Microbiology (2001), **147**, 2635–2642 Printed in Great Britain

REVIEW ARTICLE

Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives

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Keywords: *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, organic acid stress, oxidative stress, food spoilage

Overview

Frequently the decomposing plant materials where fungi grow as saprophytes will contain high concentrations of weak organic acids. Not only are acetate and lactate products of bacterial fermentation, but acetate is also secreted in high levels by certain yeasts, such as the *Brettanomyces* and *Dekkera* that have attracted attention as spoilage agents of wine fermentations (Pretorius, 2000). *Saccharomyces cerevisiae* is frequently inhibited by these acids produced by competitor microbes. To counteract their effects, it is endowed with a stress response that acts to reduce the possibility that the weak acid will accumulate within its cells to high, potentially toxic, levels.

Although weak acid adaptation probably evolved to facilitate growth at low pH in the presence of weak organic acids, it poses problems for the food industry as it leads to substantial increases in resistance to the major organic acid food preservatives. As a result, it is often necessary to use these preservatives at millimolar rather than micromolar levels in order to prevent yeast spoilage of low pH foods and beverages. This review summarizes the current knowledge of the mechanisms of weak acid resistance in *S. cerevisiae* and *Zygosaccharomyces bailii*, two important food spoilage yeasts. Both organisms are able to maintain lower intracellular levels of weak acid than would be expected on the basis of a free equilibration across the cell membrane. Nevertheless, it is unlikely they achieve this by identical strategies. *S. cerevisiae* expends considerable energy in actively extruding acid from the cell, high levels of a specific ATP binding cassette (ABC) transporter (Pdr12) being induced in its plasma membrane in order to catalyse this efflux. *Z. bailii*, in contrast, does not show major changes to its plasma membrane protein composition, but may place more reliance instead on limiting the initial diffusional entry of the acid to the cells. *Z. bailii*, unlike *S. cerevisiae*, can also catalyse oxidative degradation of two of the most commonly used food preservatives, sorbate and benzoate.

The problems posed by weak acid resistance

For centuries, man has applied natural preservatives and preservation methods, generally without knowing how these have helped to protect food from spoiling. Many of the natural compounds in herbs and spices, also plants such as onion, garlic and chives, influence microbiological stability in addition to being responsible for better-known characteristics of taste and smell (Lawson, 1996). Often active essential oils and related substances will act to make the cell membranes of fungi permeable, causing the contents to leak out. Other naturally occurring preservatives are weak organic acids. They include benzoic, acetic and sorbic acids. These, together with propionate and sulphite, nowadays constitute the acid preservatives most widely used in large-scale food and beverage preservation. It is possible though to trace the practical use of weak acid preservatives back many centuries, to the burning of sulphur to sterilize the wooden vessels used in cider making and the use of acetic acid in vinegars and pickles (Stratford & Anslow, 1996).

The conditions imposed by many preserved food materials [low water activity (a_w); low pH; the presence of high preservative levels, carbon dioxide or ethanol; or the absence of oxygen] do not represent the ideal environments for microbial growth. Yeasts and fungi pose a major spoilage threat for many materials preserved at low pH, low a_w and/or with high levels of

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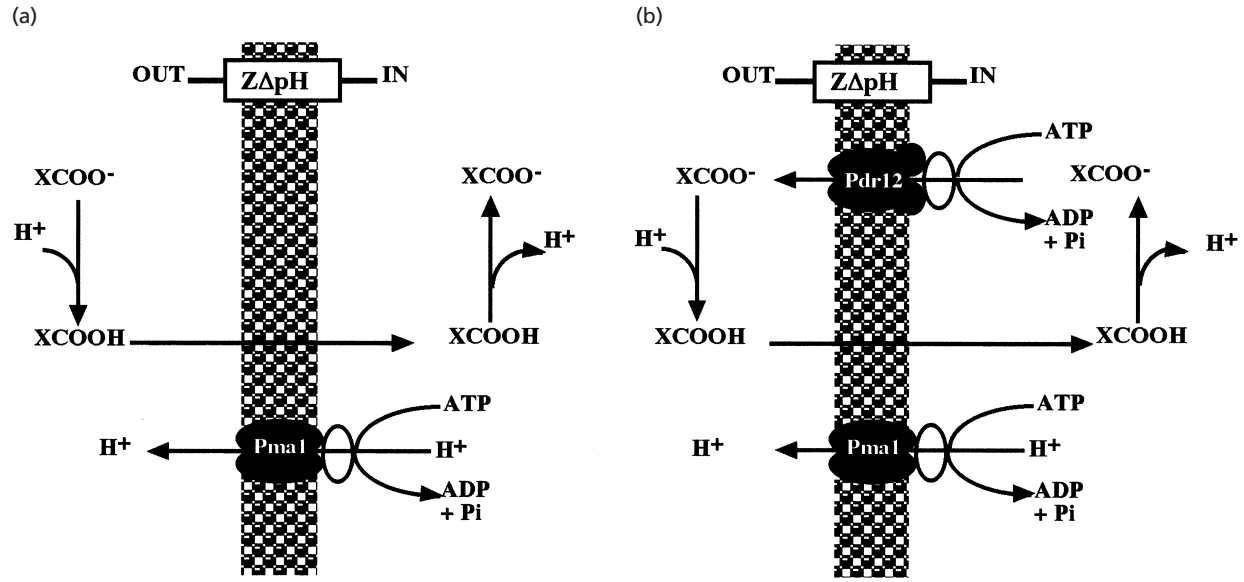

Fig. 1. Schematic model of how the Pdr12 ABC transporter may help *S. cerevisiae* counteract the inhibitory effects of water-soluble weak organic acids. In both unadapted cells (a) and cells which have adapted to grow in the presence of the weak acid (b), the protonated, uncharged form of the acid (XCOOH) is shown as freely permeable to the cell membrane and readily entering by diffusion. In unadapted cells (a), the concentration of XCOOH inside and outside should be the same, governed by the pH either side of the membrane and the dissociation constant of the acid. A higher pH on the cytosolic side of the membrane will cause a substantial fraction of this acid to dissociate to the anion ($XCOO^-$), a form which is relatively membrane-impermeant and that therefore will accumulate inside the cell. In weak-acid-adapted cells (b), it is necessary also to invoke some limitation to the free diffusional entry of weak acid into the cell (not shown). The electrochemical potential difference across the plasma membrane (*Z*ΔpH) is maintained largely through plasma membrane H^+-ATPase (Pma1)-catalysed proton extrusion.

preservative (Deak, 1991; Fleet, 1992). Nevertheless, it is only a tiny fraction of the very many characterized yeast species that are of major importance in this regard and which are therefore classified as 'spoilage yeasts' (see Steels *et al*., 2000, and references cited therein). They include a number of *Zygosaccharomyces*, as well as some isolates of *S. cerevisiae*. Often *Zygosaccharomyces rouxii* is considered the most important *Zygosaccharomyces* from the standpoint of an ability to grow at very low a_w, while *Z. bailii* and *Zygosaccharomyces lentus* are the most important from the standpoint of weak acid preservative resistance. The latter yeasts can sometimes grow in the presence of the highest levels of those acids allowed in food preservation, at pH values below the pK_a values of these acids (Thomas & Davenport, 1985; Deak, 1991; Fleet, 1992; Steels *et al*., 1999, 2000). *Z. bailii* has also attracted attention as a spoilage agent of wine, a property that accrues from its high ethanol tolerance (Kalathenos *et al*., 1995) and ability to metabolize acetic acid in the presence of the complex mixtures of sugars often found in wine fermentations (Sousa *et al*., 1996, 1998).

In any *Z. bailii* culture, the individual cells differ very considerably in their sorbate resistance, a small fraction being remarkably resistant (Steels *et al*., 2000). The minimum sorbate levels needed to inhibit the growth of *Z. bailii* therefore increase with the size of the inoculum, making it difficult to place numerical values on the weak acid resistances of this organism, or the extents to which these resistances exceed those of *S. cerevisiae*. Nevertheless, *S. cerevisiae* isolated from instances of food spoilage can frequently adapt to levels of sorbate and benzoate only slightly lower than the levels inhibitory to *Z. bailii* and *Z. lentus*.

Apart from a number of studies on the uptake and utilization of acetate by *Z. bailii* (Sousa *et al*., 1996, 1998), comparatively little is known about how *Z. bailii* and *Z. lentus* acquire their remarkable weak acid resistances. Considerably more is known about weak acid adaptation in *S. cerevisiae*, as this is a species amenable to genetic analysis. This article will therefore focus mainly on *S. cerevisiae*, where the acquisition of weak organic acid resistance appears to involve a stress response quite distinct from other, more widely studied, stress responses, such as those induced by osmostress or heat shock. A picture is emerging of an adaptation that acts to limit accumulation of the weak organic acid in the cells of the adapted yeast. Resistance to sulphite, a non-organic weak acid preservative (Pilkington & Rose, 1988), is not discussed.

Weak acid stress in *S. cerevisiae*

At low pH, acetic acid (pK_a 4·75), sorbic acid (pK_a 4·76) or benzoic acid (pK_a 4·19) will exist substantially in the undissociated state (XCOOH; Fig. 1), a form in which

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they are potent growth inhibitors. Fig. 1(a) shows the general perception of how they inhibit microbial growth. The undissociated acid, being uncharged, readily diffuses across the cell membrane only to dissociate in the higher pH environment of the cytosol. Such dissociation generates protons and the acid anion ($XCOO^-$; Fig. 1). The acid anion will tend to accumulate intracellularly to very high levels as, being charged, it cannot very readily diffuse from the cell. This high anion accumulation may generate an abnormally high turgor pressure. It can also influence free radical production, leading to the severe oxidative stress that is a major component of weak organic acid stress in aerobic *S. cerevisiae* (see below). The proton release can potentially acidify the cytosol. This acidification, if it occurs, will inhibit many metabolic functions (Krebs *et al.*, 1983). Reductions in *S. cerevisiae* intracellular pH (pH_i) have been demonstrated following the addition of acetate (see Arneborg *et al.*, 2000 for literature) and benzoate (Krebs *et al.*, 1983) though, as discussed later, a reduction in pH_i is not always a feature of organic acid stress.

The antimicrobial effects of weak organic acids at low pH have often been attributed to this intracellular acidification and anion accumulation (Krebs *et al.*, 1983; Russell, 1991; Salmond *et al.*, 1984). It is most improbable though that this represents the complete explanation of their actions, or that these acids are all operating identically to inhibit growth. There have been a number of side-by-side comparisons of the effects of acetate and sorbate on *S. cerevisiae* (Stratford & Anslow, 1996, 1998; Bracey *et al.*, 1998). These indicate that, while acetate might possibly be acting mainly as in Fig. 1(a), the more hydrophobic sorbate is mainly inhibiting cells through a disordering of membrane structure. Quite high concentrations of acetic acid (80–150 mM) are needed to totally inhibit the growth of *S. cerevisiae* at pH 4·5. In contrast, only 1–3 mM of the more liposoluble sorbate, an acid with an identical pK_a and therefore degree of dissociation to acetate, can achieve this same degree of inhibition (Stratford & Anslow, 1996; Piper *et al.*, 1998). Also, while decreases in pH_i have been observed with the addition of acetate to *S. cerevisiae* (Arneborg *et al.*, 2000), they are not seen with sorbate addition (Bracey *et al.*, 1998). Instead, sorbate-stressed yeast may be suffering more from the effects of appreciable disruption to membrane organization and the oxidative stress caused by associated effects on respiratory chain function (Piper, 1999). A strong action of many acid preservatives on membranes is also apparent from the strong propensity of monocarboxylic acids to become more inhibitory as they become more lipophilic (Piper *et al.*, 1998; Stratford & Anslow, 1998; Holyoak *et al.*, 1999).

Sorbate- and benzoate-stressed *S. cerevisiae* and *Z. bailii* are, in addition, experiencing a very severe energy (ATP) depletion (Warth & Nickerson, 1991; Piper *et al.*, 1997). At least in *S. cerevisiae* this energy crisis is partly caused by strong inhibitory effects of sorbate and benzoate on glycolysis, an inhibition exerted mainly at the phosphofructokinase (Pfk) reaction (Krebs *et al.*, 1983; Pearce *et al.*, 2001). The trehalose accumulation with sorbate treatment of *S. cerevisiae* is probably in response to this Pfk inhibition (Cheng *et al.*, 1999). In the presence of oxygen, this energy crisis is exacerbated still further by the severe influences of the more lipophilic weak acid preservatives on membrane transport processes and energy coupling. The associated mitochondrial electron transport chain dysfunction increases free radical formation, causing sorbate- and benzoate-treated *S. cerevisiae* to suffer an excessively high endogenous production of superoxide free radicals (Piper, 1999).

Generally it is in low pH cultures that these effects of weak organic acids are most apparent. At neutral pH, residues of acetic, sorbic or benzoic acids are essentially completely dissociated. As such, they pose a much smaller threat and may even provide a potential carbon source. At neutral pH though, high sorbate levels still exert some inhibitory effects on *S. cerevisiae* (Stratford & Anslow, 1996) and a strong transcriptional response to sorbate is still apparent (Martinez-Pastor *et al.*, 1996; Piper *et al.*, 1998).

Weak acid adaptation in *S. cerevisiae*

pH 4·5 cultures of *S. cerevisiae* can be induced to adapt to weak acid stress with addition of around 0·5–2·5 mM sorbate or benzoate. Neither of these preservatives can be degraded by *S. cerevisiae* such that their presence effectively provides continuous weak acid stress. Immediately following this weak acid addition the cells usually exit the cell cycle and enter a long period of stasis. Eventually, after several hours, they resume growth (Piper *et al.*, 1997, 1998; Holyoak *et al.*, 1999). They are now weak-acid-adapted, in that they will not display transient growth arrest if reinoculated into fresh pH 4·5 medium containing the same levels of sorbate or benzoate. Remarkably, non-acid-pretreated cells of the *S. cerevisiae cmk1* mutant do not have this requirement for a long adaptive period of growth arrest, resuming growth almost immediately after exposure to sorbate stress (Holyoak *et al.*, 2000). The weak acid adaptation system is therefore repressed by a mechanism requiring the Cmk1p isoform of Ca^{2+}/calmodulin-dependent protein kinase, a repression that must be relieved in wild-type cells as they undergo adaptation (Holyoak *et al.*, 2000).

Our interest in weak acid stress was originally generated by the chance discovery that these acids act as inhibitors of yet another stress response, the heat-shock response (Cheng & Piper, 1994). We subsequently discovered that an alternative stress response was being induced, a response leading to strong induction of two plasma membrane proteins, Pdr12 and Hsp30 (the latter so called because it is also a heat-shock protein) (Panaretou & Piper, 1992; Piper *et al.*, 1997, 1998). Pdr12 is the product of one of the 31 ABC transporter genes in the *S. cerevisiae* genome (Bauer *et al.*, 1999). So strong is its weak acid induction that it becomes one of the most

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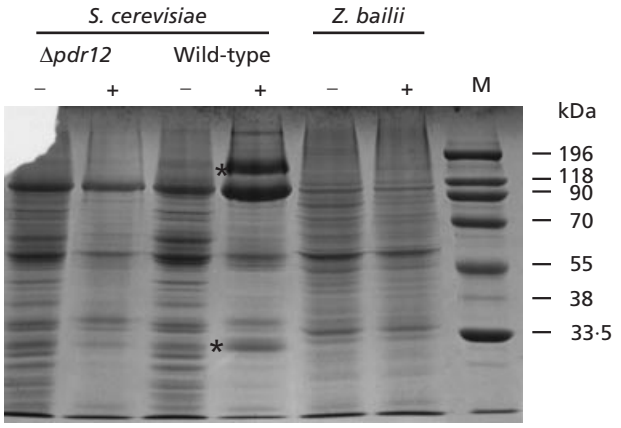

Fig. 2. Plasma membrane proteins of *pdr12* and wild-type *S. cerevisiae* FY1679-28c and also *Z. bailii* NCYC 1427, grown for 16 h on liquid pH 4·5 YPDA, either in the absence (−) or the presence (+) of 1 mM sorbate. In wild-type *S. cerevisiae*, such conditions cause strong induction of Pdr12 and Hsp30 (upper and lower asterisked bands, respectively), whereas only very weak induction of a protein of similar size to Pdr12 is apparent in the plasma membranes from *Z. bailii*. The intense 100 kDa band in the *S. cerevisiae* samples, corresponding to plasma membrane H^+-ATPase (Pma1), is much less abundant in the membrane samples from *Z. bailii*. Thirty micrograms of total protein from sucrose-gradient-purified plasma membranes was loaded in each lane of this 12·5 % SDS gel; these proteins were purified and detected by staining with Coomassie blue as described earlier (Panaretou & Piper, 1992).

abundant plasma membrane proteins in adapted cells (Fig. 2).

A full characterization of weak acid adaptation must await detailed analyses of the changes to the *S. cerevisiae* transcriptome and proteome in response to challenges with different weak organic acids. This adaptation may involve some of the *S. cerevisiae* open reading frames (ORFs) of unknown function, since we recently isolated two such ORFs (*YOR114w* and *YDR246w*) as multicopy suppressors of the sorbate sensitivity of the *pdr12* mutant (unpublished results).

Pdr12 induction is important for *S. cerevisiae* to adapt to growth in the presence of weak organic acids

Cells lacking the Pdr12 transporter (the *pdr12 S. cerevisiae* mutant) are hypersensitive to water-soluble monocarboxylic acids of relatively short aliphatic carbon chain length (Piper *et al*., 1998; Holyoak *et al*., 1999). They are also sensitive to short-chain alkanols (n-propanol, n-butanol and n-pentanol), although any effect of Pdr12 on ethanol resistance is marginal (our unpublished data). However, *pdr12* cells are not sensitive to dicarboxylic acids, or those highly lipophilic, long-chain fatty acids and alcohols whose toxic effects are thought to be mainly due to a detergent disruption effect on membranes (Holyoak *et al*., 1999; Weber & de Bont, 1996). It appears, therefore, that Pdr12 imparts resistance to those organic acids or alcohols that can, to a reasonable degree, partition into both lipid and aqueous phases (Holyoak *et al*., 1999, 2000). Its loss does not render cells hypersensitive to the known substrates for other well-characterized *S. cerevisiae* ABC transporters, drug pumps such as Pdr5 or Snq2 (Bauer *et al*., 1999).

The ability of Pdr12 to give resistance to short-chain alkanols, compounds whose toxic effects are thought to be due mainly to their ability to dissolve in membranes (Weber & de Bont, 1996), suggests that this ABC transporter may bind acid anions or alcohol molecules *actually incorporated in the inner leaflet of the plasma membrane* (rather than dissolved in the cytosol as shown in Fig. 1b). One can surmise that Pdr12 then transports these to the opposite (periplasmic) side of the membrane, in order to release them into the aqueous phase of the periplasm. Such active efflux may be able to lower the intracellular level of the acid anion or alcohol, on the basis that the polar groups on these carboxylate anion or alcohol substrates will slow their diffusion back across the cell membrane and consequent re-equilibration between the extracellular and intracellular milieu.

It is possible to visualize Pdr12 activity *in vivo* as the ability of cells to catalyse an active extrusion of fluorescein. Experimentally this involves loading the cells with fluorescein diacetate, then observing the energy-dependent efflux of the fluorescent weak acid produced by the actions of intracellular esterases on this fluorescein diacetate. Such fluorescein extrusion is competitively inhibited by the presence of weak organic acid preservatives (Holyoak *et al*., 1999). Even though wild-type cells cultured at pH 4·5 in the absence of weak acids contain Pdr12 in their membranes (Piper *et al*., 1998), they do not display this active fluorescein efflux (Holyoak *et al*., 2000). Pdr12 may not therefore be an active transporter in the absence of weak acid stress. In contrast, pH 4·5 cultures of *cmk1* cells do display an active efflux of fluorescein, even though their Pdr12 levels are no higher than in $CMK1^+$ cultures (Holyoak *et al*., 2000). This indicates that the Cmk1 protein kinase maintains the Pdr12 transporter in an inactive state until there is the need for a *catalysed* acid efflux.

The plasma membrane H^+-ATPase in weak acid adaptation

The plasma membrane H^+-ATPase, an ATP-driven proton efflux pump (Pma1; Figs 1 and 2), is yet another important activity counteracting weak acid stress. In all fungal and plant cells, this constitutes the main activity contributing to the generation of the electrochemical potential across the cell membrane ($Z\Delta pH$; Fig. 1), a potential that drives nutrient uptake and that regulates ion and pH balance (Serrano, 1991). Any acidification of the cytosol due to the intracellular dissociation of weak organic acid could, in principle, be counteracted by increasing H^+-ATPase-catalysed proton extrusion from the cell (Fig. 1). A strong activation of plasma membrane

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H^+-ATPase does indeed occur in weak-acid-stressed cells, as revealed from measurements of H^+-ATPase activity in purified plasma membranes (Piper *et al.*, 1997; Viegas & Sa Correia, 1991) and proton extrusion by intact cells (Holyoak *et al.*, 1996). A lowered expression of the gene encoding H^+-ATPase (*PMA1*) also increases the sensitivity of cells to weak acids (Holyoak *et al.*, 1996).

According to the model in Fig. 1(a), acid influx will act to dissipate the pH, though not the charge (*Z*) component of the electrochemical potential at the plasma membrane. The same would also occur with the addition of a classical uncoupler such as 2,4-dinitrophenol (the effects of sorbate and 2,4-dinitrophenol on *S. cerevisiae* being remarkable in their similarity; Stratford & Anslow, 1996). The extents to which increased H^+-ATPase activity *alone* can counteract any intracellular acidification that accompanies weak acid influx (Fig. 1) may be limited, since there is a finite limit to the extent that H^+-ATPase action can enhance the charge component (*Z*) of the electrochemical potential ($Z\Delta pH$). One way to avoid this problem is to ensure the movement of a charge that compensates for the charge on a H^+-ATPase-extruded proton. Anion ($XCOO^-$) exit from the cell, as through a membrane pore, could satisfy this requirement and could be driven by the membrane potential. However, the movement of this compensating charge in adapted *S. cerevisiae* cells is also satisfied with Pdr12-transporter-catalysed extrusion of an acid anion (Fig. 1b). There might thus be two benefits of *catalysed* anion extrusion in organic-acid-stressed *S. cerevisiae*: (i) a lowering of intracellular acid levels; and (ii) movement of a charge that balances the charge on a H^+-ATPase-extruded proton, thereby facilitating higher levels of *catalysed* proton extrusion. The *combined* actions of H^+-ATPase and Pdr12 may therefore be needed for acid-stressed *S. cerevisiae* to restore homeostasis to the point where growth can resume (Fig. 1b). This is undoubtedly extremely expensive in terms of energy consumption, at least 2 ATPs being consumed for each weak acid molecule that enters the cell (Fig. 1b). This very high energy requirement of counteracting weak acid stress is reflected in the dramatic reductions in biomass yield for cultures grown in the presence of this stress (Warth, 1988; Viegas & Sa Correia, 1991; Verduyn *et al.*, 1992; Stratford & Anslow, 1996; Piper *et al.*, 1997).

What is the role played by the other weak-acid-induced plasma membrane protein, Hsp30? Cells of the *hsp30* mutant are considerably less sensitive to sorbate than *pdr12* cells, revealing Hsp30 to be less important for weak acid resistance than Pdr12. With the loss of Hsp30 the sorbate activation of the H^+-ATPase is enhanced, indicating that Hsp30 acts in some manner to limit the activation of H^+-ATPase by stress (Piper *et al.*, 1997). Also *hsp30* mutant cells take longer to adapt to weak acid stress and, when stressed, have abnormally low ATP levels that might be a reflection of their excessive H^+-ATPase activity. We have therefore suggested that Hsp30 might serve an energy conservation function in stressed cells (Piper *et al.*, 1997).

Multiple plasma membrane transporters influence weak acid resistance

Pdr12 is not the only membrane transporter that influences the weak acid resistances of *S. cerevisiae*. It may not even be the only transporter capable of catalysing benzoate efflux in adapted *S. cerevisiae*, since some active benzoate efflux is still measurable in *pdr12* cells (Piper *et al.*, 1998). Also, while Pdr12 confers some resistance to acetate (Piper *et al.*, 1998), a plasma membrane transporter of the major facilitator superfamily (Azr1p) is also a contributor to this resistance (Tenreiro *et al.*, 2000). However, Azr1p does not appear to be providing resistance by catalysing the active efflux of acetate from cells. Also, while Azr1p confers acetate and propionate (also azole drug) resistance, it confers no resistance to either sorbate or benzoate (Tenreiro *et al.*, 2000).

The sorbate resistance of *S. cerevisiae* is *elevated* with the loss of Pdr1, a transcription factor that regulates a number of the genes for ABC transporters (though not it appears *PDR12*) (Piper *et al.*, 1998). A number of the Pdr1 target genes, including genes for ABC transporters other than Pdr12, may therefore *counteract* weak acid resistance. Thus weak acid adaptation, while it involves *inducing* Pdr12, may also require the *downregulation* of other plasma membrane transporters. The mRNA for Pdr5, a major determinant of drug resistance in yeast (Bauer *et al.*, 1999), has been observed to disappear in response to sorbate stress (Piper *et al.*, 1998).

What is the *PDR12*-inducing signal?

The *PDR12* gene is induced very strongly by weak organic acid stress (maximally by 1 mM sorbate in pH 4·5 cultures and by 8 mM sorbate in pH 6·8 cultures; unpublished data). It is nevertheless relatively unresponsive to a wide range of other stresses (heat shock, ethanol, osmostress, oxidative stress) (Piper *et al.*, 1998), suggesting the existence of a specific system for sensing weak acid stress. Consistent with this, the sorbate induction of *PDR12* is independent of Pdr1p/Pdr3p, Yap1p, Msn2p/Msn4p (Piper *et al.*, 1998) or Yrr1p (unpublished), transcription factors that direct the induction of several stress genes as well as a number of the other ABC transporters of yeast (Bauer *et al.*, 1999). The sorbate induction of Hsp30 is also independent of known stress gene activators of *S. cerevisiae* (Seymour & Piper, 1999). The transcription factor(s) that directs the weak acid induction of *PDR12* and *HSP30* therefore remain to be identified. Nevertheless, the lack of any involvement of known stress regulators in this induction, also the strong selectivity of weak acid stress as the major inducer of *PDR12*, indicates the weak acid response to be quite distinct from other, more extensively characterized, stress responses of *S. cerevisiae*.

What is the signalling mechanism leading to *PDR12* induction? This is not known, but it appears not to require MAP kinase signalling pathways or Ca^{2+}/

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calmodulin signalling mediated via calcineurin, since none of the appropriate mutants affect sorbate sensitivity (unpublished observations). Conceivably the inducing signal could simply be a high intracellular level of acid anion, acting through a direct binding to the relevant transcription factor.

How do yeasts avoid a futile cycle of diffusional entry and active extrusion of organic acids?

The induction of high levels of *active* proton and acid anion extrusion from cells would seem pointless without some limitation to the diffusional entry of the undissociated acid (XCOOH; Fig. 1b). Without such limitation, acid could diffuse into the cells as fast as it is pumped out in a futile, and energetically very expensive, cycle. Nevertheless, Pdr12 action clearly enhances sorbate, benzoate and acetate resistance (Piper *et al*., 1998; Holyoak *et al*., 1999). Also it has long been known that *acid-adapted S. cerevisiae* and *Z. bailii* can maintain an intracellular versus extracellular distribution of benzoate that is not in equilibrium (Warth, 1977, 1988, 1989; Henriques *et al*., 1997; Piper *et al*., 1998). This indicates the induction of a system whereby adapted cells can reduce their intracellular weak acid pool. It is unlikely that active acid extrusion alone would be sufficient to achieve this. Instead, the adapted cells must somehow restrict diffusional entry of weak acid across the cell envelope. This may be a most critical part of the weak acid response mechanism. How it occurs is essentially unknown, though it is established that cell wall mannoproteins can limit the porosity of the yeast cell wall (De Nobel & Barnett, 1991). *Z. bailii* may be more adept at enforcing this limitation to acid entry than *S. cerevisiae* (see below). When it adapts to the presence of 2 mM benzoic acid there is a 40% reduction in the permeability of the cells to benzoate but, remarkably, no decrease in permeability to propionate (Warth, 1989).

The influences of oxygen on weak acid resistance

S. cerevisiae cells are more weak acid sensitive when oxygen is present. Under such conditions, these acids generate severe oxidative stress by strongly enhancing endogenous production of superoxide free radicals by the mitochondrial electron transport chain (Piper, 1999). The extreme sensitivity of low-pH cultures of the *pdr12* mutant to sorbate and benzoate is due to this excessive free radical production, as it is substantially reversed with loss of superoxide dismutase activities (Piper, 1999). It would appear less harmful for this superoxide to diffuse from weak acid stressed cells [as probably occurs as the protonated form ($HO_2^{\bullet}$) in the *pdr12,sod1* mutant] than for it to be dismutated to hydrogen peroxide (as would normally occur in *pdr12,SOD1*$^+$ cells). This reflects the potential of hydrogen peroxide to engage in Fenton chemistry, leading to production of the extremely damaging hydroxyl radical. Free radical production is also the reason that sorbate and benzoate are strongly mutagenic towards the mitochondrial genome, as reflected in the high rates of petite segregation by acid-stressed aerobic *S. cerevisiae* (Piper, 1999).

Z. bailii is a petite-negative yeast. Aerobic, weak-acid-stressed *Z. bailii* does not therefore segregate respiratory-deficient cells. Instead it is able to oxidatively degrade sorbate and benzoate and use these compounds as sole carbon source (Mollapour & Piper, 2001). In contrast, *S. cerevisiae* is unable to utilize benzoate as it lacks a benzoate 4-hydroxylase (Mollapour & Piper, 2001). We recently isolated a small *Z. bailii* gene (*ZbYME2*) that, when heterologously expressed in *S. cerevisiae*, confers the ability to catabolize benzoate, sorbate and phenylalanine (Mollapour & Piper, 2001). *ZbYME2* encodes a protein with high (74%) sequence similarity to the N-terminal, mitochondrial matrix domain of the *S. cerevisiae* Yme2p (Leonhard *et al*., 2000) and the product of *ZbYME2* expressed heterologously in *S. cerevisiae* is also mitochondrial (unpublished data). Probably, therefore, *ZbYME2* confers a broad-specificity monooxygenase function with benzoate 4-hydroxylase activity in the mitochondrion of *Z. bailii*, a function that may have been lost by its *S. cerevisiae* homologue, *YME2*.

We recently deleted the two *ZbYME2* gene copies in the *Z. bailii* genome. The resulting mutant lacks any ability to utilize benzoate or sorbate as carbon sources and is, in addition, more sensitive to benzoate and sorbate inhibition on pH 4·5 glucose plates (Mollapour & Piper, 2001). *ZbYME2* therefore contributes to the weak acid resistance of *Z. bailii*, probably as a mitochondrial monooxygenase that facilitates preservative degradation.

Z. bailii and *S. cerevisiae* may not use identical strategies for acquiring weak organic acid resistance

So strong is the Pdr12 induction in weak-acid-stressed *S. cerevisiae* that levels of this transporter in the plasma membrane approach those of the most abundant plasma membrane protein, the H^+-ATPase (Fig. 2). There is no equally strong induction of a putative weak acid transporter in the plasma membrane of sorbate-stressed *Z. bailii* (Fig. 2). Why, therefore, is *Z. bailii* more weak acid resistant than *S. cerevisiae*? One explanation appears to be its ability to degrade preservatives even when in the presence of high sugar concentrations. Thus *Z. bailii* can consume acetate whilst growing on fermentable sugars (Sousa *et al*., 1998), whereas the acetate uptake (proton and acetate anion symport) and utilization systems of *S. cerevisiae* are all glucose-repressed (Casal *et al*., 1996). Similarly the (*ZbYME2*-dependent) capacity of *Z. bailii* to degrade sorbate and benzoate causes a significant enhancement of its resistances to these preservatives (Mollapour & Piper, 2001).

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Z. bailii might also have developed much more efficient ways of changing its cell envelope so as to limit the diffusional entry of the acid. This, in turn, will dramatically reduce any need for active extrusion of protons and acid anions. Indications that this may be the case come from observations that *Z. bailii* is much more resistant than *S. cerevisiae* to any short-term decrease in pH_i induced by acetate (Arneborg *et al.*, 2000). Also the trehalose induction of sorbate-stressed *S. cerevisiae*, symptomatic of glycolysis becoming inhibited at the Pfk step, is not observed with *Z. bailii* (Cheng *et al.*, 1999). Instead, conditions of sorbate and benzoate stress that lead to Pfk inhibition in *S. cerevisiae* tend to stimulate, rather than inhibit, *Z. bailii* glycolytic flux (Cole, 1987) and lead to no induction of trehalose (Cheng *et al.*, 1999). However, it is unclear if this represents the glycolytic flux of *Z. bailii* being more resistant to acid inhibition, or a reduced permeation of the acid into *Z. bailii* cells relative to the cells of *S. cerevisiae*. When the benzoate sensitivities of several different yeast species were compared, these were, to a rough approximation, *inversely* proportional to rates of diffusional entry of propionate into the cells (Warth, 1989). Reducing diffusional entry of the acid into the cells is therefore probably a key mechanism of resistance.

The strategy adopted by *S. cerevisiae*, whereby resistance is conferred largely through high levels of proton and acid extrusion (see Fig. 1b), potentially has one fundamental flaw. Active (mainly Pdr12-catalysed) anion extrusion at the plasma membrane can only export the weak acid as far as the periplasm (Fig. 1b). From there, undissociated acid can possibly just as readily diffuse back into the cell as out through the cell wall. Ensuring that the *initial* diffusion of the acid through the cell wall or membrane is much more restricted, so that lower amounts initially reach the periplasm or cytosol, should be a much better strategy for achieving resistance. It is conceivable that *Z. bailii* puts more reliance on the latter strategy and that therein lies the secret of its extreme weak acid resistance. This would explain why acid-adapting *Z. bailii* has no apparent need for any dramatic induction of a weak acid transporter (Fig. 2) and suffers no reductions in pH_i with acetate challenge.

Acknowledgements

We are indebted to Karl Kuchler, Peter Coote, Stanley Brul, Frans Klis, Graeme Walker and Aharon Abeliovich for many stimulating discussions. Our work on weak acid resistance has been supported by BBSRC grants 31/D10371 and 31/13918.

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