* mutant and wild-type cells, to sorbate inhibition of pH 4.5 37°C growth (unpublished observations). This effect is largely lost at 25°C. Indeed the effects of PFK1 loss, as in the *pfk1* mutant, appear to be more marked at higher temperatures. We have observed that this mutant, whose basal trehalose is only slightly higher than normal during pH 6.6 25°C growth, maintains 2.5-fold higher trehalose during growth at 30°C and 6-fold higher trehalose at 37°C. Higher trehalose levels during normal growth of *pfk1* mutant cells have also been noted by Sur et al. [19] and are consistent with the elevated glucose-6-phosphate and fructose-6-phosphate levels reported by Heinisch [18].

In enzyme assays, the PFK of *Z. bailii* shows less dramatic inhibition in response to low pH [3] as compared to the PFK1 of *S. cerevisiae* [1,2]. It is possible that this may better equip *Z. bailii* to maintain glycolytic flux when pH is lowered by weak-acid treatment. The lack of a trehalose induction during weak-acid exposure of *Z. bailii* at low pH (Fig. 1B) might therefore be a further indication that the PFK
of this organism is less inhibited in weak-acid-stressed cells.

3.3. Effects of sorbate and external pH on the thermotolerance of Z. bailii

Preliminary experiments showed that a 10-min 50–52°C heat treatment of a mid-exponential 25°C Z. bailii culture resulted in approximately 90% cell inactivation. Remarkably this killing was essentially unaffected by external pH (Fig. 3), unlike in S. cerevisiae where thermotolerance at 50–52°C is maximal when culture pH approximates to physiological pH values (6–7) and considerably lower at extremes of pH [9]. This pH effect on S. cerevisiae thermotolerance may be due to lowered disturbances to homeostasis when cells are heated at close to physiological pH values. The lack of a similar influence of extracellular pH on the thermotolerance of Z. bailii, at least over the pH range 3.5–5.5 (Fig. 3), might reflect a greater capacity in the latter yeast for pH maintenance during stress.

The effects of sorbic acid on the thermal inactivation of Z. bailii at 52°C are, however, pH-dependent. Close to neutral pH, 25°C preservative treatment rendered cells more resistant to thermal killing at 52°C (Fig. 3). Only at the lowest pH tested (pH 3.5) did it have the converse effect, causing a reduction in thermotolerance. In S. cerevisiae cultures of pH > 5 weak-acid treatment at 25°C also acts as a strong inducer of thermotolerance, measured as ability to survive subsequent 52°C challenge [9]. However, in neither S. cerevisiae nor Z. bailii do these 25°C sorbate treatments at pH > 5 cause appreciable trehalose accumulation (Fig. 1), or enhance the small increases in trehalose seen during the 10-min 52°C incubations used for thermotolerance determination (not shown). Thermotolerance increases due to sorbate treatment at pH > 5 25°C appear therefore not to be associated with elevation of trehalose as a
stress protectant, unlike thermotolerance increases occurring during sublethal heat shock [12,13].

The presence of sorbic acid causes more than 40% of the survivors of brief (5 min, 50°C), lethal heating of low-pH S. cerevisiae cultures to be respiratory-deficient petites [9]. Such heating probably enhances the sorbate-induced damage to yeast mitochondria that has been seen in electron micrographic cross-sections of cells [3]. In contrast, none of the survivors of heating Z. bailii in the presence of sorbate at pH 4.5 in Fig. 3 were respiratory-deficient. Culturing Z. bailii under the conditions often used for petite selection in S. cerevisiae (3 days at 30°C in YPD containing 0.2 mg ml⁻¹ ethidium bromide) causes complete cell inactivation (unpublished observations). Loss of mitochondrial DNA is probably therefore a lethal event in Z. bailii. S. cerevisiae petites cultured aerobically on glucose are slightly more weak-acid resistant [9], but the loss of respiratory competence does not seem to be an option for increasing the weak-acid resistance of the petite-negative Z. bailii.

4. Discussion

Weak-acid preservatives cross membranes readily only when undissociated. Sorbate (pKₐ 4.76) will therefore concentrate inside cells and lower pHᵢ in response to a higher pH on the cytosolic side of the cell membrane. The rapid pHᵢ decline due to weak-acid preservatives (see Section 1) is a plausible trigger for the trehalose induction in sorbate-treated low-pH S. cerevisiae cultures (Fig. 1A). Results with the pfk1 S. cerevisiae mutant (Fig. 2) indicate that this arises through PFK1 inhibition. However, sorbate does not cause trehalose accumulation in low-pH Z. bailii cultures at 25°C (Fig. 1B), possibly an indication that the PFK of Z. bailii is inhibited less by reduction in pHᵢ [3] than the corresponding enzyme in S. cerevisiae [1,2]. This is consistent with observations that millimolar amounts of weak-acid preservative cause practically no inhibition of fermentation in low-pH cultures of Z. bailii [3,20].

Adaptation to growth in the presence of weak-acid preservatives involves the induction of a stress response that appears to differ from all the other stress responses of yeast characterised to date [7,8]. S. cerevisiae, exposed to growth-inhibitory levels of sorbate (9 mM) at low pH, shows a rapid trehalose accumulation (Fig. 1A). However, these same cells, through the induction of the weak-acid response, can adapt to slightly lower amounts of weak acid (up to 3 mM sorbate at pH 4.5) and resume growth after a lag period of several hours [7,8]. Such adapted S. cerevisiae cells do not have elevated trehalose levels (data not shown). It is therefore improbable that trehalose is a factor in weak-acid resistance. Moreover, since weak-acid treatment at pH > 5 does not elevate trehalose (Fig. 1), the thermotolerance increases occurring during sorbate treatment of 25°C S. cerevisiae [9] and Z. bailii (Fig. 3) cultures are also unlikely to involve trehalose.

Results previously reported for S. cerevisiae are largely consistent with a lowered pHᵢ causing inhibition of the capacity of cells to mount the heat-shock response. A mutation which reduces plasma membrane ATPase activity results in both a lowered pHᵢ and reduced heat induction of heat-shock proteins [21]. Also weak-acid preservatives and uncouplers block Hsp induction by heat in low-pH S. cerevisiae cultures [9]. However, although 25°C sorbate treatment at low-pH induces trehalose in S. cerevisiae (Fig. 1A, Fig. 2), culture pH is a much more potent influence over the heat induction of trehalose in this species (Fig. 1A). In contrast, culture pH had relatively little effect on either the basal or heat-induced trehalose of the more preservative-resistant Z. bailii. Also heat-induction of trehalose in the latter yeast was only inhibited by sorbate at pH < 5 (Fig. 1B).

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References