Weak acid preservatives block the heat shock response and heat-shock-element-directed *lacZ* expression of low pH *Saccharomyces cerevisiae* cultures, an inhibitory action partially relieved by respiratory deficiency

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Inhibition of microbial growth by weak acid preservatives increases with medium acidification, since these agents enter cells in the undissociated state. Many of the effects of these acids are due to the cytoplasmic acidification they cause as they dissociate in the higher pH environment of the cytosol. Sorbic and benzoic acids, two widely used preservatives, were found to exert pronounced effects on the heat shock response and thermotolerance of *Saccharomyces cerevisiae*. These effects were strongly influenced by the pH of the culture medium. In low pH cultures sorbate inhibited the induction of thermotolerance by sublethal heat shock, causing strong induction of respiratory-deficient petites among the survivors of heat treatment. However, when the culture pH was above 5.5 sorbate acted as a powerful chemical inducer of thermotolerance in the absence of any sublethal heat treatment. Sorbate and benzoate also inhibited heat induction of the major heat shock proteins in low pH yeast cultures. This appears to result from lack of induction of the heat shock element (HSE) promoter sequence since sorbate prevented heat induction of a HSE-*lacZ* fusion at low pH. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) and the plasma-membrane-ATPase inhibitor diethylstilboestrol were identified as additional inhibitors of heat induction of heat shock proteins. Numerous chemicals induce the heat shock response in the absence of heat stress, but sorbate, benzoate, CCCP and diethylstilboestrol are the first compounds shown to act as selective inhibitors of heat-induced protein expression in yeast. In the presence of sorbate concentrations which, at low pH, totally inhibit both the heat shock response and growth of cells competent in respiration, respiratory-deficient petites still retain a limited capacity for growth and for heat induction of heat shock proteins. This restoration of a response to heat shock in acidified sorbate-treated cultures of petites might contribute to their higher capacity for growth in the presence of sorbate.

Keywords: *Saccharomyces cerevisiae*, weak acid preservatives, heat shock element, thermotolerance, intracellular pH

INTRODUCTION

Sorbic and benzoic acids are in extensive use as food preservatives since they inhibit the growth of fungi and

Abbreviations: PFK1, 6-phosphofructo-1-kinase; pH_i, intracellular pH; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; HSE, heat shock element.

bacteria. It has been clearly established that the penetration of these acids into cells and growth inhibition both increase with medium acidification, being essentially proportional to the concentration of undissociated acid (for literature see Russell, 1991). Following entry to the cells of low pH cultures as undissociated forms, sorbic and benzoic acids dissociate in the higher pH of the cytosol to cause intracellular acidification. In *Saccharomyces*

Fig. 1. Proteins labelled in *S. cerevisiae* incubated with [^{3} H]leucine for 40 min, either at 25 °C (–) or immediately after heat shock to 39 °C (+) in the presence and absence of 9 mM potassium sorbate. Prior to labelling the cells were resuspended in medium buffered at pH 4.5, 6.5 or 8.5 (see Methods). The heat shock proteins induced most strongly by heat shock (Mager & Moradas-Ferreira, 1993) are indicated to the right of the figure.

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 (Krebs *et al.*, 1983; Francois *et al.*, 1986, 1988a, b). More osmotolerant yeasts, notably *Zygosaccharomyces bailii*, are important causative agents of the spoilage of low pH foods and beverages of high sugar content (Thomas & Davenport, 1985). They are able to undergo an adaption that allows growth in the presence of preservative concentrations in excess of those permitted legally in foodstuffs (Warth, 1978, 1988; Cole & Keenan, 1986, 1987). In *S. cerevisiae* both respiration and fermentation are inhibited by weak acids (Krebs *et al.*, 1983; Francois *et al.*, 1986, 1988a, b), whereas in *Z. bailii* respiration is totally inhibited by weak acid concentrations that have little effect on fermentation (Cole, 1987).

A mild, sublethal heat stress increases the capacity of microbial cells to survive during a subsequent exposure to higher, potentially lethal temperatures. This development of the state of induced or 'acquired' thermotolerance is widespread amongst food-contaminating and pathogenic organisms and therefore a phenomenon having major

implications for microbiological safety (Piper, 1993). A mild heat stress also induces the highly conserved heat shock proteins in all cells (Lindquist & Craig, 1988; Watson, 1990; Mager & Moradas-Ferreira, 1993). The literature regarding the effects of weak acid preservatives on the heat tolerance of yeast is confusing, sorbate having been variously reported as either not affecting (Van Uden, 1984) or increasing (Coote *et al.*, 1991) *S. cerevisiae* thermotolerance. We report here that the effects of sorbate on yeast thermotolerance are strongly dependent on extracellular pH, and also that heating low pH cultures with sorbate causes strong induction of respiratory-deficient petites. This indicates that heat potentiates the sorbate-induced ultrastructural damage to yeast mitochondria seen in electron micrographs (Cole, 1987).

This study also revealed sorbate and benzoate acting as selective inhibitors of the heat induction of heat shock genes in yeast cultures of low pH. Numerous chemicals induce the synthesis of heat shock proteins in the absence of heat stress (Anathan *et al.*, 1985; Lindquist & Craig, 1988; Watson, 1990), but very few agents are known to selectively inhibit heat induction of these same proteins.

Fig. 2. Proteins labelled in *S. cerevisiae* incubated with [^{3} H]leucine for 40 min, either at 25 °C (–) or immediately after heat shock to 39 °C (+) in the absence and presence of benzoate. Cells had previously been resuspended in medium buffered at pH 4.5 or 7.5.

The only other selective inhibitors reported are flavonoids, which cause hypersensitization to heat and a block to the expression of heat shock proteins in mammalian cells (Hosokawa *et al.*, 1990). Other agents, including a flavonoid, were therefore tested for their ability to block heat shock protein induction in yeast.

Incubation of low pH *S. cerevisiae* cultures in the presence of sorbate can provide a strong selection for respiratory-deficient petites. This is because the petite mutation confers the capacity for slow growth at sorbate levels that cause a total inhibition of the growth of cells competent for respiration. This mutation also partially restores the capacity for a heat shock response at low pH in the presence of sorbate.

METHODS

Strains and materials. All experiments were conducted using *S. cerevisiae* strain SUB62 (a *leu2*, *trp1*, *ura3*, *his3*, *lys2*; gift of D. Finley) or the SUB62-derived transformant SUB62-pHSE2 [SUB62 transformed with pHSE2, a *URA3*-bearing plasmid that carries a heat-shock-inducible *lacZ* gene (Kirk & Piper, 1991)]. pHSE2 possesses a tandem copy of the heat shock element (HSE) sequence that directs heat shock induction of many yeast genes, inserted within a *CYC1* promoter-*lacZ* gene

fusion (HSE-*lacZ*) in place of the normal *CYC1* upstream activating sequences (Sorger & Pelham, 1987). Spontaneous petites derived from SUB62 and SUB62-pHSE2 were isolated as small colonies on YPD medium [2% Bactopectone, 1% yeast extract, 2% glucose (all %, w/v)] plates containing 1.5% agar, cells being plated after high temperature (38 °C) growth for five generations, a condition which enriches for petites (Van Uden, 1984). Sorbate-induced petites were survivors from the pH 4.5 sorbate-treated cells of the experiment in Fig. 4(b). Petites were scored as colonies capable of fermentative growth on YPD but not respiratory growth on YPG (2% Bactopectone, 1% yeast extract, 2% glycerol, 1.5% agar) plates.

Yeast culture. Cultures of SUB62 were grown either on liquid YPD medium or, for protein labelling, on SD minimal dextrose medium [0.67%, w/v, yeast nitrogen base (Difco), 2%, w/v, glucose plus uracil, leucine, histidine, tryptophan and lysine supplements (Sherman *et al.*, 1983)]. SUB62-pHSE2 was grown on the same medium minus uracil to ensure maintenance of the pHSE2 plasmid. Flask cultures were shaken at 25 °C to ensure aeration and used for experiments when in early exponential phase at 5×10^6 – 1×10^7 cells ml $^{-1}$. CCCP, potassium benzoate and potassium sorbate (all from Sigma) were added from pH 7.0–1 M, 0.6 M and 0.9 M stock solutions respectively; and diethylstilboestrol (Sigma) from a 2.7 mg ml $^{-1}$ solution in methanol.

Protein labelling at different pH values. For protein labelling, SD medium SUB62 cultures were centrifuged (5 min, 2500 g)

Fig. 3. Influences of medium pH and sorbate on the expression of a HSE-*lacZ* fusion in SUB62-pHSE2 (a); in a spontaneous petite derived from SUB62-pHSE2 (b); and in the same petite grown for several generations in the presence of 1 mM sorbate (c). Cells were resuspended in media buffered at different pH values from 4.5 to 8.5, with or without sorbate. After these cultures had been maintained at 25 °C or heat shocked to 39 °C β -galactosidase levels were determined (see Methods). β -Galactosidase is given as fold-induction relative to the constant β -galactosidase level of 2.3 Miller units (Miller, 1972) in uninduced 25 °C SD medium SUB62-pHSE2 cultures. Basal HSE-*lacZ* expression in the absence of heat shock was unaffected by either medium pH or sorbic acid, as shown by the low constant β -galactosidase level at 25 °C in the absence (○) or the presence of sorbate (□, 1 mM; △, 9 mM). *lacZ* expression after heat induction of HSE activity showed an appreciable dependence on culture pH even in the absence of sorbic acid (●). The influences of 1 mM (■) or 9 mM (▲) sorbate on this heat-induced expression are much more pronounced at lower pH values. Also while 9 mM sorbate totally inhibited heat induction of HSE-*lacZ* expression in SUB62-pHSE2 below pH 6.0 (a), this inhibition was only partial in the petite (b, c).

and resuspended at 5×10^7 – 1×10^8 cells ml⁻¹ in 0.5 × SD medium containing 50 mM of the appropriate buffer (sodium acetate pH 4.5; MES/KOH pH 6.5; TES/KOH pH 7.5 or TES/KOH pH 8.5). Cells were incubated for 20 min at 25 °C in this buffered medium prior to the addition of 0.05 mCi ml⁻¹ (1.85 MBq ml⁻¹) [4,5-³H]leucine [53 Ci mmol⁻¹ (1.96 GBq mmol⁻¹) New England Nuclear] and, where indicated, sorbate, benzoate, CCCP or diethylstilboestrol. Labelling was by further incubation for 40 min at either 25 °C or, for the heat shocked samples, at 39 °C. Preparation of samples of total *in vivo* pulse-labelled protein, analysis of these samples on one-dimensional 12.5 % SDS-polyacrylamide gels and detection of labelled proteins by fluorography was as in Panaretou & Piper (1990, 1992). Protein determinations were performed using the Bio-Rad protein assay kit and bovine serum albumin as standard.

Effects of extracellular pH and of sorbic acid on β -galactosidase expression. SD medium cultures of SUB62-pHSE2 were centrifuged and resuspended at 5×10^6 – 1×10^7 cells ml⁻¹ in 0.5 × SD medium containing 50 mM of the appropriate buffer (sodium acetate pH 4.5 or 5.0; MES/KOH pH 5.5, 6.0 or 6.5; TES/KOH pH 7.0, 7.5 or 8.5). These buffered SUB62-pHSE2 cultures were incubated for 20 min at 25 °C, before being divided into four aliquots which were subjected to alternative treatments prior to measurement of β -galactosidase activity: (i) 60 min further incubation at 25 °C; (ii) 60 min heat shock at 39 °C; (iii) addition of 1 mM or 9 mM potassium sorbate, followed by 60 min further incubation at 25 °C; and (iv) addition of 1 mM or 9 mM potassium sorbate, followed by 60 min heat shock at 39 °C. β -Galactosidase levels were measured as in Miller (1972).

Effects of extracellular pH and of sorbic acid on thermo-

tolerance. SD and YPD medium SUB62 cultures in early exponential phase at 5×10^6 – 1×10^7 cells ml⁻¹ were used for these studies. SD cultures were diluted with an equal volume of a 100 mM solution of the appropriate buffer (sodium acetate pH 3.5 or 4.5; MES/KOH pH 5.5 or 6.5; TES/KOH pH 7.5 or 8.5). YPD cultures were centrifuged and resuspended at 5×10^6 – 1×10^7 cells ml⁻¹ in YPD previously adjusted to pH 3.5, 4.5, 5.5, 6.5, 7.5 or 8.5 with HCl or NaOH. After either 5 min or 60 min incubation at 25 °C or 38 °C in these buffered media, cultures were immediately heated 5 min at 50 °C (for data in Figs 4 and 5), or recentrifuged and resuspended in pH 6.0 sorbate-free YPD prior to 5 min 50 °C heat treatment (for data in Fig. 6). Cell survival, measured as the ability to form colonies on YPD plates at 28 °C, is expressed in Figs 4–6 relative to the survival of the starter SD or YPD cultures subjected to an identical 50 °C 5 min stress. For each time point > 50 colonies were counted, the data shown being the combined results of two separate experiments.

RESULTS

Sorbate and benzoate inhibit heat induction of major heat shock proteins in *S. cerevisiae* cultures of low pH

S. cerevisiae cells were resuspended in buffered 0.5 × SD media of pH 4.5, 6.5 or 8.5. Shortly afterwards they were pulse-labelled with [³H]leucine, either at 25 °C or after a heat shock to 39 °C, and in the absence or presence of 9 mM potassium sorbate (see Methods). One-dimensional gel electrophoresis of proteins labelled under these

Fig. 4. Influence of the pH of a pre-incubation in buffered SD medium, and also the presence and absence of sorbate, on thermotolerance. SUB62 cells were grown to early exponential phase (5×10^6 cells ml^{-1}) on pH 6.8 YPD medium at 25 °C. They were then centrifuged and resuspended in buffered 0.5% SD medium at pH 3.5, 4.5, 5.5, 6.5, 7.5 or 8.5 (see Methods) either without (open symbols) or with (filled symbols) 9 mM sorbic acid. After pre-incubation in these buffered media for 5 min at 25 °C (a), 1 h at 25 °C (b) or 1 h at 38 °C (c) cultures were immediately heated for 5 min at 50 °C and then chilled. Survival was measured as ability to form colonies on plates at 28 °C, relative to the survival of the original pH 6.8 YPD starter culture given an identical 5 min 50 °C heat stress. The percentage of survivors of heat stress in (a)–(c) that were respiratory-deficient petites is indicated below the thermotolerance data.

conditions (Fig. 1) revealed that major heat shock proteins had been induced in all the 39 °C cultures, with the exception of the pH 4.5 culture heat shocked in the presence of sorbate. Proteins pulse-labelled at 39 °C in this pH 4.5 culture appeared similar to those labelled at 25 °C (Fig. 1). The block to heat shock protein expression in these cells was therefore not the result of a total inhibition of protein synthesis, suggesting that sorbate may be acting to selectively inhibit heat shock gene expression. Inhibition of the heat induction of heat shock proteins in pH 4.5 cultures, but not pH 7.5 cultures, was also obtained in a similar pulse-labelling experiment in which 3 mM or 6 mM potassium benzoate was employed in place of sorbate (Fig. 2). When sorbate or benzoate was present at concentrations of 1 mM or less the inhibition of heat shock protein induction in pH 4.5 cultures was only partial (data not shown).

Effects of sorbate and culture pH on the activity of a heat shock element (HSE)–*lacZ* fusion

Heat induction of many *S. cerevisiae* heat shock genes is due to the same HSE promoter sequence as is used in higher eukaryotes, a series of repeating units of the 5 bp sequence nGAAn, arranged in alternating orientations at each half-turn of the DNA helix (Sorgers, 1991). This HSE is the binding site for the well-characterized yeast heat shock factor, a trimeric *trans*-activator of transcription which is needed at all growth temperatures but which increases its activity in response to temperature upshift (Sorgers, 1990, 1991).

The optimum temperature for induction of HSE sequences in *S. cerevisiae* is 39 °C (Sorgers & Pelham, 1987; Kirk & Piper, 1991). Using cells carrying a plasmid-borne *E. coli lacZ* gene under HSE control (transformant

Fig. 5. Influence of the pH of a pre-incubation in YPD medium, and also the presence and absence of sorbate, on thermotolerance. Cells were grown to early exponential phase (5×10^6 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, either without (open symbols) or with (filled symbols) 9 mM sorbic acid. After pre-incubation for 5 min at 25 °C (a), 1 h at 25 °C (b) or 1 h at 38 °C (c) cultures were immediately heated for 5 min at 50 °C and then chilled. Survival is given relative to the survival of the original YPD starter culture given an identical heat stress. The percentage of the survivors that were respiratory-deficient petites is indicated in the lower panels.

SUB62-pHSE2; see Methods), the influences of medium pH and of sorbate on both basal and heat-induced levels of HSE activity were investigated (Fig. 3a). The low basal HSE-*lacZ* expression in the absence of heat stress was unaffected by either culture pH or the presence of sorbate (Fig. 3a). Sorbate is therefore not a HSE inducer. Heat-induced HSE-*lacZ* expression in the absence of sorbate was influenced by the pH of the culture, being maximal at pH 6.5 and less at higher or lower pH (Fig. 3a). Sorbate caused this heat-induced β -galactosidase expression to be dramatically suppressed at low pH. Sorbic acid (1 mM) inhibited induction when the culture pH was below 6.0 and 9 mM sorbic acid inhibited when culture pH was below 7.0. In contrast, sorbate had relatively little effect on HSE-*lacZ* expression at higher pH values (Fig. 3a). This indicates that the sorbate inhibition of the heat shock response (Figs 1 and 2) occurs through a block to the heat

induction of the HSE sequence of heat shock gene promoters.

Effects of sorbate and external pH on thermotolerance

An increased thermotolerance is one of the major consequences of a mild sublethal heat shock (see Introduction). To investigate the effects of sorbate and external pH on thermotolerance, cells were transferred to media of different pH for either 5 or 60 min at 25 °C, or 60 min at 38 °C, either without preservative or in the presence of 9 mM sorbate. They were then subjected to a potentially lethal heat treatment (50 °C, 5 min) without change to medium pH or sorbate level (Figs 4 and 5). Their survival of lethal heat stress in the absence of sorbate showed that thermotolerance was influenced by

the external pH during lethal heat treatment. This was irrespective of whether they were on complex medium (YPD; Fig. 4) or defined medium (SD; Fig. 5), and whether pH preconditioning had been at 25 °C or 38 °C. This effect of medium pH on thermotolerance is not unexpected, since external pH is known to influence pH_i even in unstressed cells (Borst-Pauwells, 1981). The pH of heating affects thermotolerance, tolerance being maximal at a pH approximating to physiological pH_i values (6–7) and considerably lower at extremes of pH (Figs 4 and 5). This probably reflects a lowered disturbance to homeostasis when cells are heated at physiological pH values.

Figs 4 and 5 also reveal that 9 mM sorbate exerted strongly pH-dependent effects on thermotolerance. At higher pH values, it caused small increases in thermotolerance whereas at low pH it had the converse effect, reducing thermotolerance and preventing the usual thermotolerance increases with a 38 °C pre-incubation. A brief heat treatment at a potentially lethal temperature (50 °C), when applied at low pH in conjunction with the presence of sorbate, was found to strongly induce respiratory-deficient petites. Such petites were a substantial fraction of the survivors of pH 4.5 cultures heated in the presence of 9 mM sorbate (see lower panels; Figs 4 and 5). It is well-established that *S. cerevisiae* cultures, when briefly heated at a potentially lethal temperature or grown at supraoptimal temperatures (37–39 °C), accumulate a greater proportion of their cells as respiratory-deficient petites (Van Uden, 1984). This is thought to reflect sensitivity of the mitochondria to heat damage. Figs 4 and 5 indicate that this damage is enhanced considerably by the presence of intracellular sorbic acid, an effect more pronounced in low pH cultures due to the higher uptake of acid by the cells of these cultures (see Introduction).

The experiment in Fig. 5(b, c) was repeated, but changing the procedure such that cells were transferred to sorbate-free medium of a single, defined pH *immediately after* the 1 h incubation at 25 °C or 38 °C in media of different pH and *prior to* the 50 °C 5 min lethal heat treatment (Fig. 6). Survival of the cells heated in the absence of sorbate shows the well-established thermotolerance induction with mild heat (38 °C) pretreatment (compare Fig. 6a, b). Also it reveals that the pH of pre-incubation does not have a marked effect on thermotolerance, the pH of lethal heating being relatively much more important (compare Fig. 6a, b with 5b, c). Pretreatment at 25 °C of higher pH cultures with sorbate increased thermotolerance (Fig. 6a). Sorbate is therefore a chemical inducer of thermotolerance at higher pH. However, 25 °C sorbate treatment at lower pH caused relatively little thermotolerance change (Fig. 6a). Pretreatment with both mild heat (38 °C) and sorbate at higher pH values increased thermotolerance only slightly above the values achievable with heat shock alone, while sorbate largely prevented the normal increase in thermotolerance with sublethal heat at lower pH (Fig. 6b). The removal of sorbate prior to lethal heating eliminated the strong selection of petites seen in Figs 4 and 5, less than 5% of the survivors of lethal heating in Fig. 6 being petites irrespective of the pH of pre-incubation (not shown).

Fig. 6. Influences of sorbate pretreatment and pre-incubation pH on thermotolerance, when the subsequent lethal heating was conducted at a single defined pH and in the absence of sorbate. Cells were grown to early exponential phase (5×10^6 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 (see Methods) either without (open symbols) or with (filled symbols) 9 mM sorbic acid. After pre-incubation for 1 h at either 25 °C (a) or 38 °C (b) at these pH values, cultures were harvested by centrifugation, resuspended in pH 6.0 sorbate-free YPD, immediately heated for 5 min at 50 °C and then chilled. Survival is indicated relative to the survival of the original YPD starter culture given an identical heat stress.

Effects of the petite mutation on heat shock protein induction and on the activity of a HSE-*lacZ* fusion

We have observed that petites derived from *S. cerevisiae* strain SUB62 (whether spontaneous or sorbic-acid-induced) are capable of fermentative growth at low pH in the presence of concentrations of sorbic acid that totally inhibit the growth of respiration-competent cells. On pH 4.5 YPD plates petites are capable of slow growth in the presence of 4.5 mM sorbic acid, whereas the growth of respiration-competent cells becomes totally inhibited when sorbic acid exceeds approximately 1 mM (data not shown). The small proportion of petites in *S. cerevisiae* SUB62 cultures can therefore be selected as the only cells capable of appreciable growth on pH 4.5 plates containing 4.5 mM sorbate. Adaption to pH 4.5 fermentative growth at this concentration of weak acid preservative therefore involves selective outgrowth of the growth-arrested respiration-competent cells by the petites which are always present as a small proportion of the cells of *S. cerevisiae* cultures.

Millimolar concentrations of sorbate and benzoate inhibit both growth and the heat shock response (Figs 1, 2 and 3a) of respiration-competent cells at low pH. We were therefore intrigued to know whether such concentrations caused a similar inhibition of heat shock gene expression in the more sorbate-resistant petites derived from these cultures. At the lowest pH at which cultures of petites

Fig. 7. The petite mutation restores the capacity for synthesis of heat shock proteins in low pH cultures. Cells of a spontaneous petite derived from strain SUB62 (S) were resuspended in medium buffered at pH 4.5 or 7.5; also, cells of a petite derived by heating SUB62 in the presence of sorbic acid (i) were resuspended at pH 4.5. The cultures were then labelled with [^{3} H]leucine for 40 min either at 25 °C or immediately after heat shock to 39 °C in the presence and absence of 9 mM sorbic acid. Major heat shock proteins are indicated to the right of the figure.

could incorporate labelled amino acid into protein (pH 4.5) the presence of 9 mM sorbate reduced incorporation of [^{3} H]leucine into protein (Fig. 7). However, both a spontaneous petite and a petite induced by heating a SD medium culture in the presence of sorbate (Fig. 4b) showed detectable heat induction of heat shock proteins at pH 4.5 in the presence of sorbate (Fig. 7). Also a petite displayed limited heat-inducibility of a HSE-*lacZ* fusion in pH 5.0, 5.5 or 6.0 cultures heat shocked in the presence of 9 mM sorbate (Fig. 3b, c), irrespective of whether it had been cultured on SD medium for several generations in the absence (Fig. 3b) or the presence (Fig. 3c) of 1 mM sorbate. It remains to be seen whether this partial recovery of a heat shock response in low pH sorbate-treated cultures of petites contributes to the greater capacity of petites for fermentative growth in the presence of this preservative.

Other inhibitors of heat shock protein induction in yeast

Prior to this study only flavonoids had been shown to be selective inhibitors of the heat shock response (see Introduction), acting to block heat shock protein ex-

pression in mammalian cells (Hosokawa *et al.*, 1990). A flavonoid (quercetin) was tested to see whether it would act, like sorbic and benzoic acids (Figs 1 and 2), to inhibit heat shock protein induction in yeast. By protein pulse-labelling, as in Figs 1, 2 and 7, the addition of 50 μ M or 200 μ M quercetin to pH 4.5 and pH 7.5 *S. cerevisiae* cultures was found not to influence heat shock protein induction by heat shock (data not shown). It is possible that this result reflects a lack of permeability of yeast cells to quercetin, a point that was not investigated further.

The rapid pH_i decline due to weak acid preservatives (see Introduction) is a possible cause of sorbate- and benzoate-treated cultures of low pH being unable to mount a response to heat shock (Figs 1 and 2). Maintenance of pH_i in yeast largely occurs through plasma-membrane-ATPase-catalysed protein extrusion and the excretion of organic acids (Serrano, 1991). We have previously shown that a mutant with reduced plasma-membrane ATPase activity shows reduced heat shock protein induction by heat shock (Panaretou & Piper, 1990). We therefore tested an inhibitor of plasma membrane ATPase as a possible inhibitor of the heat shock response. This inhibitor, diethylstilboestrol, is known to hypersensitize

Fig. 8. Diethylstilboestrol, a plasma-membrane-ATPase inhibitor, inhibits heat induction of heat shock proteins. SUB62 cells were transferred to SD medium buffered to pH 4.5, 6.5 or 8.5. Diethylstilboestrol ($50 \mu\text{g ml}^{-1}$) was added immediately before addition of labelled leucine. Labelling was for 40 min, either at 25°C (–) or immediately after heat shock to 39°C (+). For the labelling of the control cells not subject to diethylstilboestrol treatment see Fig. 1. M is a marker track containing a sample of the proteins normally labelled in yeast at 39°C .

yeast to thermal death (Coote *et al.*, 1991). When used at a concentration ($50 \mu\text{M}$) that totally inhibits plasma-membrane-ATPase action (Coote *et al.*, 1991) it rapidly rendered cells incapable of a heat shock response, diethylstilboestrol-treated cells displaying no alteration in their protein synthesis pattern with heat shock to 39°C (Fig. 8). However, this inhibitory action differed from that caused by sorbic and benzoic acids in being apparently independent of external pH (compare Fig. 1 with Fig. 8).

Another way to cause a decline in pH_i in low pH fermentative cultures is to add an uncoupler such as 2,4-dinitrophenol or CCCP. Uncouplers cause membrane depolarization. They differ from sorbic and benzoic acids in that they permeate membranes in both their dissociated and protonated states with the result that uncouplers, unlike these preservatives, do not show pronounced intracellular concentration when pH_i values are higher than the extracellular pH. In pH 4.5 cultures $75 \mu\text{M}$ CCCP abolished heat induction of heat shock proteins, while $50 \mu\text{M}$ CCCP caused a greatly reduced induction of these

proteins (Fig. 9). This inhibition was not evident at pH 7.5 (Fig. 9). The concentrations of CCCP which were effective in inhibiting the heat shock response were considerably lower than the concentrations of sorbate or benzoate needed for similar levels of inhibition (data not shown). These results with weak acid preservatives, diethylstilboestrol and CCCP suggest that a substantial drop in pH_i and/or depolarization of the plasma membrane may prevent yeast cultures from mounting a heat shock response.

DISCUSSION

Weak acids block HSE induction in low pH cultures

This study has shown that weak acid preservatives inhibit the normal induction of major heat shock proteins in low pH cultures by heat shock (Figs 1 and 2). Measurements of HSE-*lacZ* activity (Fig. 3) show this to be due to lack of induction of the HSE promoter sequence. This inhibition of the heat shock response may reflect the depression of pH_i that weak acids cause, since a similar inhibition is also seen with uncouplers (Fig. 9) and an inhibitor of plasma-membrane ATPase (Fig. 8). It is an inhibition partially suppressed by respiratory deficiency (Figs 3 and 7).

Even though weak acids inhibit *heat induction* of the heat shock response in acidified yeast cultures, we find that they also act as *chemical inducers* of certain heat shock genes in the same cells. Northern blot analysis of heat shock gene mRNAs has shown that the mRNAs for HSP30, HSP26 and HSP12 are all induced at 25°C in the *absence of heat shock* after cells are treated with the concentrations of sorbate, benzoate and CCCP shown in this paper to inhibit the response to heat shock (unpublished results). Not all heat shock genes are induced under these conditions. Also, since the HSE promoter sequence is not induced by sorbate treatment at any medium pH (Fig. 3), another promoter element must be involved in these inductions of heat shock mRNAs at 25°C . Recently, an upstream activator (UAS) element unrelated to the HSE was demonstrated to cause the activation of the *CTT1* gene in response to a variety of stresses, including heat shock, osmotic stress, oxidative stress and nitrogen starvation (Marchler *et al.*, 1993). This alternative heat shock promoter element is one possible candidate for the sequence directing heat shock gene induction by weak acids in the absence of heat stress.

Weak acid preservatives and uncouplers may inhibit the heat shock response by lowering pH_i

Weak acid preservatives differ from weak acid uncouplers in that the former cross membranes readily only when undissociated, while uncouplers are lipophilic in both their protonated and unprotonated forms. Sorbate and benzoate will therefore concentrate inside cells and lower pH_i in response to a higher pH on the cytosolic side of the cell membrane (see Introduction) while uncouplers, in contrast, will depolarize membranes (Thevelein *et al.*, 1987) but not concentrate in the cytosol. Weak acid preservatives and uncouplers also have very different

Fig. 9. An uncoupler (CCCP) inhibits heat induction of heat shock proteins in low pH cultures. *S. cerevisiae* cells were incubated with [^{3} H]leucine for 40 min either at 25 °C (–) or after heat shock to 39 °C (+) in the absence and presence of CCCP. They had previously been resuspended in medium buffered at pH 4.5 or 7.5 as in Methods. At 75 μ M, CCCP appears to cause a total inhibition of heat shock protein induction, whereas 50 μ M CCCP produces more partial inhibition.

effects on yeast glycolytic flux. In low pH cultures preservatives inhibit (Krebs *et al.*, 1983; Francois *et al.*, 1986) while uncouplers stimulate glycolysis (see Francois *et al.*, 1986, 1988a, b, for literature).

In this study we have observed that weak acid preservatives (sorbate and benzoate) and an uncoupler (CCCP) have similar inhibitory effects on the response of low pH yeast cultures to heat shock (Figs 1, 2 and 9). It is improbable therefore that these inhibitions are linked to the dramatically opposite effects of these agents on glycolytic flux. Instead, they are more likely to reflect the common action of preservatives and uncouplers in causing pH_i decline in low pH cultures. The electrochemical gradient at the yeast plasma-membrane, largely established and maintained through the action of plasma-membrane ATPase (Serrano, 1991), is essential for nutrient uptake, maintenance of potassium balance and regulation of pH_i . The action of this ATPase also has a strong influence over the thermotolerance of *S. cerevisiae* and *Schizosaccharomyces pombe* (Panaretou & Piper, 1990; Coote *et al.*, 1991; Piper, 1993; Coote, 1993) and influences the ability of *S. cerevisiae* cells to synthesize heat shock

proteins in response to heat shock. A mutation which reduces ATPase activity (*pma1.1*) results in both a lowered pH_i (Ulazewski *et al.*, 1987) and reduced induction of heat shock proteins with heat shock (Panaretou & Piper, 1990). The similarities of uncouplers and weak acid preservatives in preventing a response to heat shock in low pH cultures (Figs 1, 2 and 9) might reflect their common action in reducing pH_i . Consistent with this, the plasma-membrane ATPase inhibitor diethylstilboestrol rapidly rendered cells incapable of a heat shock response even though their capacity for protein synthesis was not lost (Fig. 8). While the results in Figs 1–3, 8 and 9 are largely consistent with lowered pH_i preventing a response to heat shock, there may be other explanations for our results, including the direct inhibition of a stress-signalling pathway by the various chemical agents that we have employed.

The effects of sorbate on thermotolerance

Prior to this study N. Van Uden had reported that sorbate has no effect on the thermotolerance of *S. cerevisiae*, but shifts the Arrhenius plots and T_{max} of growth to lower

temperatures (Van Uden, 1984). It is difficult to assess this study as the conditions, notably pH, of growth were not reported. Coote *et al.* (1991) showed thermotolerance increasing during a 9 mM sorbic acid treatment, both at pH 4.5 and at pH 6.0. However, a partial thermotolerance increase was also seen in cells exposed to pH 4.5 in the absence of sorbate. In their study, Coote *et al.* (1991) conducted lethal heating after resuspending the cells in sorbate-free medium of defined pH (as in Fig. 6 of this paper). Our results clearly show that a 25 °C sorbate treatment at a pH below about 5.5 slightly reduces thermotolerance (Fig. 6a) and also that sorbate prevents the usual thermotolerance increase with sublethal heat treatment (Fig. 6b). However, in cultures of pH values above about 5.5 sorbate acts as a strong chemical inducer of thermotolerance even at 25 °C (Fig. 6a), there being little additional increase in thermotolerance when this treatment is carried out at a heat stress temperature (38 °C; Fig. 6b). Figs 4 and 5 show the pH of lethal heating exerting an appreciable influence on basal (25 °C) and sublethal heat-induced thermotolerance levels in the absence of sorbate. They also reveal the strong influence that medium pH exerts over the effects of sorbate on thermotolerance.

Heating low pH cultures with sorbate selects cytoplasmic petites

Upon entry to yeast cells sorbate concentrates within mitochondria, causing pronounced disruption of mitochondrial structure (Cole, 1987). Heating in the presence of sorbate strongly induces petites (Figs 4 and 5) indicating that this sorbate-induced damage to mitochondria is enhanced considerably by heat. This effect is probably more pronounced in low pH cultures because the acid penetrates cells more effectively in such cultures (see Introduction). Also, it occurs in glucose-grown *S. cerevisiae* in which mitochondrial functions are repressed. However, mitochondrial repression is never absolute and even on high sugar substrates respiration can provide as much as 34%, 48% and 88% of the total ATP yield during the aerobic fermentation of glucose, maltose and galactose respectively (Lagunas, 1986).

Possible reasons for the higher resistance of petites to growth in the presence of sorbate

The higher sorbate resistance of petites is unlikely to be due to the loss of any residual respiratory activity present in glucose-repressed cells. While weak acids inhibit respiration in *S. cerevisiae*, they also cause an inhibition of fermentation (Krebs *et al.*, 1983; Francois *et al.*, 1986, 1988a, b). In the more preservative-tolerant *Z. bailii* long-term adaptation to growth in the presence of weak acids involves a switch from a predominantly aerobic to a predominantly anaerobic metabolism, millimolar amounts of preservative causing almost immediate inhibition of oxidative respiration in *Z. bailii* yet, unlike in *S. cerevisiae*, practically no inhibition of fermentation (Cole, 1987; Cole & Keenan, 1987).

It has been proposed that the adaption of *Z. bailii* to

growth in the presence of weak acids involves the induction of an energy-requiring system for extrusion of the acid (Warth, 1978, 1988). While such active extrusion has not yet been discounted, it would probably be futile in conferring resistance since concentration of benzoate by *Z. bailii* cells is exactly as would be predicted from considerations of pH_i and the pK of the acid (Cole & Keenan, 1986, 1987). Instead, increases in plasma-membrane-ATPase-catalysed proton extrusion and a decreased protoplast volume undoubtedly contribute to maintaining the pH_i of *Z. bailii* cells that have adapted to growth in the presence of weak acids (Cole & Keenan, 1987). In addition, the PFK1 of *Z. bailii* shows less dramatic inhibition in response to pH_i depression (Cole, 1987) as compared to the PFK1 of *S. cerevisiae* (Krebs *et al.*, 1983; Francois *et al.*, 1986). This may better equip *Z. bailii* to maintain glycolytic flux in the presence of pH_i -depressing amounts of weak acids.

There is no evidence to indicate that adaption of yeasts to growth in the presence of weak acids can occur through reduced acid entry, the result of a permeability barrier at the cell membrane. However, uptake of certain molecules by *S. cerevisiae* is partly controlled by the functional state of mitochondria. Conversion of cells to petites, or treatment with inhibitors affecting mitochondrial function and biogenesis, causes deficiencies in the utilization of galactose, maltose and α -methyl-D-glucoside that are the result of reduced uptake of these sugars (Evans & Wilkie, 1976; Mahler & Wilkie, 1978). Also, the mitochondrial genome of *S. cerevisiae* controls cell permeation by several cytotoxic drugs, petites being considerably more resistant to killing by these agents (Mahler & Wilkie, 1978; L. Cheng & D. Wilkie, unpublished results). It will be interesting to determine if the enhanced resistance of *S. cerevisiae* petites to growth in the presence of weak acids has the same genetic basis. It will also be interesting to determine if this enhanced resistance is associated with the partial recovery of a response to heat shock in preservative-treated petite cultures of low pH (Figs 3 and 7).

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