Plasma membrane H\(^+\) and K\(^+\) transporters are involved in the weak-acid preservative response of disparate food spoilage yeasts

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The food spoilage yeasts *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae* have been proposed to resist weak-acid preservative stress by different means; *Z. bailii* by limiting influx of preservative combined with its catabolism, *S. cerevisiae* by active extrusion of the preservative weak-acid anion and H\(^+\). Measurement of H\(^+\) extrusion by exponential-phase *Z. bailii* cells suggest that, in common with *S. cerevisiae*, this yeast uses a plasma membrane H\(^+\)-ATPase to expel H\(^+\) when challenged by weak-acid preservative (benzoic acid). Simultaneous measurement of *Z. bailii* net H\(^+\) and K\(^+\) fluxes showed that net K\(^+\) influx accompanies net H\(^+\) efflux during acute benzoic acid stress. Such ionic coupling is known for *S. cerevisiae* in short-term preservative stress. Both yeasts significantly accumulated K\(^+\) on long-term exposure to benzoic acid. Analysis of *S. cerevisiae* K\(^+\) transporter mutants revealed that loss of the high affinity K\(^+\) uptake system Trk1 confers sensitivity to growth in preservative. The results suggest that cation accumulation is an important factor in adaptation to weak-acid preservatives by spoilage yeasts and that *Z. bailii* and *S. cerevisiae* share hitherto unsuspected adaptive responses at the level of plasma membrane ion transport.

**INTRODUCTION**

The ability of certain yeasts to grow at low ambient pH in the presence of lipophilic weak-acid preservatives (such as benzoic acid, propionic acid and sorbic acid) inflicts heavy losses on the food and beverage industries (Thomas & Davenport, 1985). Understanding the mechanisms of weak-acid resistance is central to the development of more effective food and beverage preservation protocols. Consequences of preservative exposure include membrane destabilization, oxidative damage and reduced ATP production (Krebs et al., 1983; Stratford & Anslow, 1998; Piper, 1999; Piper et al., 2001). At low ambient pH, undissociated lipophilic preservatives may permeate the plasma membrane and then dissociate in the more alkaline yeast cytosol (Cole & Kiernan, 1987). Preservative dissociation in the cytosol can lead to intracellular acidification and deleterious accumulation of acid anion (Cole & Kiernan, 1987; Krebs et al., 1983). The ability to resist intracellular acidification is likely to be a survival determinant, given that pH homeostasis is critical to cellular function (Krebs et al., 1983; Cole & Kiernan, 1987; Holyoak et al., 1996). Preservative-resistance mechanisms are thought to include decreased plasma membrane permeability to diffusional preservative influx (Warth, 1989), active extrusion of the weak-acid anion (Holyoak et al., 1999; Piper et al., 1998) and ability to catabolize preservatives (Mollapour & Piper, 2001).

*Zygosaccharomyces bailii* exhibits generally greater preservative resistance than any other spoilage yeast and consequently is a greater economic threat. Despite this, far more is known about the resistance mechanisms of *Saccharomyces cerevisiae* than those of *Z. bailii*. The plasma membrane H\(^+\)-extruding H\(^+\)-ATPase is firmly implicated in the ability of *S. cerevisiae* to resist intracellular acidification caused by weak organic acids (Holyoak et al., 1996; Viegas & Sa-Correia, 1991). Activity (and possibly abundance) of this enzyme is stimulated by exposure to weak organic acid (Holyoak et al., 1996; Viegas & Sa-Correia, 1991; Piper et al., 2001) and an *S. cerevisiae* mutant with suboptimal plasma...
membrane H\(^{+}\)-ATPase activity adapts poorly to sorbic acid (Holoyoak et al., 1996). If the Z. bailii plasma membrane H\(^{+}\)-ATPase plays an important role in resisting intracellular acidification, then this well-conserved fungal protein could provide a common target in future control strategies applicable to both Z. bailii and S. cerevisiae. However, abundance of the Z. bailii plasma membrane H\(^{+}\)-ATPase does not appear to respond to weak-acid stress (Piper et al., 2001) and its role, if any, in the stress response is unknown. Moreover, it has been proposed that Z. bailii generally relies on limiting entry of weak-acid preservatives, whereas S. cerevisiae relies on plasma membrane efflux systems to rid the cell of the H\(^{+}\) and organic acid anions resulting from preservative permeation (Piper et al., 2001).

A difference in resistance strategy would have profound implications for the development of control methods common to both yeasts. To resolve the question of whether Z. bailii plasma membrane H\(^{+}\)-ATPase responds to weak-acid preservative stress, the acute responses of exponential-phase cells to benzoic acid exposure have been examined using initial rates of glucose-induced medium acidification as the standard diagnostic test for plasma membrane H\(^{+}\)-ATPase activity (Serrano, 1980; Holoyoak et al., 1996; Northrop et al., 1997). Acute exposure to butyric or propionic acids has been shown previously to stimulate H\(^{+}\) efflux and K\(^{+}\) influx in S. cerevisiae, with K\(^{+}\) counter-transport probably acting to regulate charge balance (Ryan et al., 1971; Ryan & Ryan, 1972; Boxman et al., 1985). If Z. bailii were also to rely on K\(^{+}\) as a counter-ion during the preservative stress response, then plasma membrane K\(^{+}\) transporters may form a target for a control strategy common to both yeasts. To test for K\(^{+}\) uptake, the MIFE technique (non-invasive ion-selective vibrating microelectrode; Newman, 2001; Shabala et al., 2001a, b, 2002) has been applied to Z. bailii for the first time to measure simultaneous net H\(^{+}\) and K\(^{+}\) fluxes in response to challenge by benzoic acid.

No previous study has examined whether K\(^{+}\) uptake as an acute response by either S. cerevisiae or Z. bailii manifests as K\(^{+}\) accumulation on chronic exposure to preservative (a scenario of greater relevance to food preservation). Here the effect of long-term benzoic acid exposure on cellular K\(^{+}\) content of both Z. bailii and S. cerevisiae has been examined as a test of the importance of K\(^{+}\) uptake in adaptive growth. Finally, as a test of the molecular identity of the K\(^{+}\) transporters involved in preservative adaptation, the effect of benzoic acid exposure on growth of S. cerevisiae mutants defective in plasma membrane K\(^{+}\) transporters has been investigated.

**METHODS**

**Culture maintenance.** Zygosaccharomyces bailii (strain 563, UK National Collection of Yeast Cultures, Institute of Food Research, Norwich) was maintained at 23 °C on YPD agar comprising 2% (w/v) glucose, 1% (w/v) bactopeptone, 0.5% (w/v) yeast extract and 2% (w/v) bactoagar (Difco). Saccharomyces cerevisiae strains were obtained from the EUROSCARF knockout collection (http://www.uni-franfurt.de/fb15/mikro/euroscarf). The diploid strain used was BY4743 (MATa/MATa; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; met15Δ0/ MET15; lys2Δ2/lys2Δ0; ura3Δ0/strα3Δ0). The MIC of benzoic acid for BY4743 on YPD agar at pH 4.5 was 5 mM, establishing it as a resistant strain (Warth, 1989). BY4743 and its diploid null mutants for TRK1 (strain Y37000; ORF YIL129c), TRK2 (strain Y35121; ORF YKR050w) and TOK1 (strain Y31330; ORF YIL093c) were maintained on YPD agar at 23 °C. Diploid strains of S. cerevisiae were used to allow valid comparisons to diploid Z. bailii.

**Experimental cultures.** Cells were grown aerobically at 27 °C with orbital shaking at 160 strokes min\(^{-1}\) in batch culture; 30 ml medium in 100 ml flasks. YPD medium comprised 2% (w/v) glucose, 2% (w/v) bactopeptone and 1% (w/v) yeast extract, adjusted to pH 4.5 with 2 M HCl. The K\(^{+}\) content of control YPD medium was 15 mM (determined by flame photometry). For adaptation, 2 mM potassium benzoate was added to YPD; this concentration has been used previously against Z. bailii 563 (Warth, 1989) and is above the maximum permitted level of 1.6 mM used in non-alcoholic beverages in the European Union (Directive 95/2/EC). In inhibitor studies using YPD, inhibitors (Sigma) were dissolved in growth medium and filter-sterilized. Inoculation density was to an OD\(_{600}\) of 0.03. Growth was determined as the increase in OD\(_{600}\) (1 cm light path cuvette; Pye Unicam SP6-300). Specific growth rate (\(\mu\)) was estimated for the exponential-phase culture. For spot assays of growth, cells were grown to saturation (OD\(_{600}\) > 2.0) in control YPD (pH 4.5), washed and resuspended to an OD\(_{600}\) of 4.0 in SDAP (Rodríguez-Navarro & Ramos, 1984) supplemented with 3 mM KCl and auxotrophic amino acids (100 µg ml\(^{-1}\)) and adjusted to pH 5.5 with arginine base. Aliquots (10 µl) of serially diluted suspension were spotted onto SDAP solidified with 2% (w/v) bactoagar with or without 2 mM benzoic acid. Growth was assessed after 3 days at 30 °C.

**Glucose-induced acid efflux.** Glucose-induced medium acidification was used as a diagnostic of Z. bailii plasma membrane H\(^{+}\)-ATPase activity and the method was adapted from Serrano (1980) and Northrop et al. (1997). Exponential-phase (6 h) cells grown in YPD or YPD with 2 mM potassium benzoate (pH 4.5) were harvested and washed twice with assay buffer (1 mM MES, adjusted to pH 5.5 with Tris base). After resuspension in assay buffer, acid efflux was initiated by the addition of glucose (total assay volume 10 ml; final glucose concentration 2 mM, final cell suspension 10 mg fresh weight ml\(^{-1}\)). Inhibitors were incorporated in the assay to final concentrations of 1 mM [N-ethylmaleimide (NEM), sodium azide] or 2 mM (sodium orthovanadate). Medium acidification was recorded using a Corning 240 pH meter connected to a Servocor 220 chart recorder. Calibration was by the addition of 1 µmol H\(^{+}\) equivalents. Assays were performed at room temperature.

**Immobilization of cells.** Acid-cleaned glass coverslips were coated with 0.1% (w/v) poly-L-lysine. A 1 ml aliquot of exponential-phase Z. bailii cells grown in YPD without benzoate was harvested as described above and the cells were resuspended in 30 µl assay solution (2 mM KCl, 1 mM MES, ± 2 mM benzoic acid, adjusted to pH 5.5 with Tris). Cells were applied to a treated coverslip and left for 3 min. Unattached cells were washed off with three applications of 2.5 ml assay solution. This procedure resulted in a monolayer of attached cells (Shabala et al., 2002). The coverslip was placed in the experimental chamber and 2.5 ml assay solution was added. Cells were analysed within 20–40 min of harvesting.

**Ion-selective flux measurements.** Net K\(^{+}\) and H\(^{+}\) fluxes were measured using an ion-selective vibrating microelectrode (the MIFE technique) as described previously (Shabala et al., 2001a, b, 2002). Microelectrode blanks were pulled from borosilicate glass capillaries, oven dried and silanized with tributylchlorosilane (Fluka
Transport events in yeast weak-acid adaptation

**Results**

Glucose addition stimulated medium acidification by control-grown *Z. bailii*

Exponential-phase *Z. bailii* cells grown in YPD at pH 4.5 exhibited a mean ± SEM glucose-induced initial H⁺ extrusion rate of 1.72 ± 0.37 μmol H⁺ (g fresh wt)⁻¹ min⁻¹ (n = 4 independent trials), determined at pH 5.5. The effects of test compounds are shown in Table 1 (‘Control-grown cells’). Inclusion of 1 mM sodium azide in the assay as a respiratory inhibitor caused a mean 88% reduction of H⁺ extrusion rate. NEM inhibits the fungal plasma membrane H⁺-ATPase and depolarizes fungal plasma membrane potential difference (Brooker & Slayman, 1982; Davies *et al.*, 1990). When this was incorporated into the medium acidification assay at 1 mM it caused a mean reduction in H⁺ extrusion rate of 95% (Table 1). Application of the widely used plasma membrane H⁺-ATPase inhibitor sodium orthovanadate (2 mM) did not inhibit medium acidification, nor (in contrast to *S. cerevisiae* Holyoak *et al.*, 1996) did it significantly inhibit growth (control K, 0.27 ± 0.03, n = 3; 2 mM sodium orthovanadate, 0.23 ± 0.04, n = 3). However, vanadate inhibits the plasma membrane H⁺-ATPase at its intracellular face (Scarborough & Addison, 1984) and therefore must enter the cell to take effect. Vanadate uptake is facilitated by phosphate transporters and so vanadate insensitivity at the whole-cell level may be due to lack of permeation (Bowman *et al.*, 1983). Sodium orthovanadate (2 mM) caused 70% inhibition of ATP hydrolysis in a partially purified plasma membrane preparation from *Z. bailii* control-grown cells (H. Rooney and J. M. Davies, unpublished data), which tends to confirm the premise that vanadate failed to penetrate whole cells. Overall the results indicate that glucose-induced medium acidification by control-grown cells was ultimately dependent on mitochondrial ATP production and likely to be mediated by a plasma membrane H⁺-ATPase.

The presence of 2 mM potassium benzoate in the assay

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**Table 1. Glucose-induced acidification rates of *Z. bailii* exponential-phase cells**

Mean ± SEM values are presented with the number of independent trials in parentheses. The control medium was YPD, pH 4.5, and benzoate-grown cells had 2 mM potassium benzoate added to the control medium. Cells were treated with respiratory inhibitors (azide), H⁺-ATPase inhibitors (vanadate, NEM) and acute benzoate challenge.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acidification rate [μmol H⁺ (g fresh wt)⁻¹ min⁻¹]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control-grown cells</td>
</tr>
<tr>
<td>Basal medium</td>
<td>1.72 ± 0.37 (4)</td>
</tr>
<tr>
<td>1 mM Azide</td>
<td>0.20 ± 0.20 (3)</td>
</tr>
<tr>
<td>2 mM Vanadate</td>
<td>2.09 ± 0.49 (4)</td>
</tr>
<tr>
<td>1 mM NEM</td>
<td>0.09 ± 0.09 (3)</td>
</tr>
<tr>
<td>2 mM Potassium benzoate</td>
<td>2.62 ± 0.10 (3)</td>
</tr>
</tbody>
</table>

**Replication and statistics.** Results are reported from independent trials and statistically significant differences have been tested for using Student’s t-test.
medium resulted in a 52% increase in mean glucose-induced acidification rate by control-grown cells (Table 1). This enhanced rate of medium acidification in the presence of 2 mM potassium benzoate was also inhibited by 1 mM NEM; there was no detectable glucose-induced acidification (n=3). Thus it appears that at the phenomenological level, the acute response of Z. bailii resembles that of S. cerevisiae in that weak-acid exposure enhances H+ efflux. The response to NEM suggests that this efflux may also be mediated by a plasma membrane H+-ATPase.

**Benzoate-grown Z. bailii shows enhanced glucose-induced medium acidification**

To test whether longer-term exposure to benzoic acid resulted in enhanced H+ efflux, Z. bailii cells were grown in YPD at pH 4-5 with 2 mM potassium benzoate and harvested during exponential phase. When assayed at pH 5-5 the glucose-induced medium acidification rate was 2.80±0.23 µmol H+ (g fresh wt)⁻¹ min⁻¹ (n=4; Table 1, 'Benzoate-grown cells'). This is significantly greater than the mean rate observed in control-grown cells (95% confidence level). Mean acidification rate was inhibited by 1 mM sodium azide (86% inhibition; Table 1) and 1 mM NEM (98% inhibition) but not by 2 mM sodium orthovanadate (Table 1). These results are comparable with control-grown cells and suggest the operation of a plasma membrane H+-ATPase.

The mean acidification rate of benzoate-grown cells in the presence of 2 mM benzoate was 2.90±0.11 µmol H+ (g fresh wt)⁻¹ min⁻¹ (n=3; Table 1) and this was completely inhibited by 1 mM NEM. Thus, the presence of benzoate in the assay increased the rate of glucose-induced acidification by only 4%. This is in contrast to the 52% mean increase in acidification rate when control-grown cells were exposed to benzoate in the assay medium. Growth in benzoate therefore appears to enhance basal levels of H+ extrusion and under assay conditions this basal level may be close to maximal potential activity.

**Net K⁺ influx accompanies net H⁺ efflux in control-grown Z. bailii**

Having demonstrated that H⁺ extrusion from Z. bailii was stimulated by glucose, the effect of glucose on K⁺ flux (as a candidate counter-ion) was then examined. Application of the MIFE technique permitted simultaneous measurement of net K⁺ and net H⁺ flux. Whereas bulk phase measurements of ions in the medium report gross concentration changes relative to the amount of yeast, MIFE allows net flux to be estimated on a surface area basis (and incorporates a bulk phase ion measurement). Previous studies have confirmed that K⁺ is lost from a medium when MIFE reports net K⁺ influx to cells, so the technique is secure in this respect (Shabala & Newman, 1999). Here, recordings were made in 2 mM KCl to render conditions comparable with the growth studies and acidification assay reported previously. However, such a high K⁺ concentration is incompatible with a good signal to noise ratio from the K⁺-selective microelectrode and hence recordings of net K⁺ fluxes do show greater fluctuations than those of H⁺. Qualitatively similar results with smaller fluctuations in net K⁺ flux were obtained in 0.2 mM KCl—a concentration which affords an improved signal to noise ratio (data not shown). The mean responses of control-grown exponential-phase cells to glucose addition (2 mM KCl, pH 5.5) are shown in Fig. 1. At the start of recording and in the absence of benzoate (Fig. 1a), a mean net influx of H⁺ (i.e. positive values) into the monolayer of cells was observed (net H⁺ influx of 4±1 nmol m⁻² s⁻¹ at the time of glucose addition, t=9.5 min; n=6), concurrent with a net efflux (negative values) of K⁺ (−25±4 nmol m⁻² s⁻¹ net efflux at glucose addition; n=6). These fluxes are consistent with a de-energized state in which the activity of

![Fig. 1. Mean±SEM glucose-induced net H⁺ and K⁺ fluxes from immobilized monolayers of Z. bailii cells. Exponential-phase cells grown in the absence of benzoic acid were assayed at pH 5.5 in a buffer containing (a) 2 mM KCl or (b) 2 mM KCl and 2 mM benzoic acid. Glucose was added to a final concentration of 2 mM at (a) 9.5 min and (b) 8 min (indicated by the arrow). Simultaneous recording of net H⁺ (crosses) and K⁺ (squares) fluxes was resumed after mixing of solutions. Results in (a) are from six independent trials in which both H⁺ and K⁺ fluxes were recorded simultaneously. In (b), six independent trials were conducted but in two of those, only H⁺ was recorded. Polarity convention: efflux negative, influx positive.](image-url)
the plasma membrane $\text{H}^+\text{-ATPase}$ is low and both $\text{H}^+$ and $K^+$ are being translocated down their respective electrochemical gradients. On the addition of 2 mM glucose, a net efflux of $\text{H}^+$ and simultaneous net influx of $K^+$ were observed. At the peak of the response there was a mean net $\text{H}^+$ efflux of $-132 \pm 32 \text{nmol m}^{-2} \text{s}^{-1} (t=16.5 \text{ min}; n=6)$. Overall this represents a mean glucose-induced change of $136 \text{nmol m}^{-2} \text{s}^{-1}$. There was a peak mean net $K^+$ influx of $129 \pm 25 \text{nmol m}^{-2} \text{s}^{-1} (t=17.5 \text{ min})$, yielding an overall mean glucose-induced change of $154 \text{nmol m}^{-2} \text{s}^{-1}$ (ratio of mean glucose-induced change of $\text{H}^+$ flux to $K^+$ flux $=1:1$). Net fluxes then declined but net $\text{H}^+$ efflux and net $K^+$ influx were maintained. These responses are consistent with the rapid activation of the $\text{H}^+\text{-ATPase}$ followed by a new apparent steady-state.

The mean responses of control-grown cells to glucose with 2 mM benzoic acid in the assay medium (in addition to 2 mM KCl) are shown in Fig. 1(b). Again, glucose-induced net $\text{H}^+$ efflux occurred with net $K^+$ influx. However incubation of de-energized cells with 2 mM benzoic acid resulted in different basal mean net flux values at the time of glucose addition ($t=8 \text{ min}$); both net $\text{H}^+$ influx and net $K^+$ efflux were greater ($H^+$, $13 \pm 7 \text{nmol m}^{-2} \text{s}^{-1}$ net influx, $n=6$; $K^+$, $-121 \pm 22 \text{nmol m}^{-2} \text{s}^{-1}$ net efflux; $n=4$). This is perhaps consistent with intracellular acidification caused by the preservative. The mean peak $\text{H}^+$ response to glucose occurred at $t=14 \text{ min}$; mean net $\text{H}^+$ efflux was $-284 \pm 30 \text{nmol m}^{-2} \text{s}^{-1}$. The mean peak $K^+$ response to glucose occurred at $t=15.5 \text{ min}$; net $K^+$ influx was $64 \pm 39 \text{nmol m}^{-2} \text{s}^{-1}$. Even at the end of the experiment, net $K^+$ influx was observed. The overall mean glucose-induced changes in flux (from basal level at addition of glucose to peak response) were: $\text{H}^+$, $297 \text{nmol m}^{-2} \text{s}^{-1}$; and $K^+$, $185 \text{nmol m}^{-2} \text{s}^{-1}$ (cf. 136 and 153 nmol m$^{-2}$ s$^{-1}$, respectively, without benzoic acid in the assay medium).

Thus, in agreement with the previous bulk assay of glucose-induced acidification, the presence of benzoic acid increased the rate of $\text{H}^+$ extrusion by control-grown exponential-phase cells. However, with benzoic acid, the ratio of mean glucose-induced change of $\text{H}^+$ flux to $K^+$ flux changed from 1:1:1 to 1:0:6.

**Effect of preservative on growth and intracellular $K^+$ content of *Z. bailii***

The MIFE experiments demonstrated that net $K^+$ influx accompanies net $\text{H}^+$ extrusion during acute benzoic acid challenge of *Z. bailii*. The cellular $K^+$ status on more prolonged benzoate exposure was then examined to test the hypothesis that continued net $K^+$ influx (as a consequence of stimulated $\text{H}^+$ efflux) would manifest as $K^+$ accumulation. Table 2 summarizes the results of 12 paired tests between control- and benzoate-grown *Z. bailii* (YPD). Growth in 2 mM benzoate produced a significant reduction in specific growth rate ($K$) and fresh weight yield at stationary phase. Mean values for $K^+$ content lie within the range reported for yeast species (Jones & Gadd, 1990). Despite the mean 15% decline in yield from benzoate exposure, there was a mean 33% increase in cellular $K^+$ content. In each pair of cultures $K$ was always lower, but $K^+$ content was always higher in benzoate-grown cells. For both populations, $K^+$ content tended to increase as $K$ increased but in instances of comparable $K$ values, the $K^+$ content of benzoate-adapted cells was greater than those of the control (data not shown). In preliminary experiments, inclusion of 2 mM KCl rather than potassium benzoate produced no significant effect on *Z. bailii* growth or $K^+$ content of cells (data not shown), demonstrating that growth effects were due to benzoate rather than $K^+$.

**Table 2. Effect of benzoic acid on growth rate ($K$) and intracellular $K^+$ content**

Data are from 12 paired *Z. bailii* experiments, 6 paired WT *S. cerevisiae* experiments and 6 paired trk1Δ experiments of control- and benzoate-grown cells harvested at early stationary phase from YPD, pH 4.5. $P$ is the probability value of the difference in means not resulting from chance. All values are mean ±SEM.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Benzoate</th>
<th>$P$ (%)</th>
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<tbody>
<tr>
<td><strong>Z. bailii</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fresh weight (g)</td>
<td>0.197 ±0.007</td>
<td>0.168 ±0.008</td>
<td>99</td>
</tr>
<tr>
<td>$K$</td>
<td>0.31 ±0.017</td>
<td>0.27 ±0.017</td>
<td>95</td>
</tr>
<tr>
<td>$K^+$ [μmol (g fresh wt)$^{-1}$]</td>
<td>85 ±3</td>
<td>113 ±3</td>
<td>99-9</td>
</tr>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh weight (g)</td>
<td>0.154 ±0.008</td>
<td>0.091 ±0.004</td>
<td>99-9</td>
</tr>
<tr>
<td>$K$</td>
<td>0.37 ±0.023</td>
<td>0.26 ±0.025</td>
<td>98</td>
</tr>
<tr>
<td>$K^+$ [μmol (g fresh wt)$^{-1}$]</td>
<td>116 ±5</td>
<td>134 ±8</td>
<td>90</td>
</tr>
<tr>
<td><strong>trk1Δ</strong></td>
<td></td>
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</tr>
<tr>
<td>Fresh weight (g)</td>
<td>0.139 ±0.008</td>
<td>0.062 ±0.002</td>
<td>99-9</td>
</tr>
<tr>
<td>$K$</td>
<td>0.28 ±0.01</td>
<td>0.195 ±0.01</td>
<td>99-9</td>
</tr>
<tr>
<td>$K^+$ [μmol (g fresh wt)$^{-1}$]</td>
<td>98 ±11</td>
<td>104 ±10</td>
<td>Not significant</td>
</tr>
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</table>
These results suggest that chronic exposure and adaptation to benzoate causes accumulation of K\(^+\). It follows that increasing availability of K\(^+\) could enhance growth in benzoate. Trials were therefore performed in which the K\(^+\) content of YPD was increased up to 150 mM (by KCl addition). The mean K values for normal YPD were: control, 0·24 ± 0·01 (n = 3); benzoate, 0·20 ± 0·01 (n = 5). For YPD with 150 mM KCl the values were: control, 0·26 ± 0·01 (n = 3); benzoate, 0·24 ± 0·004 (n = 5). Increasing K\(^+\) availability in the presence of benzoate caused an increase in K that was statistically significant at the 99·8% confidence level, but did not produce a significant difference in K in the absence of benzoate.

**Effect of preservative on growth and intracellular K\(^+\) content of S. cerevisiae**

Growth of WT *S. cerevisiae* in YPD batch culture was significantly inhibited by 2 mM benzoate at pH 4·5 (Table 2). A mean decrease in yield (41%), but increase in mean intracellular K\(^+\) (16%) at stationary phase was observed (n = 6) (cf. 15% and 33% for *Z. bailii*, respectively). Accumulation of K\(^+\) during chronic preservative stress appears to be conserved between these disparate spoilage yeasts.

**Sensitivity of *S. cerevisiae* K\(^+\) transporter mutants to preservative**

*S. cerevisiae* plasma membrane K\(^+\) transporter knockout strains were initially assessed by spot growth assay on solid SDAP medium supplemented with 2 mM benzoic acid (pH 4·5 and 3 mM KCl). As shown in Fig. 2, deletion of either TRK2 (encoding a plasma membrane moderate-affinity K\(^+\) uptake transporter; Ramos et al., 1994) or TOK1 (encoding a plasma membrane outwardly rectifying K\(^+\) channel capable of mediating influx; Ketchum et al., 1995; Fairman et al., 1999) did not confer sensitivity to chronic benzoic acid exposure (72 h). However, deletion of TRK1 (encoding the plasma membrane high/moderate-affinity K\(^+\) uptake transporter; Haro & Rodriguez-Navarro, 2002) resulted in impaired growth in the presence of benzoic acid compared to control. Similar growth responses were observed with sorbic acid as the preservative stress (data not shown).

Benzoic acid inhibition of *trk1Δ* growth was confirmed in liquid batch culture. In SDAP supplemented with 2 mM benzoic acid (pH 4·5 and 3 mM KCl), the mean OD\(_{600}\) of WT at early stationary phase (108 h) was 1·41 ± 0·04, n = 3; mean OD\(_{600}\) of *trk1Δ* at early stationary phase (126 h) was 0·59 ± 0·05, n = 3. In YPD, 2 mM benzoate (pH 4·5) caused a mean 30% decrease in growth rate, a mean 55% decrease in yield and a statistically insignificant mean 6% increase in intracellular K\(^+\). Thus, as WT showed a mean 16% increase in K\(^+\) content during chronic benzoate exposure, *TRK1* is implicated in K\(^+\) accumulation during growth in this preservative.

**DISCUSSION**

Weak organic acid preservative resistance by spoilage yeasts is likely to be a multi-component process that may vary between genera and even species. The greater preservative resistance exhibited by *Z. bailii* (compared to *S. cerevisiae*) has been postulated to result from greater ability to limit diffusional entry of the undissociated acid and greater ability to degrade preservatives (Piper et al., 2001). Such adaptations would tend to prevent cytosolic acidification simply by reducing the amount of preservative reaching the cytosol. In contrast, *S. cerevisiae* appears more prone to perturbations in cytosolic pH caused by preservatives and its main adaptive strategy is proposed to be H\(^+\) extrusion by the plasma membrane H\(^+\)-ATPase coupled with acid anion extrusion by the ABC transporter.

![Fig. 2. Spot growth assay of *Z. bailii* (Zb), diploid wild-type *S. cerevisiae* (Sc) and diploid potassium transport mutants (*trk1Δ*, *trk2Δ* and *tok1Δ*). Strains were grown for 72 h on SDAP (pH 4·5, 3 mM KCl); ba indicates incorporation of 2 mM benzoic acid. Serial dilutions are indicated. This experiment is representative of three independent trials.](image)
Pdr12 (Holyoak et al., 1999; Piper et al., 1998, 2001). Here, the possible involvement of the plasma membrane H^+-ATPase in preservative adaptation by Z. bailii has been examined.

The plasma membrane H^+-ATPase of Z. bailii has been neither reconstituted nor cloned. However, analysis of a plasma membrane fraction has revealed the presence of a 100 kDa polypeptide that is the hallmark of this conserved fungal enzyme (Piper et al., 2001). The results of growth and glucose-induced medium acidification experiments in the present study support plasma membrane H^+-ATPase activity in both control and preservative-adapted exponential-phase Z. bailii. The initial rate of H^+ extrusion was comparable with other yeasts (Northrop et al., 1997) and was sensitive to respiratory blockade and NEM (Brooker & Slayman, 1982; Davies et al., 1990). Stimulation of glucose-induced H^+ efflux from control-grown cells by benzoic acid (measured both in bulk assay by pH meter and from an immobilized monolayer using MIFE) suggests increased turnover or more efficient H^+ : ATP coupling of the enzyme (Warnke & Slayman, 1980) in response to preservative challenge.

The observation that growth to exponential phase in benzoate manifested in a greater basal glucose-induced H^+ efflux than control cells may be due to an increased abundance of the H^+-ATPase, increased turnover or more efficient coupling ratio. Qualitatively the increase in basal rate resembles the behaviour of late-exponential-phase S. cerevisiae grown in sorbic acid (Holyoak et al., 1996). Although not directly comparable, glucose-induced acid extrusion rates of the latter increased by 20% when grown in 0.9 mM sorbic acid at pH 4.5 (Holyoak et al., 1996).

How such adapted S. cerevisiae cells then responded to sorbic acid challenge in the glucose assay has not been reported, but whereas H^+ efflux of control-grown Z. bailii was stimulated 52% by benzoate under assay conditions (bulk pH meter assay), efflux of benzoate-grown cells was stimulated by only 4%. This could mean that the system is already operating at near maximum capacity and cannot be further stimulated. It is feasible that, as cells adapted to benzoic acid undergo a 40% reduction in cellular permeability to this preservative (Warth, 1989), less preservative is going into the cell to stimulate efflux. This would in turn mean that, as the cells grew in benzoate, enhanced H^+ efflux was effected early on by the presence of the preservative and maintained even in the face of its reduced permeability. Studies on Z. bailii must now aim to establish the mechanistic basis of H^+-ATPase upregulation. The introduction of Z. bailii haploid strains (Rodrigues et al., 2003) promises to make production of mutants far easier and those for the plasma membrane H^+-ATPase would provide definitive evidence for the role of this enzyme in adaptation to preservatives.

Acute challenge of S. cerevisiae with butyric or propionic acid has been found to stimulate H^+ efflux with concomitant K^+ influx and affect short-term K^+ accumulation (Boxman et al., 1985; Ryan et al., 1971; Ryan & Ryan, 1972). Here, stimulation of net H^+ efflux in both the presence and absence of benzoate was accompanied by net K^+ influx by Z. bailii. It is reasonable to assume that net K^+ uptake by Z. bailii serves the same (initial) purpose in S. cerevisiae in that it counters the loss of positive charge from the cell due to H^+-ATPase activity. However, it is interesting to note that the mean glucose-induced changes in net H^+ : K^+ flux (basal to peak response) were approximately 1:1 for the control assay but 1:0.6 in the presence of benzoic acid. This suggests that net K^+ influx may be insufficient to maintain charge balance on increased H^+ efflux in the presence of preservative and that another transport pathway could also participate. Charge balance could be affected not only by net K^+ uptake, but also by anion efflux, perhaps even that of the preservative acid anion.

Previous studies on S. cerevisiae only monitored K^+ accumulation during the first 2 h of preservative stress. The findings here that long-term preservative exposure leads to K^+ accumulation by both S. cerevisiae and Z. bailii (and that increased K^+ availability improves growth in preservative) suggests that it is a conserved adaptive response conferring stress-specific physiological advantage. Indeed, K^+ uptake by yeast has been linked to regulation of oxidative phosphorylation (Aiking et al., 1977), intracellular pH and the cell cycle (Yenush et al., 2002), all of which would be critical to weak-acid preservative resistance. The sensitivity to preservative of S. cerevisiae lacking the Trk1p high/moderate-affinity K^+ uptake transporter confirms the observation from a mutant screen by Mollapour et al. (2004) that the trk1Delta mutant grows poorly in sorbic acid. It has previously been shown that K^+ uptake by the Trk system helps alkalinize cytosolic pH by promoting H^+ extrusion (Yenush et al., 2002). The failure of the trk1Delta mutant to accumulate K^+ in response to benzoic acid observed here could impair its ability to counteract the cytosolic acidification caused by the preservative. However, sensitivity may not be the simple consequence of reduced K^+ uptake capacity; trk mutants exhibit more hyperpolarized plasma membrane voltage (Madrid et al., 1998) which could affect growth by disturbing other transport processes. A more negative membrane voltage could promote increased divalent cation uptake and disturb homeostasis of, for example, Ca^2+. More detailed studies are now required to elucidate the role of Trk1p in weak-acid preservative resistance, but it is clear that the trk1Delta strain is a good candidate for complementation cloning of Z. bailii K^+ transporters relevant to growth in preservative.

Overall, this study demonstrates that weak-acid resistance of Z. bailii has greater mechanistic parallels with that of S. cerevisiae than previously suspected. Increased plasma membrane H^+-ATPase activity is common to both yeasts and identifies this enzyme as a rational target in spoilage prevention. Additionally, short-term K^+ influx and long-term K^+ accumulation are common responses to preservative stress and suggest that K^+ transporters may also
serve as control targets. Restricting K⁺ availability in food and beverages may not be a realistic control strategy, particularly for fruit-derived drinks such as wines. These tend to have high (mM) and variable K⁺ content set by the K⁺ accumulation of the fruit (Núñez et al., 2000). Rather, a pharmacological approach appears warranted.

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REFERENCES


