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The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast

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Exposure of *Saccharomyces cerevisiae* to sorbic acid strongly induces two plasma membrane proteins, one of which is identified in this study as the ATP-binding cassette (ABC) transporter Pdr12. In the absence of weak acid stress, yeast cells grown at pH 7.0 express extremely low Pdr12 levels. However, sorbate treatment causes a dramatic induction of Pdr12 in the plasma membrane. Pdr12 is essential for the adaptation of yeast to growth under weak acid stress, since Δ*pdr12* mutants are hypersensitive at low pH to the food preservatives sorbic, benzoic and propionic acids, as well as high acetate levels. Moreover, active benzoate efflux is severely impaired in Δ*pdr12* cells. Hence, Pdr12 confers weak acid resistance by mediating energy-dependent extrusion of water-soluble carboxylate anions. The normal physiological function of Pdr12 is perhaps to protect against the potential toxicity of weak organic acids secreted by competitor organisms, acids that will accumulate to inhibitory levels in cells at low pH. This is the first demonstration that regulated expression of a eukaryotic ABC transporter mediates weak organic acid resistance development, the cause of widespread food spoilage by yeasts. The data also have important biotechnological implications, as they suggest that the inhibition of this transporter could be a strategy for preventing food spoilage.

Keywords: ABC protein/adaptation/*Saccharomyces cerevisiae*/stress response/weak organic acid tolerance

Introduction

Weak acid preservatives are generally considered safe antimicrobials, consistent with the long history and now widespread use of these compounds for the preservation of foods and beverages. For instance, the use of sulfite for the sterilization of wine vessels is centuries old and still used in wine making. In solution, these acids are in a dynamic, pH-dependent equilibrium between their undissociated molecules and anionic states. An acidic pH favours the undissociated, uncharged state, a state in which weak acid preservatives exert much stronger antimicrobial action. This is probably because such action largely involves the uncharged acid diffusing through the plasma membrane into the cytoplasm, where it encounters a more neutral pH and consequently dissociates. This dissociation releases protons, the resulting intracellular acidification inhibiting several metabolic processes (Krebs *et al.*, 1983).

In yeast, weak acid preservatives characteristically cause an extended lag phase and cell stasis, rather than cell death. The ability of certain yeast species to grow at low pH in the presence of weak organic acid food preservatives enables them to act as important agents of food spoilage which can cause considerable economic losses (Deak, 1991; Fleet, 1992). Certain strains of *Saccharomyces cerevisiae* will grow in the presence of up to 3 mM sorbic acid at pH 4.5, although the presence of the preservative causes both a drastic lag phase extension and a reduction of final biomass yield (Stratford and Anslow, 1996; Piper *et al.*, 1997). Although *S.cerevisiae* is sometimes identified as a food spoilage organism, other even more weak acid-tolerant and osmotolerant yeasts such as *Zygosaccharomyces bailii* are more frequently found causing food spoilage. These yeast species are sometimes capable of adapting to growth in the presence of the highest levels of weak organic acids allowed in commercial food preservation, at pH values less than the pK_as of these acids (Deak, 1991; Fleet, 1992).

We have been investigating whether weak acid adaptation by *S.cerevisiae* involves a novel stress response or is the manifestation of an already identified stress response pathway. In this yeast, we found that weak organic acid treatment at low pH rapidly renders cells refractory to the well-studied heat shock response, inhibiting both heat shock protein (Hsp) and thermotolerance induction by sublethal heat stress (Cheng *et al.*, 1994). Instead, sorbic acid treatment at pH 4.5 stimulates a hitherto unknown stress response pathway, leading to a strong induction of two plasma membrane proteins, one of which was identified earlier as Hsp30, a protein that is also induced by heat shock and ethanol (Piper *et al.*, 1997). Hsp30 assists weak acid adaptation, since cultures lacking Hsp30 show reduced biomass yields and take longer to adapt to growth in the presence of sorbate (Piper *et al.*, 1997).

In this study, we identify the larger sorbate-induced protein as the ATP-binding cassette (ABC) transporter Pdr12, a homologue of the Snq2 (Servos *et al.*, 1993) and Pdr5 (Balzi *et al.*, 1994; Bissinger and Kuchler, 1994) ABC drug efflux pumps. We demonstrate that the induction of Pdr12 plays a pivotal role in the acquisition of tolerance to weak organic acid preservatives such as sorbate and

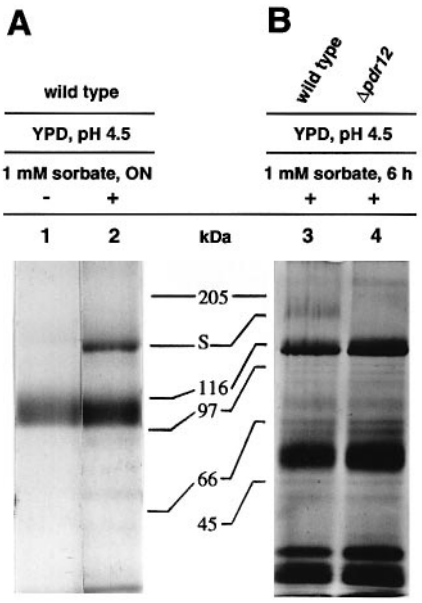

Fig. 1. Purified plasma membrane fractions from sorbate-treated yeast cells show a highly induced (S) membrane protein. (**A**) Wild-type cells were cultured overnight (ON) in pH 4.5 YPD in the absence (1) and presence (2) of 1 mM sorbate. About 40 μg total plasma membrane protein per lane were separated through a 9% SDS–polyacrylamide gel and stained with Coomassie blue. (**B**) About 8 μg of total plasma membrane proteins from wild-type (3) and Δ*pdr12* (4) cells grown for 6 h in pH 4.5 YPD in the presence of 1 mM sorbate were analysed by SDS–PAGE and silver-staining. The main 100 kDa band represents the Pma1 plasma membrane H^+-ATPase.

benzoate. Weak acid-mediated Pdr12 induction and concomitant development of tolerance is independent of the Yap1 (Kuge and Jones, 1994) and Msn2/Msn4 (Martinez-Pastor *et al.*, 1996) transcription factors, all of which are important stress response regulators. Surprisingly, sorbate resistance was enhanced in Δ*pdr1* and Δ*pdr1* Δ*pdr3* deletion mutants, implying a functional cross-talk between a yet unknown sorbate response pathway and the pleiotropic drug resistance (PDR) network (Decottignies and Goffeau, 1997; Kuchler and Egner, 1997).

Results

We have been investigating plasma membrane proteins induced in *S.cerevisiae* during adaptation to growth at pH 4.5 in the presence of sorbic acid, a non-metabolized weak acid food preservative. Microsequencing of a highly induced 170 kDa protein (Figure 1B, lane 3) in purified plasma membrane fractions from sorbate-treated cells yielded four peptide sequences that were perfect matches to the regions 287–300, 366–383, 838–859 and 1062–1078 of a large open reading frame, YPL058c, present in the yeast Proteome Database. YPL058c residing on chromosome XVI encodes the 1511-residue protein Pdr12, a typical member of the ABC protein superfamily (Decottignies and Goffeau, 1997; Kuchler and Egner, 1997). The predicted topology of the Pdr12 transporter includes 12 putative transmembrane-spanning α-helices and two highly conserved nucleotide binding domains, the hallmark domains of all ABC proteins (data not shown). Pdr12 is highly homologous to two previously identified yeast ABC drug efflux pumps, Snq2 (Servos *et al.*, 1993; Decottignies *et al.*, 1995; Mahé *et al.*, 1996b) and Pdr5 (Balzi *et al.*, 1994; Bissinger and Kuchler, 1994), sharing 46% and 37% primary sequence identity with these latter parameters, respectively.

Next, a Δ*pdr12* deletion strain was constructed and the protein patterns of plasma membrane fractions of both wild-type and isogenic Δ*pdr12* cells were analysed. It was apparent from silver-stained gels (Figure 1B, lane 4) that a sorbate-induced protein of 170 kDa (S) was completely absent in Δ*pdr12* cells, whereas it was found as a prominent band in sorbate-treated wild-type cells (Figure 1A, lane 2). Based on these results, we investigated the effects of different stress conditions on the mRNA levels of three ABC transporter genes, namely *PDR5*, *SNQ2* and *PDR12*.

Weak acid stress strongly induces PDR12 mRNA levels

Northern analysis of total yeast RNA showed that the *PDR12* mRNA was increased in response to ethanol treatment or severe osmostress (2 M sorbitol, 1 M NaCl or 1 M KCl) at pH 7.0 (Figure 2A, lanes 4, 6, 8 and 9). Consistent with earlier studies (Miyahara *et al.*, 1996), *PDR5* and *SNQ2* mRNAs were also slightly induced in response to various stresses (Figure 2A). *PDR12* mRNA was also detectable in pH 4.5 cultures in the absence of weak acid, but became much more strongly induced by addition of either 1 mM or 9 mM sorbate (Figure 2B). The quantification of Northern blots by laser-scanning densitometry indicated that *PDR12* mRNA was induced at least 15-fold in wild-type cells following 9 mM sorbate treatment. Surprisingly, *PDR5* mRNA levels were severely reduced in response to sorbate stress, while *SNQ2* mRNA levels remained essentially unchanged (Figure 2B). *HSP30* encoding the second known sorbate-induced plasma membrane protein (Piper *et al.*, 1997) required higher sorbate levels for a strong induction than *PDR12* (Figure 2B). However, *HSP30* was more strongly heat shock-inducible and less osmostress-inducible than *PDR12* (Figure 2A).

These results show that *PDR12* mRNA is stress-inducible, with a particularly strong induction in response to sorbic acid treatment. To test whether the transcription factors implicated in cellular stress response or PDR development contribute to *PDR12* regulation, we analysed stress induction of *PDR12* in appropriate yeast strains deleted for the *YAP1* (Moye-Rowley *et al.*, 1989; Wemmie *et al.*, 1994; Li *et al.*, 1996), *PDR1* (Balzi *et al.*, 1987) and *PDR3* (Delaveau *et al.*, 1994) genes. The levels of *PDR5*, *SNQ2* and *PDR12* mRNAs in response to sorbate exposure were investigated by Northern analysis of RNAs isolated from wild-type and isogenic Δ*yap1* and Δ*pdr1* Δ*pdr3* cells. In agreement with our earlier work (Mahé *et al.*, 1996b), *PDR5* expression was almost abolished and *SNQ2* mRNA levels were reduced in the Δ*pdr1* Δ*pdr3* mutant (Figure 2B). Notably, normalizing for RNA amounts indicated slightly elevated *PDR12* mRNA levels in Δ*pdr1* Δ*pdr3* cells treated with sorbate (Figure 2B). However, *PDR12* mRNA was essentially unchanged in response to low pH and sorbate in Δ*yap1* cells when compared with wild-type *YAP1* cells. These results suggest that the induction of Pdr12 by weak organic acids such as sorbate does not require the transcriptional regulators Pdr1, Pdr3 or Yap1.

Finally, a strong Pdr12 induction was also found in

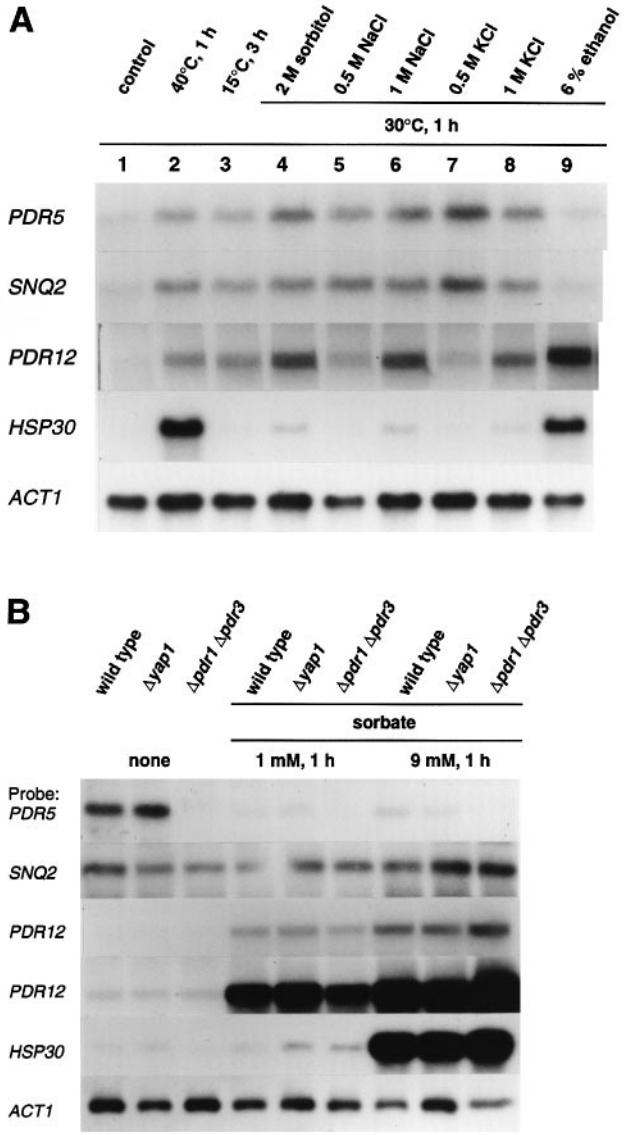

Fig. 2. Northern analysis of total RNA from yeast cells subjected to different stresses. Hybridization to radiolabelled probes specific for the genes indicated to the left of the figure panels was carried out by routine methods. An actin-specific probe (*ACT1*) served as a control for equal RNA loading. (**A**) About 20 μg total RNA each from unstressed control cells (lane 1); or cells heat-shocked at 40°C for 1 h (lane 2); cold-shocked at 15°C for 3 h (lane 3); or osmostressed at 30°C for 1 h with either 2 M sorbitol (lane 4), 0.5 M or 1 M NaCl (lanes 5, 6), 0.5 M or 1.0 M KCl (lanes 7, 8), and 6% (w/v) ethanol at 30°C for 1 h (lane 9) were fractionated through agarose gels as described in Materials and methods. (**B**) Northern analysis of total RNA from wild-type (FY1679-28C) and isogenic Δ*yap1* and Δ*pdr1* Δ*pdr3* strains grown in pH 4.5 YPD medium and treated with 0, 1 or 9 mM sorbate for 1 h. RNA samples of 10 μg per lane were separated through a 1% agarose formaldehyde gel. Both short (10 min) and long (1 h) exposures of the blot hybridized to the *PDR12*-specific probe are shown.

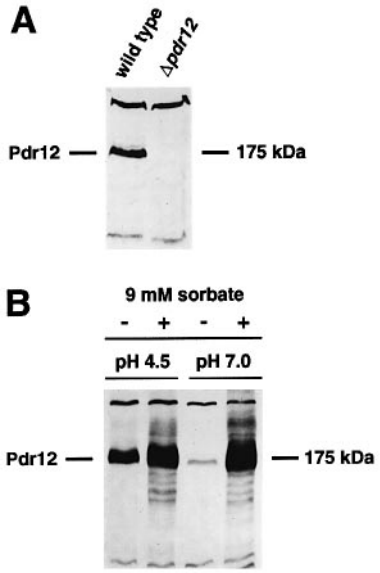

Fig. 3. Immunological detection of Pdr12 in wild-type and sorbate-treated cells. (**A**) Total cell extracts of wild-type and Δ*pdr12* cells grown at pH4.5 were immunoblotted using a polyclonal antiserum raised against a GST–Pdr12 fusion protein. (**B**) Cell extracts from untreated (–) and 9 mM sorbate-treated (+) pH 4.5 and pH 7.0 FY1769-28C cultures were analysed for Pdr12 expression by immunoblotting. The non-specific cross-reaction at higher molecular mass serves as an internal standard for equal protein loading in each lane.

cells lacking Msn2 and Msn4 (data not shown), both of which are transcriptional regulators of a stress response pathway acting through a promoter motif known as STRE (for 'stress response element'; Ruis and Schüller, 1995; Martinez-Pastor *et al.*, 1996). Taken together, these data show that the induction of *PDR12* by weak organic acid stress does not require the transcriptional regulators Pdr1, Pdr3, Yap1, Msn2 and Msn4. Moreover, the results indicate that as yet unidentified stress-responsive transcription factors are required for the response to weak organic acid stress in *S.cerevisiae.*

Low pH and sorbate-mediated induction of Pdr12

The *PDR12* open reading frame of 4533 bp potentially encodes a 1511-residue protein with a predicted molecular mass of 171 kDa. To demonstrate that *PDR12* is over-expressed at the protein level following weak acid treatment, a polyclonal anti-Pdr12 antiserum was raised in rabbits using a bacterially expressed GST–Pdr12 fusion protein as the antigen. Total cellular extracts were prepared from both wild-type and isogenic Δ*pdr12* cells and subjected to immunoblotting (Figure 3A). A polypeptide band with an expected molecular mass of ~175 kDa was specifically recognized by the antiserum in wild-type cell extracts, whereas no protein in this molecular mass range was detectable in extracts from Δ*pdr12* cells (Figure 3A). A possible sorbate-mediated induction of Pdr12 was also tested by immunoblotting. Cells from an overnight culture of wild-type FY1679-28C were inoculated into fresh pH 4.5 and pH 7.0 YPD medium. Both cultures were then grown to an OD_{600} of 0.7–1.0, whereupon sorbate was added to a final concentration of 9 mM to half of each culture. After another 2 h incubation, extracts were analysed for Pdr12 expression by immunoblotting (Figure 3B). Pdr12 expression was extremely low at pH 7.0 in the absence of sorbate. However, sorbate addition to such pH 7.0 cultures resulted in 50-fold higher levels of Pdr12 (Figure 3B). Notably, pH 4.5 cultures, when compared with pH 7.0 cultures, also displayed a 10-fold elevated Pdr12 expression even in the absence of sorbate (Figure 3B). Thus, both sorbate exposure and low pH can dramatically induce Pdr12 protein levels. No signal in the Pdr12

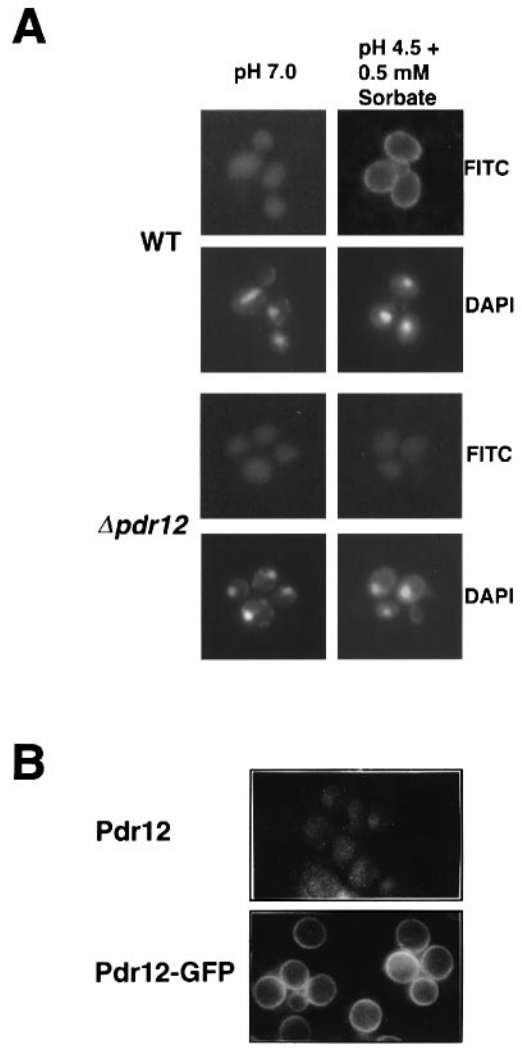

Fig. 4. Subcellular localization of Pdr12. (**A**) Wild-type and Δ*pdr12* cells were grown in pH 7.0 YPD in the absence (pH 7.0) and presence of sorbate (pH 4.5 + 0.5 mM sorbate). After fixation of cells, Pdr12 localization was analysed by indirect immunofluorescence using the FITC filter set. Nuclear DNA was stained and visualized with DAPI. (**B**) The fluorescence of a Pdr12–GFP fusion was visualized microscopically in living cells of strain YYMMI-2 grown in pH 7.0 YPD to mid-logarithmic growth phase.

size range was observed in extracts from the Δ*pdr12* strain, even after sorbate treatment (data not shown), demonstrating that the induced protein is Pdr12.

Subcellular localization of Pdr12

To determine the subcellular localization of Pdr12 in wild-type and in sorbate-treated cells, we performed subcellular fractionation and indirect immunofluorescence experiments. Wild-type cells were grown in complete YPD medium in the absence and presence of sorbic acid. A ring-like fluorescence staining in sorbate-induced cells was apparent, revealing a cell surface localization of Pdr12 (Figure 4A). The antibodies failed to detect Pdr12 in non-treated wild-type cells, presumably because Pdr12 expression under these conditions is too low to allow for a detection by this method. As expected, no fluorescence was observed in non-induced or induced control Δ*pdr12* cells (Figure 4A). However, sucrose gradient fractionation experiments of cell-free extracts did confirm a plasma membrane localization of Pdr12 in uninduced wild-type cells (data not shown).

Finally, we used the Δ*pdr5* Δ*snq2* strain YYMMI-2 to genomically tag *PDR12* at the C-terminus with green fluorescent protein (GFP), yielding a Pdr12–GFP fusion that is fully functional *in vivo* (data not shown). Again, a ring-like fluorescence showed that the Pdr12–GFP fusion protein was localized in the plasma membrane of living cells, while no fluorescence was observed in control cells expressing Pdr12 without the GFP tag (Figure 4B). In summary, these results show unequivocally a plasma membrane localization of Pdr12, and demonstrate that increased Pdr12 levels are due to a sorbate-induced *PDR12* overexpression, rather than regulated cell surface targeting of pre-existing intracellular Pdr12 pools.

A Δpdr12 deletion strain is hypersensitive to weak acids

The sorbate induction of Pdr12 is remarkably strong, raising the possibility that Pdr12 may be required for adaptation to growth in the presence of weak acid stress. Thus, both wild-type and isogenic Δ*pdr12* strains were analysed for their growth phenotypes on pH 4.5 YPD plates containing various commonly used food preservatives. Furthermore, we have also tested the sorbate resistance phenotypes of isogenic strains carrying Δ*yap1*, Δ*pdr1*, Δ*pdr3* and Δ*pdr1* Δ*pdr3* deletions (Figure 5). This analysis revealed a striking hypersensitivity of Δ*pdr12* cells to sorbate at pH 4.5 when compared with the wild-type strain, as Δ*pdr12* mutants failed to grow in the presence of 0.5 mM sorbate (Figure 5). In separate experiments, we have also determined the IC_{50} values for sorbate, benzoate and acetate. The results showed that Δ*pdr12* cells gave IC_{50} values of ~0.20 mM for sorbate and benzoate, and 20 mM for acetate, while isogenic wild-type cells exhibited 4- to 6-fold higher IC_{50} values for weak acid inhibition, respectively (data not shown). Similar experiments also revealed a hypersensitivity of Δ*pdr12* cells to propionate at pH 4.5, though not to sulfite (data not shown). Surprisingly though, loss of Pdr1, but not Pdr3 or Yap1, led to an increased sorbate resistance, implying that under these conditions perhaps other so far unknown Pdr1 target genes can also contribute to weak acid resistance development (Figure 5).

Next, we investigated in more detail the effects of different sorbate (pK_a 4.76), benzoate (pK_a 4.19) and acetate (pK_a 4.75) concentrations on the growth behaviour of wild-type and Δ*pdr12* cells at pH 4.5 (Figure 6), pH 3.8 and pH 5.7 (data not shown). At all three pH values, Δ*pdr12* cells grew slightly slower in the absence of weak acid when compared with the wild-type. This subtle slow-growth phenotype was manifested as a longer lag-phase period (Figure 6). At pH 5.7, a pH at which all tested weak acids are almost completely dissociated and relatively non-toxic to cells, the presence of 0.8 mM benzoate or 0.9 mM sorbate produced little extension to the lag phase; moreover, they caused practically no difference to the growth of Δ*pdr12* and wild-type cells (data not shown). In contrast, at pH 4.5, where an appreciable fraction of each acid is undissociated, the same amounts of benzoate and sorbate severely reduced both growth rate and biomass yield of wild-type cells (Figure 6A and C). Furthermore,

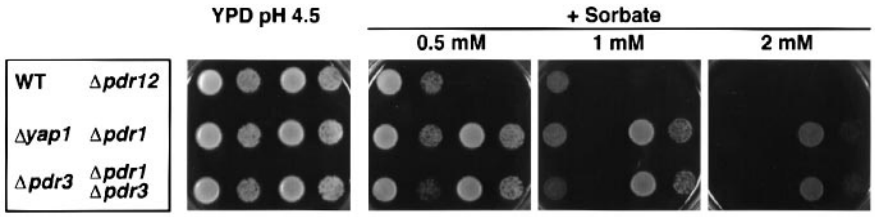

Fig. 5. *PDR12* is essential for adaptation of yeast cells to growth in the presence of weak acids. Growth of wild-type and Δ*pdr12* cells and isogenic strains carrying Δ*yap1*, Δ*pdr1*, Δ*pdr3* and Δ*pdr1* Δ*pdr3* deletions was monitored on sorbate plates. Cell suspensions of OD_{600} = 0.025 as well as 1:10 serial dilutions were spotted onto pH 4.5 YPD plates with the indicated concentrations of sorbate. The plates were photographed after 2.5 days incubation at 30°C.

Δ*pdr12* cells displayed a marked hypersensitivity to weak acids at this pH, since they were unable to grow at benzoate levels >0.2 mM (Figure 6B). While Δ*pdr12* cells could still adapt to 0.45 mM sorbate at pH 4.5, they failed completely to grow in the presence of 0.9 mM sorbate (Figure 6D).

Although acetic acid can be used as a carbon source by non-glucose-repressed yeast, high acetate levels are inhibitory in glucose-grown cultures (Figure 6E and F). Wild-type cells, although totally inhibited by 90 mM acetate, grew in the presence of 45 mM acetate at pH 4.5 (Figure 6E) and at pH 3.8 and pH 5.7 (data not shown). However, no growth was observed when Δ*pdr12* cells were grown for 65 h in the presence of 45 mM acetate at pH 4.5 (Figure 6F), as well as at pH 3.8 and pH 5.7 (data not shown). Thus, Δ*pdr12* cells are defective in glucose growth in the presence of high levels of acetate. Under these conditions the monocarboxylate uptake systems of *S.cerevisiae* are repressed (Casal *et al.*, 1996), so that acetate will enter the cells primarily by diffusion of the undissociated acid. Hence, the data (Figure 6E and F) indicate that Pdr12 is capable of catalysing an active extrusion of acetate. Finally, we also tested strongly membrane-disruptive compounds, including ethanol, the antifungal drug amphotericin B (Bolard, 1986) and decanoate, the latter a highly lipophilic weak acid with a long aliphatic chain (Stratford and Anslow, 1996). However, loss of Pdr12 had no effect on growth inhibition caused by these compounds (data not shown). This suggests that Pdr12 confers no protection against compounds that are highly liposoluble and primarily membrane-disruptive in their cytotoxic effects.

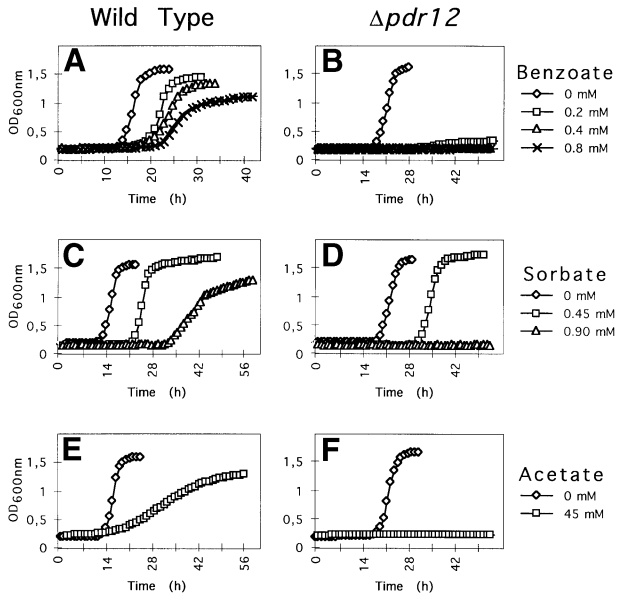

Fig. 6. Bioscreen monitoring of the growth of wild-type (A, C, E) and Δ*pdr12* (B, D, F) cells in liquid pH 4.5 YPD medium containing increasing concentrations of benzoate (A, B), sorbate (C, D) or acetate (E, F).

Δpdr12 mutants show impaired benzoate extrusion

We used [^{14}C]benzoate in efflux experiments to test whether Δ*pdr12* cells display any defects in benzoic acid extrusion. Both wild-type and Δ*pdr12* cells were cultured at pH 4.5 to the mid-exponential growth phase. Half of each culture was treated with 1 mM sorbic acid for 2 h. Cells were then harvested and resuspended in glucose-free pH 4.5 buffer. Next, [^{14}C]benzoate was added, followed 5 min later by the addition of glucose. Both the intracellular accumulation of radiolabelled benzoate and its rapid efflux after glucose addition were followed (Henriques *et al.*, 1997). The benzoate initially taken up by the cells represented one-quarter to one-third of the added radiolabel for the non-adapted cells (Figure 7A), and half of the added benzoate for the sorbate-pretreated cells (Figure 7B). Although sorbate-pretreated wild-type

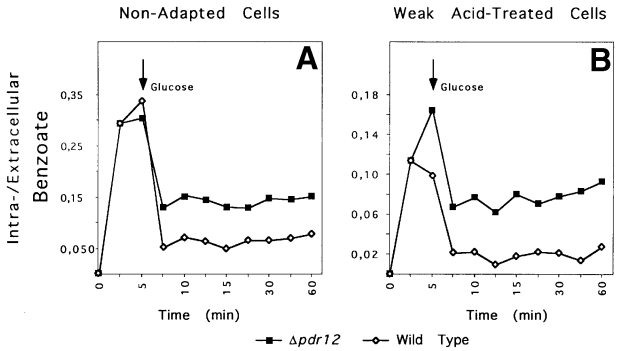

Fig. 7. Intracellular accumulation of [^{14}C]benzoate by wild-type (■) and Δ*pdr12* cells (◇) before and after glucose addition marked by an vertical arrow (at 5 min). Cells were grown at either pH 4.5 (A) or at pH 4.5, then pre-treated with 1 mM sorbate for 2 h (B) as described in Materials and methods. Each point represents the SEM of three separate measurements made on the same batch of cells.

cells accumulated less [^{14}C]benzoate, presumably because their intracellular pH was lower, they still displayed a rapid extrusion of much of this benzoate after glucose addition (Figure 7B). However, although the initial [^{14}C]benzoate accumulation of Δ*pdr12* cells was similar to that of wild-type, energy-dependent benzoate efflux by

the mutant was severely impaired (Figure 7). For both the non-adapted and the sorbate-pretreated wild-type cells, 70–80% of the accumulated [^{14}C]benzoate was rapidly extruded after glucose addition. In contrast, non-adapted and sorbate-pretreated Δ*pdr12* cells extruded only ~50% of their intracellular [^{14}C]benzoate under the same conditions (Figure 7B). These differences between the Δ*pdr12* mutant and its isogenic parent were maintained for at least 1 h, suggesting that Pdr12 was continuously effluxing [^{14}C]benzoate over this period (Figure 7). In summary, these results demonstrate that benzoate is a substrate for Pdr12-mediated extrusion, and that Pdr12 is a major catalyst of energy-dependent benzoate efflux in yeast.

Discussion

This study provides, for the first time, genetic and biochemical evidence that adaptation of yeast cells to growth in the presence of toxic weak acids involves the induction of a system for energy-dependent weak organic acid extrusion. Our studies identify Pdr12 as the ABC protein of *S.cerevisiae* strongly induced in response to sorbate exposure (Figures 1 and 3). Pdr12 is a major determinant conferring resistance to sorbate, benzoate and acetate (Figures 5 and 6) and it provides much of the cellular capacity for active benzoate extrusion (Figure 7). Pdr12 is strongly stress-inducible, its induction being essential for the development of weak acid resistance. Furthermore, our results strongly support the notion that certain yeast ABC transporters, through their actions in cellular detoxification and defence against toxic compounds in the environment, have important physiological roles in adaptation to adverse conditions (Decottignies and Goffeau, 1997; Kuchler and Egner, 1997).

Ycf1 is another example of an ABC transporter that is both stress-inducible and which assists stress survival. Ycf1 is induced by oxidative stress in a Yap1-dependent manner (Wemmie *et al.*, 1994) and it confers resistance to high cadmium levels (Szczypka *et al.*, 1994) and glutathione-conjugated molecules (Li *et al.*, 1996). Our data indicate that Pdr12 induction, at least under the experimental conditions used, is independent of the Pdr3, Yap1 and Msn2/Msn4 transcription factors, all of which are important mediators of PDR development and the general stress response pathway, respectively. Indeed, consensus 5′-TCCGCGGA-3′ PDRE (for PDR responsive element) motifs found in PDR-responsive genes such as *PDR5* (Delahodde *et al.*, 1995; Katzmann *et al.*, 1996), *YOR1* (Katzmann *et al.*, 1995), *PDR10* and *PDR15* (Wolfger *et al.*, 1997) are absent from the *PDR12* promoter. Likewise, both the STRE consensus motif 5′-AGGGG-3′ mediating the general stress response (Martinez-Pastor *et al.*, 1996) and the Yap1 consensus 5′-TGACTCA-3′ (Kuge and Jones, 1994) are absent from the *PDR12* promoter. Interestingly, a recently identified novel Yap1-binding motif, 5′-TTACTAA-3′ (Fernandes *et al.*, 1997), is found at position –64 from the initiating methionine, implying a possible role for Yap-homologues in Pdr12 regulation under weak acid stress (Fernandes *et al.*, 1997).

Unexpectedly, Δ*pdr1* and Δ*pdr1* Δ*pdr3* cells exhibited increased sorbate resistance (Figure 5), indicating that certain as yet unknown Pdr1 target genes may exert a negative influence on weak acid adaptation. Such a negative effect could perhaps operate through a degenerate PDRE-like motif (5′-TCGCCGGA-3′) at position –486 relative to the *PDR12* translational start site. The Pdr12 pump shares >37% primary sequence identity with Pdr5. Nevertheless, their expression, regulation and functions seem quite different. The reason for the drastic reduction of *PDR5* mRNA under weak acid stress (Figure 2) is unclear at the moment, but one could argue that Pdr5 could somehow interfere with Pdr12 function in stressed cells. Thus, it will be interesting to determine whether or not the same transcriptional machinery that mediates Pdr12 induction under weak acid stress is also responsible for Pdr5 repression in weak acid-treated cells.

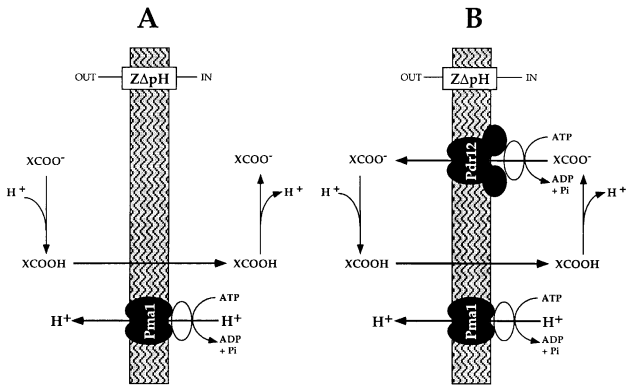

Fig. 8. Schematic representation of the effects of substantial amounts of undissociated weak organic acid (XCCOH) on unadapted yeast cells (**A**). As mentioned in the Discussion, the induction of a weak acid efflux pump (Pdr12) poses potential problems for homeostasis maintenance in cells adapted to these acids (**B**), unless there is also simultaneous induction of a system restricting free diffusional entry of the undissociated acid. Pma1 is the proton-translocating plasma membrane ATPase.

The mechanism of Pdr12 in weak acid resistance

A schematic model of how Pdr12 function might aid acidified yeast cultures in counteracting the inhibitory effects of water-soluble weak organic acids is depicted in Figure 8. In both unadapted (A) and acid-adapted cells (B), the protonated uncharged form of the acid (XCOOH) is shown as freely permeable to the cell membrane and readily entering the cell by passive diffusion. In unadapted cells (Figure 8A), the XCOOH concentration inside and outside should be about the same. However, the higher pH environment of the cytoplasm will cause a substantial fraction of the intracellular acid to dissociate to the anion ($XCOO^-$) which, being charged, is relatively membrane-impermeable and therefore accumulates inside the cell. Moreover, this dissociation also releases protons, resulting in a cytoplasmic acidification that inhibits many metabolic processes. The electrochemical potential (ZΔpH) across the plasma membrane, largely maintained through the plasma membrane ATPase (Pma1)-catalysed proton extrusion, is essential for many aspects of cellular metabolism. Thus, weak acid influx in (A) will act to dissipate the ΔpH, though not the charge (Z) component of this gradient. The extent to which the weak acid-induced cytoplasmic acidification in (A) can be counteracted by increased Pma1 activity may be severely limited, since the high levels of additional proton extrusion needed will also require greater increases to the electrostatic charge across the plasma membrane (Z) than can be generated by the Pma1 ATPase.

In weak acid-adapted cells (Figure 8B), the proposed Pdr12-catalysed anion extrusion will reduce both intracellular organic acid levels and, by moving a charge compensating for the charge on a Pma1-extruded proton, enable greater levels of catalysed proton extrusion than would otherwise be possible. The latter process, though energetically expensive, could assist weak acid-stressed cells to elevate their intracellular pH to the point where substantial metabolic activity and cell growth can resume. However, induction of Pdr12-catalysed acid anion extrusion alone would seem to be pointless without simultaneous limitation to the diffusional uptake of the undissociated acid (XCOOH). Without such a limitation, acid could potentially diffuse in as fast as Pdr12 pumps it out in a futile cycle that, besides consuming large quantities of ATP, will also cause substantial influx of protons (Figure 8B). How weak acid diffusion across the cell envelope is restricted in adapted cells, whether by cell wall or membrane alteration, is at present unknown. However, it is noteworthy that there exists an inverse correlation between the rates with which different yeast species take up benzoic acid and the resistances of these yeasts to benzoate (Warth, 1989). Thus, although our data indicate that Pdr12-mediated anion efflux is essential for weak acid adaptation, it appears likely that Pdr12 is not the only component of the adaptation system.

Whether or not the induction of a weak acid efflux pump is important for weak acid resistance by yeasts has been a contentious issue for several years (Warth, 1977; Cole and Keenan, 1987). It has now been resolved by this study. Earlier work had established that adaptation of *S.cerevisiae* (Henriques *et al.*, 1997) and *Z.bailii* (Warth, 1977) to growth in the presence of 1 mM benzoic acid caused cells to maintain an intracellular versus extracellular distribution of benzoate that is not in equilibrium. Since benzoate (Henriques *et al.*, 1997) is not metabolized by *S.cerevisiae*, these data were fully consistent with the induction of an energy-dependent extrusion system for the anion in response to benzoate exposure.

The substrate specificity and normal physiological roles of Pdr12

Because *PDR12* encodes a close homologue of the Snq2 ABC drug efflux pump (Servos *et al.*, 1993), we also tested the sensitivity phenotypes of Δ*snq2* and Δ*pdr12* strains. However, despite a high primary identity of Pdr12 and Snq2, their substrate specificity does not overlap. A Δ*pdr12* mutant is not hypersensitive to 4-nitroquinoline-*N*-oxide (4-NQO), a typical Snq2 substrate, while a Δ*snq2* strain failed to display any hypersensitivity to weak organic acids (data not shown). Instead, Δ*snq2* Δ*pdr5* double mutants even exhibited increased resistance to sorbic acid (Y.Mahé and K.Kuchler, unpublished results). Although we cannot formally exclude the possibility that Pdr12 might transport other cytotoxic drugs or toxic metabolites, it appears as if its main function is in mediating cellular efflux of weak organic acids (Figures 5–7). The substrates that we have identified to date are all water-soluble carboxylic acids, suggesting that Pdr12 primarily pumps intracellular carboxylate anions, rather than more lipophilic molecules that partition preferentially into the membrane lipid bilayer.

It follows that the normal physiological function of the Pdr12 ABC transporter may be to minimize the effects of water-soluble organic acids. These may accumulate to toxic levels within yeast cells growing in environments of slightly acidic pH (Figure 8A). Weak organic acids will often be present in plant materials, such as ripe fruits and cacti, where yeasts grow as saprophytes. These environments, with their plentiful supply of water and carbohydrates, provide niches where growth does not need a high degree of evolutionary specialization and where competition among different microbes will be extreme. Acetic acid, for example, will often be present at quite high concentrations in such situations as it is both a product of bacterial fermentation and a compound secreted in high levels by certain non-*Saccharomyces* yeasts such as *Brettanomyces* and *Dekkera*. The *S.cerevisiae* in wine mush is frequently inhibited, especially at the early stage of fermentation, by the high acetic acid levels caused by such microbes. It therefore seems plausible that the strong Pdr12 induction by weak acid stress protects *S.cerevisiae* against the toxicity of high organic acid levels under these conditions. Still further protection, on fermentative substrates, will come from the high ethanol yield of *S.cerevisiae* and the fact that this is one of the most ethanol-tolerant organisms known.

Materials and methods

Yeast strains and media

Rich medium (YPD) and synthetic medium (SD), supplemented with auxotrophic components were prepared essentially as described elsewhere (Kaiser *et al.*, 1994). Unless otherwise indicated, all yeast strains listed in Table I were grown routinely at 30°C. The Δ*pdr12::hisG* disruption strain YYM19 was constructed through a one-step gene replacement procedure (Rothstein, 1983) by transforming FY1679-28C with the *Bgl*II–*Xho*I Δ*pdr12::hisG-URA3-hisG* fragment isolated from plasmid pYM63. Transformants were grown on plates containing 5-fluoro-orotic acid (Boeke *et al.*, 1987) to select for the pop-out of the *URA3* marker. Correct genomic integration of deletion constructs and proper looping-out was confirmed by PCR analysis of genomic DNA (Mahé *et al.*, 1996a).

Plasmid constructions

A glutathione-*S*-transferase (GST)–Pdr12 gene fusion was constructed as follows. A 500 bp PCR fragment of *PDR12* was generated from a genomic DNA template using the custom primers PDR12-8: 5′-CGA-CTG-ACG-AAT-TCA-TTG-AGA-AAG-3′ and PDR12-528: 5′-CAT-TTC-ACC-GAA-T**TC**-AAC-GAC-ACC-3′. The PCR product was digested with *Eco*RI and cloned into the *Eco*RI site of plasmid pGEX-5X-1 (Pharmacia). The resulting plasmid pYM53 allowed for the bacterial expression of the N-terminal 164 Pdr12 residues (aa 8–172) fused to the C-terminus of GST.

The Δ*pdr12::hisG-URA3-hisG* deletion plasmid was constructed in two steps. First, the above-mentioned 500 bp *Eco*RI fragment obtained by PCR with primers PDR12-8 and PDR12-528 was inserted in the *Eco*RI site of plasmid pYM28, which contains the *hisG-URA3-hisG* element (Mahé *et al.*, 1996a), resulting in plasmid pYMI14. In the second step, the 3′ end of the *PDR12* gene was cloned as a 840 bp *Bam*HI–*Xho*I fragment, generated by PCR using the primers PDR12-31: 5′-CGT-GCA-TCT-CAT-GCA-GG-3′ and PDR12-32: 5′-GCC-ATT-ACT-C**GA**-GAG-TGG-GAT-AG-3, into *Bam*HI and *Xho*I-cleaved pYMI14 to yield plasmid pYM63.

Drug resistance and weak acid susceptibility assays

Drug resistance and weak acid susceptibility of yeast strains was initially tested by spotting serial dilutions of exponentially growing cultures onto YPD plates supplemented with the indicated compounds (Bissinger and Kuchler, 1994; Mahé *et al.*, 1996a). For studies of the effects of pH and weak acids on glucose batch fermentation cultures, the strains FY1679-28C and YYM19 were grown to late exponential phase at 30°C on YEPD medium containing no stress agent. Cultures were diluted to an OD_{600} of 0.8, followed by another 100-fold dilution with YPD of

Table I. Genotypes of *S.cerevisiae* strains used in this study

Strain	Genotype	Source
FY1679-28c	*MAT***a** *ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63*	Delaveau *et al.* (1994)
YYM19	*MAT***a** Δ*pdr12::hisG* (otherwise isogenic to FY1679-28c)	this study
FY1679-28c/Δ1	*MAT***a** Δ*pdr1::TRP1* (otherwise isogenic to FY1679-28c)	Delaveau *et al.* (1994)
FY1679-28c/Δ3	*MAT***a** Δ*pdr3::HIS3* (otherwise isogenic to FY1679-28c)	Delaveau *et al.* (1994)
FY1679-28c/Δ1Δ3	*MAT***a** Δ*pdr1::TRP1* Δ*pdr3::HIS3* (otherwise isogenic to FY1679-28c)	Delaveau *et al.* (1994)
YYM16	*MAT***a** Δ*yap1::hisG* (otherwise isogenic to FY1679-28c)	Wendler *et al.* (1997)
W303-1A	*MAT***a** *can1-100 ade2-1^{oc} his3-11,-15 leu2-3,-112 trp1-1 ura3-1*	Rothstein (1983)
Wmsn2msn4	*MAT***a** *msn2-Δ3::HIS3 msn4-1::TRP1* (otherwise isogenic to W303-1A)	Martinez-Pastor *et al.* (1996)
YPH499	*MAT***a** *ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63 lys2-801^{amb} ade2-101^{oc}*	Sikorski and Hieter (1989)
YYM4	*MAT***a** Δ*pdr5::TRP1* Δ*snq2::hisG* (otherwise isogenic to YPH499)	Egner *et al.* (1998)
YYMMI-2	*MAT*α Δ*pdr5::TRP1* Δ*snq2::hisG:URA3:hisG* (otherwise isogenic to YPH499)	this study

pH 5.74, pH 4.5 or pH 3.8, with or without the indicated concentrations of weak acid, giving ~5×10^3 cells/ml and placed into the wells of a Bioscreen plate. The Bioscreen plate was then placed into a Bioscreen turbidometric analyser (Labsystems OY, Helsinki, Finland) that was programmed to provide both continuous shaking at 30°C and to monitor the OD_{600}.

RNA isolation, radiolabelling and Northern analysis

Total yeast RNA was isolated, fractionated through agarose gels and hybridized to radiolabelled probes using standard methods (Piper, 1994). DNA fragments were radiolabelled using a Megaprime Labelling Kit under conditions recommended by the manufacturer (Amersham). The *PDR12*-specific probe (+8 to +4787 region of *PDR12*) was amplified by PCR from total yeast genomic DNA using the primers PDR12-8 and PDR12-32 under standard PCR conditions (Mahé *et al.*, 1996b).

Preparation of a polyclonal anti-Pdr12 antiserum

The *Escherichia coli* strain DH5α carrying plasmid pYM53 was grown at 30°C to an OD_{600} of 0.7. Expression of the GST–Pdr12 fusion protein was induced by adding 0.1 mM isopropyl β-D-thiogalactopyranoside for 4 h. Purification of the GST–Pdr12 fusion protein was done exactly as described previously (Mahé *et al.*, 1996b). Removal of glutathione and concentration of the eluted GST fusion protein was carried out in a Centricon 10 microconcentrator (Amicon Division). The purified GST–Pdr12 fusion protein was used to immunize rabbits according to routine injection regimes (Harlow and Lane, 1988).

Plasma membrane isolation, microsequencing and immunoblotting

Yeast plasma membrane fractions were partially purified and fractionated by one-dimensional SDS–PAGE exactly as described previously (Piper *et al.*, 1997). Peptide microsequencing was performed on protein samples blotted onto PVDF membranes by routine laboratory methods (Harlow and Lane, 1988). Protein extracts from whole yeast cells were isolated essentially as described elsewhere (Egner *et al.*, 1995). Proteins on immunoblots were visualized with the ECL system (Vieira *et al.*, 1994) under conditions recommended by the manufacturer (Amersham).

Indirect immunofluorescence and subcellular fractionation

Immunofluorescence of yeast cells was carried out as previously published (Kuchler *et al.*, 1993) using the following modifications. Wild-type and Δ*pdr12* cells were grown in complete YPD medium to an OD_{600} of ~0.5. After addition of 0.5 mM sorbate to one-half of each culture, cells were cultivated for another 3 h. Further treatment of cells was exactly as previously published (Egner *et al.*, 1995). Fluorescence staining of Pdr12 was visualized with a Zeiss Axiovert 10 fluorescence microscope equipped with an appropriate FITC filter set. Photomicrographs were taken with a Kodak TMY400 black and white film.

Genomic tagging of the *PDR12* C-terminus with GFP (Cubitt *et al.*, 1995) was carried out by a PCR-based method (Wach *et al.*, 1997) using the primer pair PDR12-GFPn 5′-ATT-TTC-CAA-ACA-GTT-CCA-GGT-GAC-GAA-AAT-AAA-ATC-ACG-AAG-AAA-GTC-GAC-GGA-TCC-CCG-GG-3′ and PDR12-GFPc 5′-GTA-AAA-TCA-AAT-GTA-AAA-TTA-AAA-AAA-TGA-TGT-TAA-AGG-ACG-CCA-ATC-GAT-GAA-TTC-GAG-CTC-G-3′. PCR fragments were transformed into yeast strain YYMMI-2 by electroporation as previously published (Mahé *et al.*, 1996b). GFP-fluorescence in living cells was observed microscopically using a FITC filter set.

Subcellular fractionation of yeast cells was performed following a previously published protocol (Egner *et al.*, 1998). Yeast strain YYM4 containing the plasmid pCKSF1 (Bissinger and Kuchler, 1994) was grown in synthetic medium at pH 4.5 without sorbate to logarithmic phase (OD_{600} = 2). Cell-free extracts were fractionated in a sucrose gradient and fractions were analysed by SDS–PAGE. Immunoblotting with polyclonal antisera against Pdr12, Pdr5, Pdr12 and Pma1 was performed by standard laboratory procedures (Egner *et al.*, 1995).

Measurement of benzoic acid efflux

Overnight FY1679-28c and Δ*pdr12* cultures were diluted 100-fold in water, then inoculated into two flasks with 100 ml pH 4.5 YPD and grown to an OD_{600} of 0.7–1.0. Each culture was then divided into two 50 ml portions, with the addition of 1 mM sorbic acid to one of these. After a further 2 h incubation at 30°C, the cells were harvested, washed in ice-cold water and resuspended in 5.4 ml 20 mM sodium citrate pH 4.5 at room temperature. After a 10 min incubation in this buffer, 5 μCi [7-^{14}C]benzoic acid (740 MBq/mmol; NEN) was added, followed 5 min later by the addition of 0.6 ml 20% (w/v) glucose. After several time intervals, 0.5 ml samples of the cell suspension were filtered on Whatman GF/C filters, the filters briefly washed in pH 4.5 citrate buffer. Filter-bound radioactivity of air-dried filters was determined by liquid scintillation counting.

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