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The *Saccharomyces cerevisiae* Weak-Acid-Inducible ABC Transporter Pdr12 Transports Fluorescein and Preservative Anions from the Cytosol by an Energy-Dependent Mechanism

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Growth of *Saccharomyces cerevisiae* in the presence of the weak-acid preservative sorbic acid results in the induction of the ATP-binding cassette (ABC) transporter Pdr12 in the plasma membrane (P. Piper, Y. Mahe, S. Thompson, R. Pandjaitan, C. Holyoak, R. Egner, M. Muhlbauer, P. Coote, and K. Kuchler, EMBO J. 17:4257–4265, 1998). Pdr12 appears to mediate resistance to water-soluble, monocarboxylic acids with chain lengths of from C_1 to C_7. Exposure to acids with aliphatic chain lengths greater than C_7 resulted in no observable sensitivity of Δ*pdr12* mutant cells compared to the parent. Parent and Δ*pdr12* mutant cells were grown in the presence of sorbic acid and subsequently loaded with fluorescein. Upon addition of an energy source in the form of glucose, parent cells immediately effluxed fluorescein from the cytosol into the surrounding medium. In contrast, under the same conditions, cells of the Δ*pdr12* mutant were unable to efflux any of the dye. When both parent and Δ*pdr12* mutant cells were grown without sorbic acid and subsequently loaded with fluorescein, upon the addition of glucose no efflux of fluorescein was detected from either strain. Thus, we have shown that Pdr12 catalyzes the energy-dependent extrusion of fluorescein from the cytosol. Lineweaver-Burk analysis revealed that sorbic and benzoic acids competitively inhibited ATP-dependent fluorescein efflux. Thus, these data provide strong evidence that sorbate and benzoate anions compete with fluorescein for a putative monocarboxylate binding site on the Pdr12 transporter.

Lipophilic weak acids, such as sorbic and benzoic acids, are commonly used to preserve foods and beverages. However, many species of spoilage yeasts and molds are able to adapt and grow in the presence of the maximum permitted levels of these preservatives used in manufactured foods and beverages. This results in inconvenience to the consumer and considerable economic loss (13, 18).

In solution, weak-acid preservatives exist in a pH-dependent equilibrium between the undissociated and dissociated states. Preservatives have optimal inhibitory activity at low pH because this favors the uncharged, undissociated state of the molecule, which is freely permeable across the plasma membrane and is thus able to enter the cell. Upon encountering the higher pH inside the cell, the molecule dissociates, resulting in the release of charged anions and protons which cannot cross the plasma membrane. Thus, the preservative molecule diffuses into the cell until equilibrium is reached in accordance with the pH gradient across the membrane, resulting in the accumulation of anions and protons inside the cell. Therefore, inhibition of growth by preservatives has been proposed to be due to a number of actions, including membrane disruption (8, 19), inhibition of essential metabolic reactions (25), stress on intracellular pH (pH_{in}) homeostasis (8, 11, 34), and the accumulation of toxic anions (17).

Recent research has shown that yeast cells are able to mount an adaptive response that attempts to counteract these detrimental effects and restore homeostasis. It has been shown that upon exposure to weak acids, the enzyme that regulates pH_{in} homeostasis in yeast cells, the membrane H^+-ATPase, is activated and is essential for optimal adaptation to preservatives (22, 29, 40, 41). However, because the membrane H^+-ATPase has been shown to consume up to 60% of cellular ATP (35), this adaptive mechanism was shown to be energetically expensive, resulting in the depletion of intracellular ATP (8, 22, 29). It has also been shown that a mutant with reduced expression of key glycolytic enzymes and thus reduced ability to generate ATP was unable to adapt optimally to weak-acid stress (22). Thus, it has been proposed that the actual inhibitory action of preservatives on yeast cells could be due to the induction of an energetically expensive stress response that attempts to restore homeostasis and results in the reduction of available energy pools for growth and other essential metabolic functions (8).

Recent studies have shown that exposure to weak-acid preservatives, in addition to the activation of existing proteins, also results in the induction of two plasma membrane proteins. The smaller of these two proteins is a heat shock protein, Hsp30, which was shown to assist in adaptation to weak acids by regulating the activity of the membrane H^+-ATPase (9, 29). The second, and larger, of these two proteins was identified as the ATP-binding cassette (ABC) transporter Pdr12 (30), a homologue of the Snq2 (36) and Pdr5 (1, 2) ABC drug efflux pumps. It was shown that Pdr12 was essential for the adaptation of yeast cells to growth in the presence of weak-acid preservatives, since Δ*pdr12* mutants were hypersensitive at low pH to sorbic, benzoic, and acetic acids (30). Thus, for the first time, genetic and biochemical evidence was presented showing that the adaptation of yeast cells to growth in the presence of weak-acid preservatives involved the induction of a plasma membrane protein that appeared to mediate energy-depen-

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dent weak organic acid extrusion. This supported earlier physiological studies showing that only when yeast cells were grown in the presence of benzoic acid were they subsequently able to extrude significant amounts of radiolabelled benzoate when glucose was added to the system (20, 39, 42).

The aim of the present study was to use a Δ*pdr12* mutant to gain a more precise understanding of how Pdr12 confers resistance to preservatives by studying the mode of action, substrate specificity, and transport kinetics of the protein.

MATERIALS AND METHODS

Organism. The *Saccharomyces cerevisiae* strains used in this study included FY1679-28c (*MAT***a** *ura3-52 his3-*Δ*200 leu2-*Δ*1 trp1-*Δ*63*) (15) and YYM19 (*MAT***a** Δ*pdr12*::*hisG*) (otherwise isogenic to FY1679-28c) (30). These strains were maintained on YEPD (2% [wt/vol] glucose, 2% [wt/vol] yeast extract [Betalab], 1% [wt/vol] Bacto-Peptone [Difco]) plates.

Chemicals. Unless otherwise stated all chemicals were obtained from Sigma-Aldrich.

Growth conditions. Cultures of FY1679-28c or YYM19 were grown with shaking at 30°C to late exponential phase (optical density at 600 nm of 0.8) in either YEPD medium or synthetic medium (SD) supplemented with amino acids (23). The pH values of these media were adjusted to 4.5 with HCl and, for experiments requiring induction of Pdr12, a level of sorbic acid subinhibitory for both strains was added to the growth medium (0.45 mM). These cells served as inocula for further growth studies or transport assays.

Drug and weak acid sensitivity. Cultures of *S. cerevisiae* FY1679-28c and YYM19 were diluted in fresh YEPD (pH 4.5) and inoculated into the wells of a Bioscreen microtiter plate (100-well honeycomb; Life Sciences International, Basingstoke, United Kingdom) to give an inoculum size of 5.0×10^3 cells ml^{-1}. Increasing concentrations of formic (C_1), acetic (C_2), propionic (C_3), butyric (C_4), valeric (C_5), caproic (C_6), heptanoic (C_7), octanoic (C_8), nonanoic (C_9), decanoic (C_{10}), sorbic, and benzoic acids; 4-nitroquinoline-*N*-oxide; amphotericin B; ethanol; tamoxifen; and decorticosterone were then added to the wells. Growth at 30°C with continuous shaking was then monitored by observing the change in optical density at 600 nm in a Labsystems Bioscreen automated turbidometric analyzer (Life Sciences International).

Loading cells with fluorescein diacetate. *S. cerevisiae* FY1679-28c and YYM19 were grown in YEPD (pH 4.5; with or without 0.45 mM sorbic acid) to late exponential phase. Cells were then harvested by centrifugation and washed four times in sterile distilled water and resuspended to give identical cell numbers (1.8 mg [dry weight] ml^{-1}) in 50 mM HEPES-NaOH (pH 7.0) containing 5 mM 2-deoxy-D-glucose and 50 μM fluorescein diacetate (FDA) (from a 5 mM stock in dimethyl sulfoxide). These cells were then incubated at 30°C for 3 h to allow the FDA to enter the cells by passive diffusion (6). Once inside the cells, FDA is hydrolyzed to the polar, fluorescent dye fluorescein via intracellular esterases (6). Aliquots of dye-loaded cells were then harvested, washed with 50 mM HEPES-NaOH (pH 7.0), and resuspended in the same buffer at pH 7 or 5.5.

Measurement of fluorescein efflux from whole cells. This measurement was based on a method with rhodamine as described by Kolaczkowski et al. (24). Cell suspensions of *S. cerevisiae* FY1679-28c and YYM19 loaded with fluorescein were transferred to a 50-ml magnetically stirred jacketed heating vessel at 30°C, and fluorescein efflux was started by the addition of 10 mM glucose. Samples of 1 ml (containing 1.8 mg [dry weight] of cells) were taken at set intervals over a period of 5 min, and the cells were removed by rapid centrifugation ($13,000 \times g$ for 4 min). Levels of fluorescein in the supernatant were measured in a magnetically stirred, optically clear, quartz cuvette (Helma; Fisher Scientific) by using a Shimadzu RF-1501 fluorometer (Shimadzu, Haverhill, Suffolk). To measure supernatant fluorescence, all readings were done with an excitation scan of between 400 and 500 nm with an emission set at 525 nm (bandwidths of 10 nm). Supernatant fluorescence intensity data was collected at an excitation wavelength of 435 nm (pH-independent point) (7). This was carried out over a time period of 10 min after the addition of glucose. Inhibitors, such as sodium orthovanadate, were added to the cell suspensions 5 min prior to the glucose addition.

Assay of fluorescein efflux inhibition. Assays designed to measure competition with fluorescein efflux were carried out exactly as described above except the cells were incubated with a range of FDA concentrations (from 0 to 50 μM in 10 μM increments) in order to load the cells with variable concentrations of fluorescein. Thus, the intracellular concentration of the substrate and the measurable product of Pdr12 activity were varied. A calibration curve of known fluorescein concentration versus fluorescence (constructed in the presence of 1.8 mg [dry weight] of yeast cells ml^{-1} to account for any fluorescence quenching due to the biomass) was used to determine the intracellular concentration of fluorescein. The initial rates of glucose-induced efflux of fluorescein for each concentration of substrate loaded were measured from the linear part of the fluorescein efflux curves (approximately 100 to 400 s after glucose addition). This was carried out in the presence of increasing concentrations of sorbic or benzoic acid at pH 7 and 5.5. Thus, in conjunction with the known concentrations of intracellular fluorescein (substrate), the initial rates of the efflux values were then used to construct Lineweaver-Burk plots for the determination of competitive versus noncompetitive inhibition of Pdr12 activity by sorbic or benzoic acid.

Fluorescence microscopy. To visualize levels of intracellular fluorescein and subsequent energy-dependent efflux of the dye, cells were studied by confocal scanning laser microscopy (CSLM). The cells were visualized by using a Bio-Rad MRC 600 CSLM fitted with a 20-mW krypton-argon mixed gas laser (Bio-Rad) and an objective magnification of ×60 (Nikon ×60 oil, 1.4 numerical aperture, Plan Apo objective). Split-screen images were acquired by using the dual-channel collection mode. The first channel was a transmitted illumination phase-contrast image; the second channel was an epifluorescence image of intracellular fluorescein (excitation line, 488 nm). Each image was averaged over at least three frames to reduce the level of background noise.

Determination of pH_{in}. pH_{in} measurements were carried out exactly as previously described by Bracey et al. (7, 8), except that cultures were grown in SD medium (23). Briefly, cells were grown to late exponential phase in SD medium (pH 4.5, with or without 0.45 mM sorbic acid) at 30°C with shaking. These cells were then loaded with a 100 μM concentration of the fluorescent probe 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), as described previously (7, 8). Fluorescence determinations were made on a Shimadzu RF-1501 fluorometer by using a 1.5-ml optically clear, quartz cuvette (Helma). All readings were made with an excitation scan between 400 and 500 nm, with an emission set at 525 nm (bandwidth, 10 nm). Calibration curves of CFDA-SE cleaved to the fluorescent form, CF-SE, were made in SD medium, buffered with 25 mM citric/phosphate buffer, and were composed by plotting the ratio of fluorescence intensities (emission wavelength, 525 nm) at the excitation wavelengths of 495 nm (pH-dependent point) and 435 nm (pH-independent point) as a function of pH (7). Intracellular pH was calculated from this calibration curve as described previously (7, 8).

Measurement of the effect of sorbic acid on the intracellular ATP/ADP ratio. ATP was measured by using the Celsis High-Sensitivity Bioluminescence kit (Celsis International, Cambridge, United Kingdom). This was carried out by a method adapted from that of Chapman et al. (10) and was done exactly as described by Bracey et al. (8).

RESULTS

Pdr12 confers resistance to monocarboxylic acids with chain lengths of C_1 to C_7. Piper et al. (30) showed that a Δ*pdr12* mutant was hypersensitive to the weak-acid food preservatives sorbic and benzoic acids at pH 4.5. To more clearly identify the substrate specificity of Pdr12, or the range of compounds that it confers resistance to, we tested the sensitivity of the Δ*pdr12* mutant to other weak acids and antifungal compounds.

Unlike growth of the FY1679-28c parental strain, the Δ*pdr12* mutant showed no growth after 28 days of incubation at 30°C in the presence of 20 mM formic acid (C_1), 45 mM acetic acid (C_2), 40 mM propionic acid (C_3), 20 mM butyric acid (C_4), 4 mM valeric acid (C_5), 1.5 mM caproic acid (C_6), and 1.0 mM heptanoic acid (C_7) (Fig. 1). However, the sensitivities of the Δ*pdr12* mutant to fatty acids of longer chain lengths, C_8, C_9, and C_{10}, were similar to that of the isogenic parent (MICs of 0.2, 0.3, and 0.15 mM for octanoic, nonanoic, and decanoic acids, respectively; data not shown) (Fig. 1). In addition, we observed no difference in the sensitivities of the Δ*pdr12* mutant and its isogenic parent (in YEPD [pH 4.5]) to the di- and tricarboxylates succinic acid and citric acid (data not shown).

Loss of Pdr12 had no measurable effect on the sensitivity to the membrane-active compounds amphotericin B and ethanol, the anticancer drug tamoxifen (to which the yeast ABC transporter Pdr5 confers resistance) (24), and the mutagen 4-nitroquinoline-*N*-oxide (a resistance conferred by the ABC transporter Snq2) (36) (data not shown).

Growth in the presence of sorbic acid induces Pdr12, which catalyzes the energy-dependent extrusion of fluorescein from the cytosol. Breeuwer et al. (5) demonstrated that the efflux of carboxyfluorescein from *S. cerevisiae* was dependent on an energy-dependent, carrier-mediated mechanism but did not identify the transport protein. Furthermore, it is commonly known that yeast cells extrude fluorescein from the cytosol and in this study we designed experiments to identify whether fluorescein was a substrate for Pdr12 in order to develop a fluorometric assay to study the kinetics of this transporter.

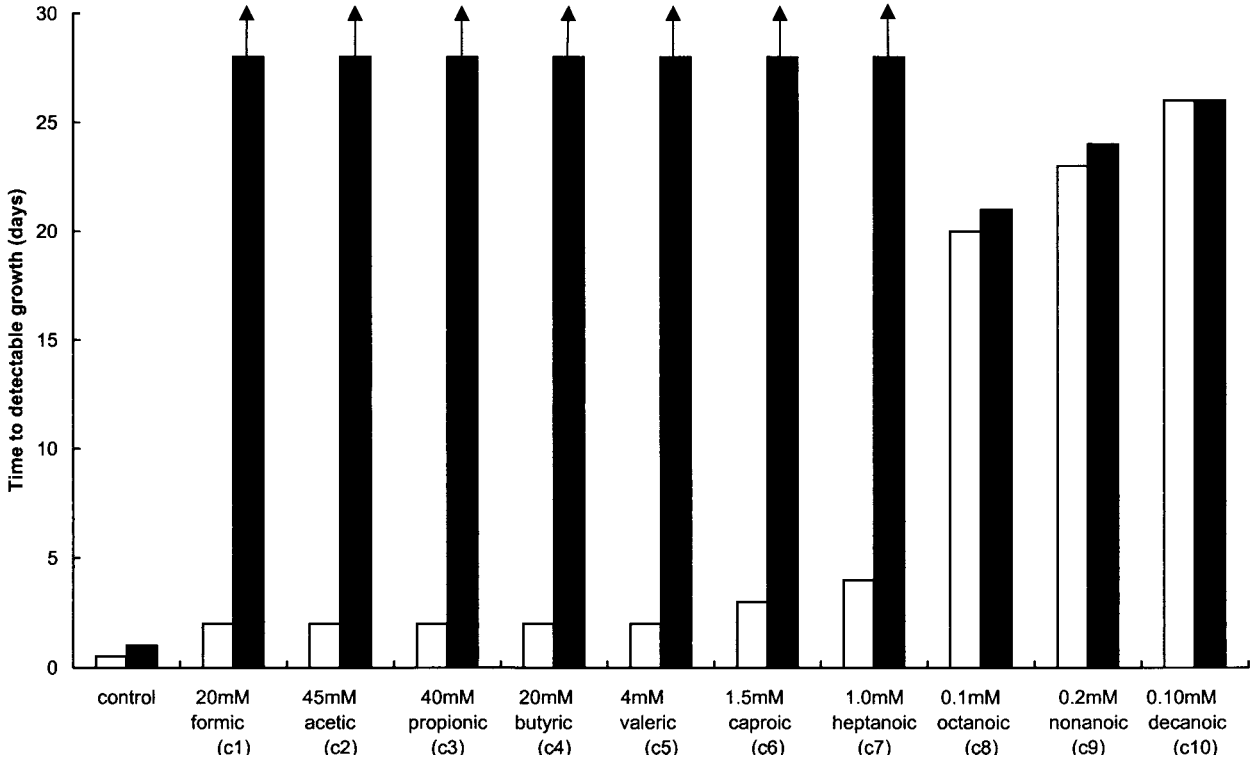

FIG. 1. Comparison of the growth inhibition of *S. cerevisiae* FY1679-28C, the isogenic parent (open bars), and YYM19, the Δ*pdr12* mutant (solid bars), upon exposure to a range of carboxylic acids with carbon chain lengths of C_1 to C_{10}. Growth was determined in a Labsystems Bioscreen apparatus as a detectable increase in optical density (600 nm) compared to the initial value. Arrows indicate that no growth was detected after 27 days of incubation at 30°C in YEPD (pH 4.5). A representative result of at least two replicate experiments is shown.

Cells of the FY1679-28c parent strain and the Δ*pdr12* mutant were grown in YEPD (pH 4.5) in the presence of a subinhibitory concentration of sorbic acid (0.45 mM) to induce strong expression of the Pdr12 transporter in the former strain (30). Both cell types were then loaded with fluorescein (see Materials and Methods). Upon the addition of an energy source in the form of glucose, the parent cells immediately effluxed fluorescein from the cytosol into the surrounding medium. However, under the same conditions, cells of the Δ*pdr12* mutant were unable to efflux any of the dye from the cytosol (Fig. 2A). This reveals that the ABC transporter Pdr12 is the protein that catalyzes energy-dependent fluorescein efflux from *S. cerevisiae*.

As an additional control, the parent and Δ*pdr12* strains were grown in YEPD (pH 4.5) without sorbic acid, conditions under which the expression of Pdr12 is considerably reduced (30), and then loaded with fluorescein as before. Upon the addition of glucose, the efflux of fluorescein from the cytosol by these unadapted cells was virtually negligible over the time course of the experiment (Fig. 2B).

To visualize the extent of intracellular labelling with fluorescein and the energy-dependent efflux of the dye, cells were examined by phase-contrast and fluorescence microscopy. Wild-type parent and Δ*pdr12* mutant cells were again grown in YEPD (pH 4.5) in the presence of sorbic acid in order to induce the expression of Pdr12 in the parent prior to loading with FDA. Before the addition of glucose, both parent and Δ*pdr12* cells were highly fluorescent due to the intracellular cleavage of FDA into fluorescein (Fig. 3). Upon the addition of glucose it can clearly be seen that the parent cells start to lose fluorescence from the cytosol (0.5 h after addition); by 2 h the majority of the intracellular fluorescein had been effluxed. In contrast, despite the addition of glucose, the intracellular levels of fluorescein in the Δ*pdr12* mutant remained virtually constant even after 2 h of incubation (Fig. 3). These visual observations clearly support the results shown in Fig. 2A.

Activity of Pdr12 results in depletion of intracellular ATP and is sensitive to the ATPase inhibitor vanadate. Many studies have employed sodium orthovanadate, a phosphate analogue, to inhibit the ATPase activity of mammalian P glycoproteins (31), the putative P-glycoprotein homologue in *Lactococcus lactis* (3), and the yeast Pdr5 ABC transporter (14).

Adapted parent cells, grown in the presence of sorbic acid and loaded with fluorescein, were exposed to 1 mM sodium orthovanadate prior to the addition of glucose. The presence of vanadate resulted in partial inhibition of the glucose-induced, Pdr12-catalyzed extrusion of fluorescein from the cytosol compared to that in the control (Fig. 4). This provides tentative evidence that the transport of fluorescein by the Pdr12 ABC transporter may use the energy obtained from ATP hydrolysis.

To study the consequences of the induction of Pdr12 on cellular energy levels, the effect of exposure to sorbic acid on the intracellular ATP/ADP ratio of parent and Δ*pdr12* mutant cultures was measured (Table 1). Exposure of the parent cells to 0.9 mM sorbic acid for 5 h resulted in a significant reduction in the growth rate (results not shown) and a depletion of intracellular ATP. This finding supports previous observations that yeast cells induce an energy-consuming stress response upon exposure to preservatives (8, 22, 29). In contrast, while exposure of the Δ*pdr12* mutant cells to sorbic acid resulted in

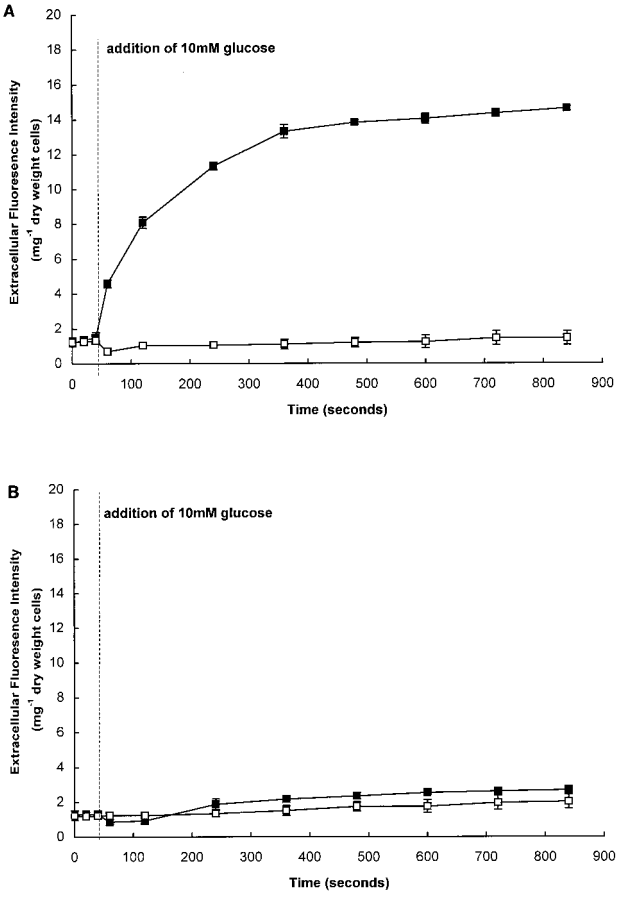

FIG. 2. Efflux of fluorescein from *S. cerevisiae* FY1679-28c, the isogenic parent (■), and YYM19, the Δ*pdr12* mutant (□), resuspended in 50 mM HEPES-NaOH (pH 7.0), upon the addition of 10 mM glucose. Prior to loading of the cells with FDA, both FY1679-28c and YYM19 were grown in either YEPD (pH 4.5) with 0.45 mM sorbic acid to induce Pdr12 (A) or YEPD (pH 4.5) alone (B). The supernatant fluorescence intensity was collected at an excitation wavelength of 435 nm (a pH-independent point for fluorescein). Each datum point represents the mean and the standard deviation of three independent measurements.

the complete inhibition of growth (data not shown), there was a significant increase in levels of ATP inside the cell (Table 1). These results are consistent with the removal of Pdr12 ATPase activity in the Δ*pdr12* mutant, resulting in the accumulation of ATP which would otherwise be consumed by Pdr12 action to remove preservative from the cell.

Pdr12-catalyzed extrusion of fluorescein is inhibited by sorbic and benzoic acids only at low pH. The fluorescein extrusion assay of Pdr12 activity (Fig. 2) allowed us to study whether compounds that inhibit the growth of the Δ*pdr12* mutant are competitive inhibitors of this activity.

At an external pH of 5.5, the addition of increasing concentrations of sorbic acid (0.9 and 1.8 mM) resulted in significant inhibition of glucose-induced fluorescein efflux from parent cells adapted to growth in the presence of 0.45 mM sorbic acid (Fig. 5A). Similarly, the addition of benzoic acid (0.9 and 1.8 mM) also resulted in the inhibition of the Pdr12-catalyzed fluorescein extrusion by these cells (Fig. 5B). We did not study the effect of sorbic and benzoic acids on fluorescein efflux at pH values lower than 5.5 because below this pH the fluorescence intensity of the dye was reduced, making accurate measurements difficult.

Interestingly, we observed greater inhibition by benzoic acid than by sorbic acid. This correlated with growth inhibition data showing that the Δ*pdr12* mutant was more sensitive to benzoic acid than sorbic acid (30). In contrast, increasing the external pH to 7.0 resulted in no significant inhibition of fluorescein efflux by 0.9 and 1.8 mM sorbic acid (Fig. 6). A similar effect was also observed at this pH for benzoic acid (data not shown).

According to the Henderson-Hasselbalch equation, at pH 5.5 sorbate and benzoate are approximately 15 and 5% undissociated, respectively. In contrast, at pH 7.0 both sorbate and benzoate are approximately 99.9% dissociated. Thus, we can postulate that weak-acid inhibition of the in vivo activity of Pdr12 occurs only when the cells are exposed to undissociated sorbic and benzoic acids, implying that inhibition requires the entry of undissociated molecules into the cells.

Pdr12-catalyzed extrusion of fluorescein is competitively inhibited by sorbate and benzoate anions. The inhibition of glucose-induced Pdr12-catalyzed extrusion of fluorescein by increasing concentrations (0.9 and 1.8 mM) of sorbic and benzoic acids at pH 5.5 was characterized kinetically. Analysis of the data in Lineweaver-Burk plots revealed that both sorbic acid and benzoic acid competitively inhibited ATP-dependent fluorescein efflux (Fig. 7A and B, respectively), displaying an unchanging V_{max} but an increasing K_m in the presence of the preservatives. From Fig. 7A, the K_m of Pdr12 for fluorescein was seen to be 5.25×10^{-5} M ($r^2 = 0.96$), increasing to 1.13×10^{-4} M ($r^2 = 0.97$) in the presence of 0.9 mM sorbic acid and 1.32×10^{-4} M ($r^2 = 0.99$) with 1.8 mM sorbic acid. From Fig. 7B, the K_m of Pdr12 for fluorescein was seen to be 3.09×10^{-5} M ($r^2 = 0.99$), increasing to 4.58×10^{-3} M ($r^2 = 0.93$) in the presence of 0.9 mM benzoic acid and 1.47×10^{-3} M ($r^2 = 0.94$) with 1.8 mM benzoic acid. These data provide strong evidence for sorbate and benzoate anions competing with fluorescein for a monocarboxylate binding site on the Pdr12 transporter.

Pdr12-catalyzed extrusion of fluorescein, sorbate, and benzoate is not due to changes in pH_{in}. Cole and Keenan (12) suggested that the efflux of benzoate observed after the addition of glucose to a suspension of starved cells could be due to a reduction in pH_{in} induced by glucose, resulting in a re-equilibration of the weak acid inside and outside the cell in accordance with the new pH gradient.

Under the conditions used in this study and using a method that we have successfully used to detect changes in pH_{in} previously (8), we were unable to detect any significant long-term reduction in pH_{in} in both the isogenic parent and the Δ*pdr12* mutant strains upon addition of glucose. In fact, the pH_{in} values for both strains were the same (data not shown). A possible explanation for this could be that we missed the pH_{in} drop, since it has been shown to be minor (0.4 of a pH unit) and of short, transient duration (38).

It has been proposed that bacteria could be more resistant to weak acids because they are able to survive with a lower pH_{in}, which could result in the efflux of preservatives from the cell (16, 33). Similarly, it could be proposed that yeast cells adapted to growth in the presence of weak acids may accumulate fewer preservative anions internally because the pH_{in} is lower. We tested this hypothesis to determine whether this mechanism could account for the efflux of preservatives from wild-type cells grown in the presence of sorbic acid.

Cells growing in SD medium (pH 4.5) maintain a constant value of pH_{in} (ca. 6.0) (Fig. 8). As we have shown previously (8), despite exposure to 0.9 mM sorbic acid resulting in the significant inhibition of growth, the pH_{in} remains virtually unchanged. As we would expect, cells preadapted to preservative (grown in the presence of 0.45 mM sorbic acid) had a faster

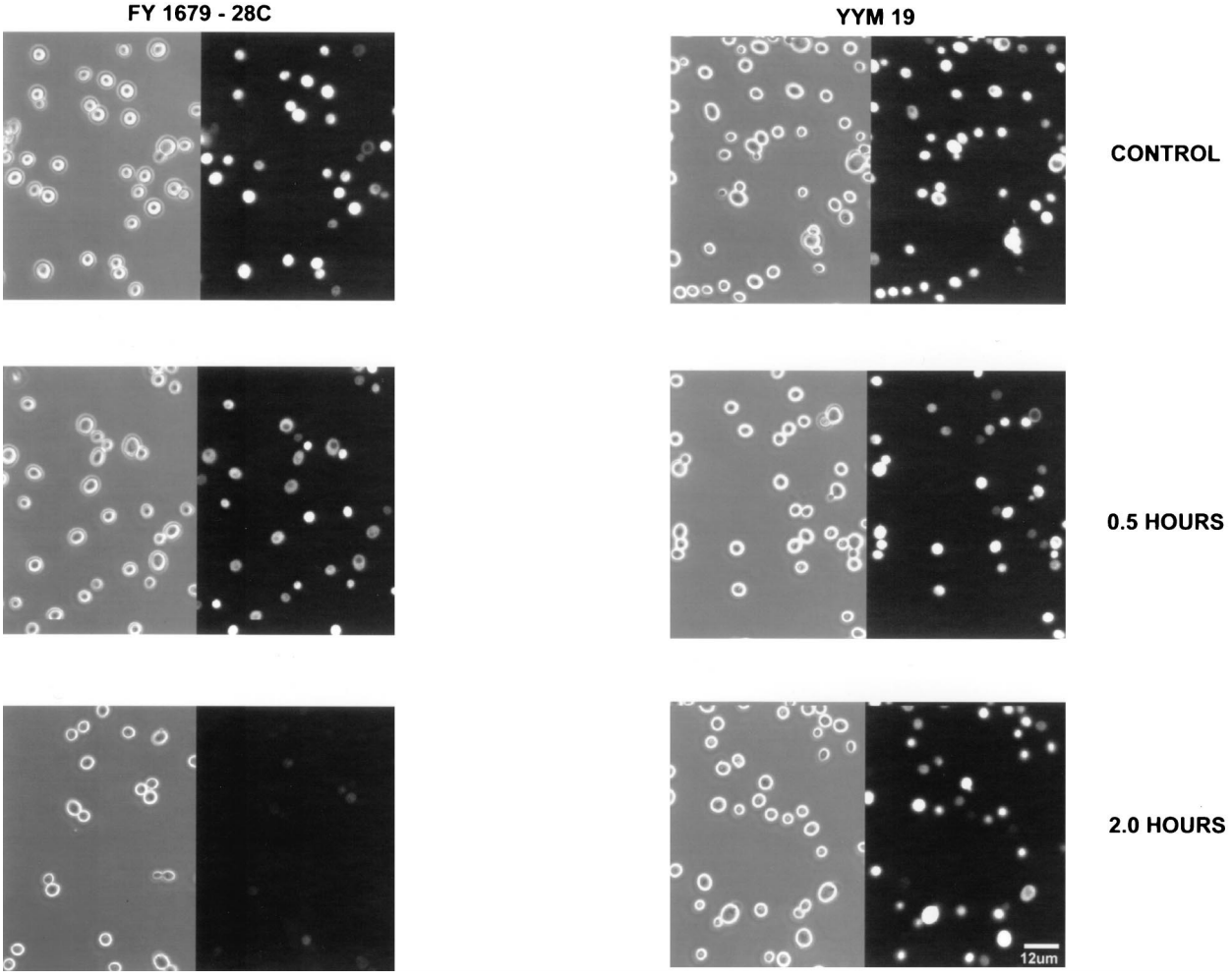

FIG. 3. Visualization of changes in the level of intracellular fluorescein after glucose addition in populations of *S. cerevisiae* FY1679-28c and YMM19 resuspended in 50 mM HEPES-NaOH (pH 7.0). Simultaneous phase-contrast and fluorescence images (excitation line, 488 nm) were obtained by CSLM. Images were taken prior to the addition of glucose (control) and at 0.5 and 2.0 h after the addition of 10 mM glucose to the cell suspensions. Both FY1679-28c and YYM19 were grown in YEPD (pH 4.5) in the presence of 0.45 mM sorbic acid prior to the loading with FDA. Representative images from a number of experiments are shown.

growth rate when reexposed to 0.9 mM sorbic acid than did unadapted cells; however, this could not be attributed to any consequences arising from differences in the pH_{in} which remained the same throughout growth.

DISCUSSION

There are three proposed models for the possible mode of action of ABC transporter proteins such as Pdr12, including transport via an aqueous pore, a lipid "flippase," or a membrane clearing action (reviewed in references 4 and 21). Previously, we demonstrated that growth in the presence of sorbic acid induces Pdr12 (30). We have now shown that this transporter, in the presence of a metabolizable energy source, extrudes fluorescein from the cytosol to the external medium. This implies that Pdr12 does not transport substrates partitioned in the membrane and thus does not operate to clear the membrane as a "hydrophobic vacuum cleaner" (4). However, from our data we cannot distinguish whether Pdr12 acts as an aqueous pore or as a lipid flippase, but there is evidence in the literature that other ABC transporters may operate in the latter fashion (28, 32). Although transport by Pdr12 is entirely dependent on the provision of an energy source, we cannot discount the possibility that transport is initiated by a glucose-activated signal transduction cascade. Similar to other ABC transporters, such as Pdr5 (14), we have demonstrated inhibition of glucose-induced transport by vanadate and accumulation of ATP in the Δ*pdr12* mutant. Together, these results are consistent with Pdr12 having ATPase activity.

We have shown that Pdr12 appears to mediate resistance to water-soluble, monocarboxylic acids with chain lengths from C_1 to C_7. The fact that fluorescein, a much larger molecule, is also a substrate of Pdr12 is compatible with this list of substrates because fluorescein is also a water-soluble, monocarboxylic acid, albeit one with a more complex structure. Exposure to acids with aliphatic chain lengths greater than C_7 resulted in no observable sensitivity of the Δ*pdr12* mutant compared to the parent. Possible explanations for this could be that fatty acids above C_7 are less water soluble and more lipophilic and thus partition into membranes to a greater extent (11, 26). Also, longer-chain carboxylic acids, such as octanoic and decanoic acid, have a more membrane-disruptive effect (37, 41) than do smaller weak acids, such as acetic acid, which tend to dissociate in the cytosol, releasing protons and anions (34). The observation that Pdr12 confers resistance only

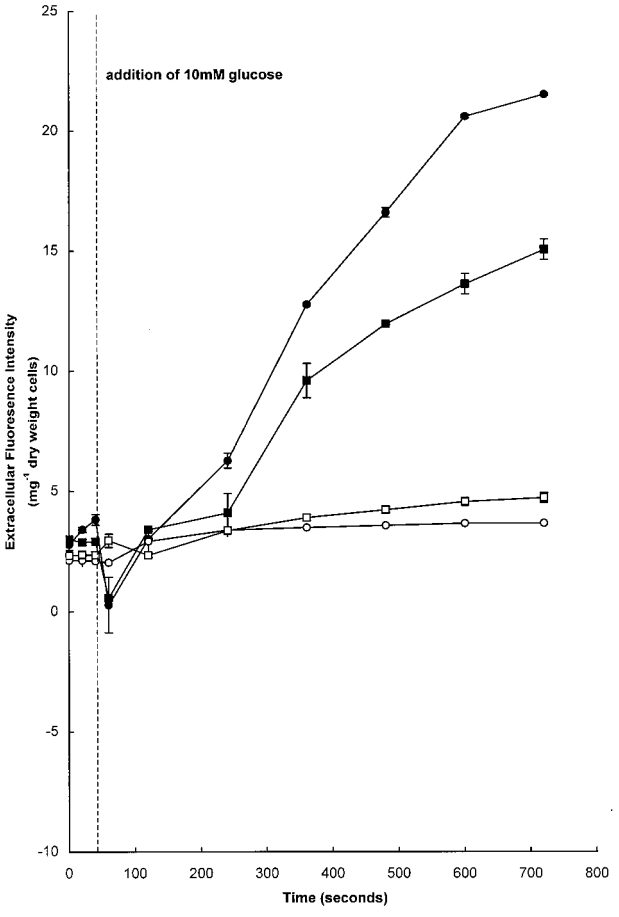

FIG. 4. Glucose-induced (10 mM) efflux of fluorescein from *S. cerevisiae* FY1679-28c (solid symbols) and YYM19 (open symbols) resuspended in 50 mM HEPES-NaOH (pH 7.0) in the absence (●, ○) or presence (■, □) of 1 mM sodium orthovanadate (added 5 min prior to the glucose addition). Both FY1679-28c and YYM19 were grown in YEPD (pH 4.5) in the presence of 0.45 mM sorbic acid prior to the loading with FDA. Each datum point represents the mean and the standard deviation of three independent experiments.

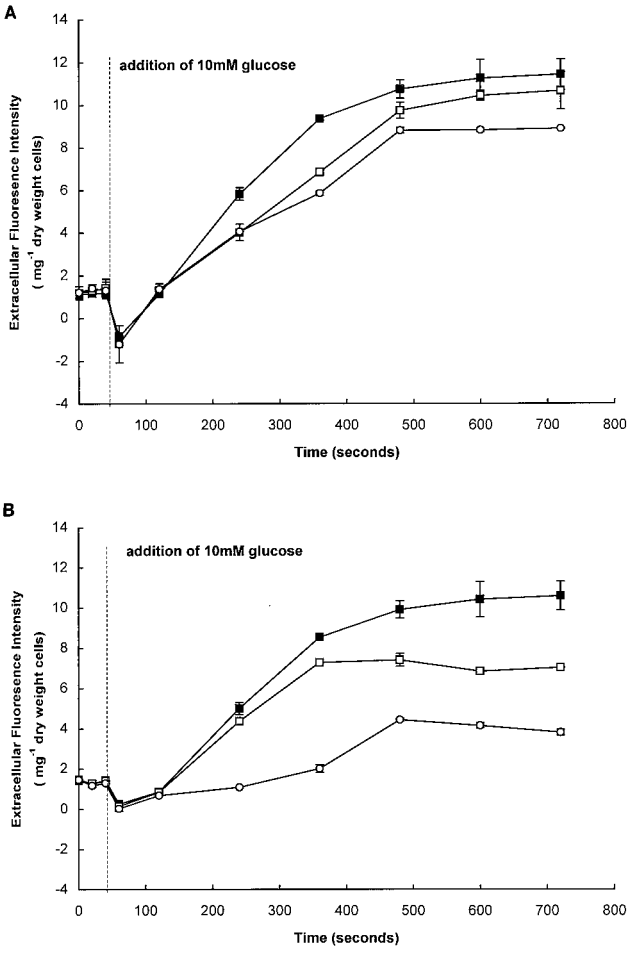

FIG. 5. Glucose-induced (10 mM) efflux of fluorescein from cells of *S. cerevisiae* FY1679-28c resuspended in 50 mM HEPES-NaOH (pH 5.5) in the presence of 0 mM (■), 0.9 mM (□), and 1.8 mM (○) sorbic acid (A) and 0 mM (■), 0.9 mM (□), and 1.8 mM (○) benzoic acid (B). Both sorbic acid and benzoic acid were added 5 min prior to the addition of glucose. FY1679-28c was grown in YEPD (pH 4.5) in the presence of 0.45 mM sorbic acid prior to the loading with FDA. Each datum point represents the mean and the standard deviation of three independent experiments.

to relatively short-chain carboxylic acids and not those of longer chain length that would be partitioned in membranes to a greater extent implies that Pdr12 is capable of transporting only weak acids that would be largely dissociated and thus in the form of anions in the cytosol.

The observation that Pdr12 transports fluorescein has allowed us to use this assay to characterize the molecular substrates and kinetics of the pump. The enzyme has a relatively low K_m value for fluorescein (between 30 and 50 μM), indicating a high degree of affinity for this substrate. This is perhaps surprising considering the structurally diverse range of carboxylic acids that are potentially transported by Pdr12. The finding that sorbic acid and benzoic acid both competitively inhibit the transport of fluorescein provides unequivocal evidence that Pdr12 transports weak acids. However, in what molecular state are the compounds transported: as dissociated anions or as undissociated acid? The fact that we observed no inhibition of Pdr12 transport activity at pH 7.0 indicates that Pdr12 probably transports anions from the cytosol to the external environment. At pH 7.0, both sorbic acid and benzoic acid are greater than 99% dissociated and thus cannot permeate the cell. However, at pH 5.5, at which a small proportion of both acids would be in the undissociated state, inhibition of fluorescein transport by Pdr12 was observed. The most likely explanation for this finding is that undissociated acid external to the cell diffuses across the membrane and, once inside the cell, dissociates into anions and protons due to the higher pH_{in}.

TABLE 1. Effect of deletion of Pdr12 on the intracellular ATP/ADP ratio after exposure to sorbic acid

Growth conditions	Intracellular ATP/ADP ratio[a] (RLU/OD_{600} U of biomass)	
	S. cerevisiae FY1679-28c	*S. cerevisiae* YYM19
0 h of growth in YEPD (pH 4.5)	2.44	2.69
5 h of growth in YEPD (pH 4.5), with no sorbic acid	2.17	2.13
5 h of growth in YEPD (pH 4.5), with 0.45 mM sorbic acid	0.98	3.91

[a] Representative results of two experiments are shown. RLU, relative light unit(s).

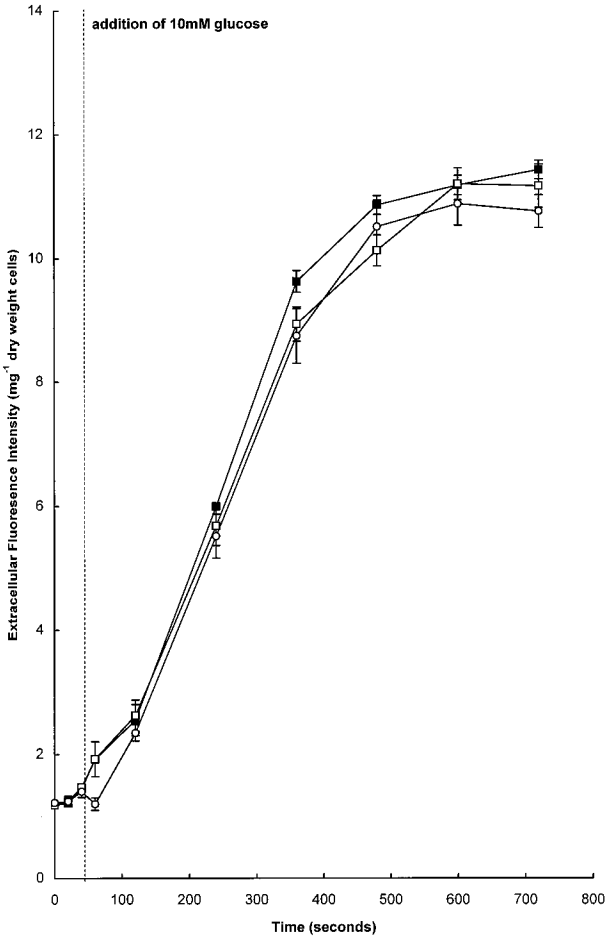

FIG. 6. Efflux of fluorescein from *S. cerevisiae* FY1679-28c resuspended in 50 mM HEPES-NaOH (pH 7.0) upon the addition of 10 mM glucose in the presence of 0 mM (■), 0.9 mM (□), and 1.8 mM (○) sorbic acid. Sorbic acid was added 5 min prior to the addition of glucose. FY1679-28c was grown in YEPD (pH 4.5) in the presence of 0.45 mM sorbic acid prior to the loading with FDA. Each datum point represents the mean and the standard deviation of three independent experiments.

In this way, intracellular preservative anions compete with intracellular fluorescein to be transported from the cell by Pdr12. The available evidence supports this mode of action because Piper et al. (30) observed increased retention of radiolabelled benzoate inside cells of the Δ*pdr12* mutant compared to the parent and Henriques et al. (20) demonstrated that cells grown in the presence of preservatives were able to extrude radiolabelled benzoic acid when a pulse of glucose was added to the cell suspension. Furthermore, to obtain competitive inhibition, it is likely that there would be competition for an active site on Pdr12 between preservative and fluorescein anions inside the cell rather than between extracellular undissociated acid and intracellular fluorescein. We believe that all of the available evidence suggests that Pdr12 transports preservative anions from the cytosol.

The demonstration that yeast cells are able to adapt to preservatives by inducing a membrane protein that transports anions from the cytosol supports the original weak-acid pumping hypothesis that was proposed by Warth (42). Furthermore, other researchers were unable to detect true equilibrium between the internal and external benzoic acid concentrations and thus proposed that anions were being actively extruded from the cells to account for the lower intracellular concentration (20, 39). An alternative explanation for this observed efflux of anions was that it could be due to a reduction in pH_{in} that may occur upon the addition of glucose to starved cells (12, 38). In theory, any decrease in pH_{in} would result in an adjustment of the equilibrium of the preservative inside the cell, resulting in reassociation of the accumulated anion and, due to the concentration gradient, flow of acid back out of the cell. However, in the present study and in one earlier study (8), we were unable to detect any significant differences between the pH_{in} values of cells exposed to preservatives and of those that were not despite observing growth inhibition. Also, in contrast to other studies (12), we were unable to detect any long-term drop in pH_{in} in cells exposed to a pulse of glucose. The most obvious explanations for these contrasting results are that in the aforementioned study the authors were studying *Zygosaccharomyces bailii* and not *S. cerevisiae* and that they were using a different method to measure pH_{in}.

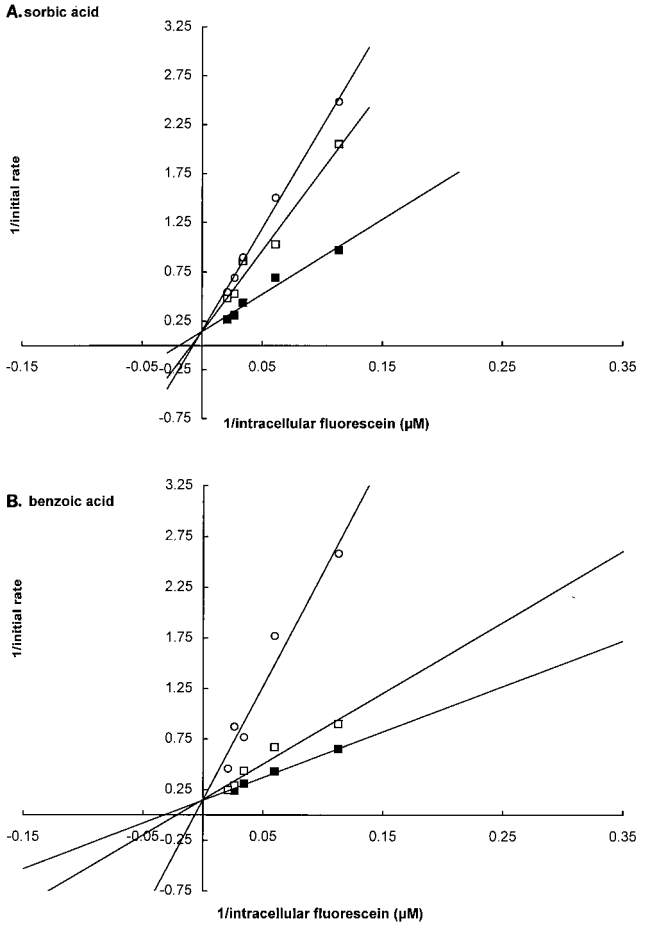

FIG. 7. Lineweaver-Burk plots illustrating competitive inhibition of glucose-induced (10 mM) efflux of fluorescein from *S. cerevisiae* FY1679-28c resuspended in 50 mM HEPES-NaOH (pH 5.5) by 0 mM (■), 0.9 mM (□), and 1.8 mM (○) sorbic acid (A) and 0 mM (■), 0.9 mM (□), and 1.8 mM (○) benzoic acid (B). Both sorbic acid and benzoic acid were added 5 min prior to the addition of glucose. FY1679-28c was grown in YEPD (pH 4.5) in the presence of 0.45 mM sorbic acid prior to the loading with FDA. Rates were calculated from the slope of the linear region of plots showing glucose-induced fluorescein efflux in the presence of increasing concentrations of preservatives. Rate data was then plotted and analyzed by linear regression (Microsoft Excel, version 5.0; Microsoft Corp.) to calculate K_m values describing Pdr12-mediated efflux of fluorescein in the presence of preservatives. Representative results are shown.

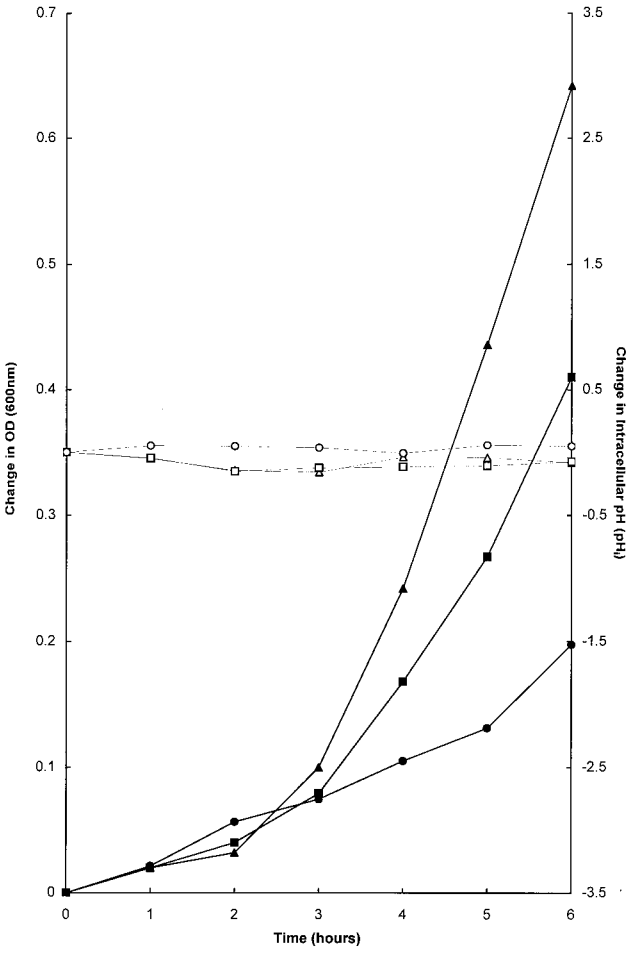

FIG. 8. The effect of exposure to 0.9 mM sorbic acid on the growth (solid symbols) and pH_{in} (open symbols) of unadapted and preservative-adapted (pregrown in SD medium [pH 4.5] in the presence of 0.45 mM sorbic acid) cells of *S. cerevisiae* FY1679-28c growing in SD medium at pH 4.5 at 30°C. At the start of the experiment, the appropriate cells were inoculated into three separate flasks, with or without 0.9 mM sorbic acid, to give an identical starting optical density (600 nm) of 0.35. The growth (monitored by measuring the change in optical density at 600 nm) and pH_{in} were measured in an untreated, control culture (▲, △), while unadapted cells were exposed to 0.9 mM sorbic acid (●, ○) and adapted cells were exposed to 0.9 mM sorbic acid (■, □). The actual value for pH_{in} at the start of the experiment was approximately 6.0. Representative results of two independent experiments are shown.

Importantly, if changes in pH_{in} were mediating the efflux of fluorescein and other carboxylic acids from the cell there is no satisfactory explanation as to why this does not occur to the same extent in the Δ*pdr12* mutant as in the isogenic parent. Furthermore, if a drop in pH_{in} due to glucose addition was mediating long-term, large-scale efflux of preservative, there is no satisfactory explanation as to why this is not also observed to the same extent in unadapted cells exposed to preservatives (20). In conclusion, while the transient reduction in pH_{in} that occurs upon addition of glucose may result in some efflux of preservative, we believe that there is little convincing evidence to suggest that the efflux of fluorescein and other carboxylic acids from adapted *S. cerevisiae* is due to changes in pH_{in} over the long term.

Any model proposing that resistance to preservatives can occur via extrusion of anions from the cell to the external environment must address the problem of futile cycling (12). In theory, if preservative anions were pumped from the cell they would immediately reassociate upon contacting the lower external pH and thus freely diffuse back into the cell, creating a futile cycle that would not confer resistance. This hypothesis assumes that the rate of diffusion of weak acids across the plasma membrane remains the same and that the cell makes no effort to alter membrane composition or structure to reduce the access of the toxic compound. In fact, a recent study by Loureiro-Dias (27) with benzoic acid has shown that adapted yeast cells reduce the diffusion coefficient of preservatives across the plasma membrane such that passage of weak acids into the cell is reduced. Therefore, an adaptive mechanism based around efflux of preservative anions by Pdr12 is no longer futile if there is a concurrent reduction in the ability of the compounds to diffuse back across the cell membrane.

In summary, we can now propose a model describing the mechanism of adaptation to weak-acid preservatives by yeast cells. Water-soluble, monocarboxylic acids diffuse across the plasma membrane, dissociate, and accumulate as anions in the cytosol. In turn, this induces a stress response that results in the energy-dependent transport of preservative anions back into the external environment by the preservative-inducible ABC transporter, Pdr12. At the same time, the activity of the plasma membrane H^+-ATPase is increased, and the energy obtained from the hydrolysis of ATP is used to transport accumulated protons from the cytosol in order to maintain pH_{in} homeostasis (8, 22). In this fashion, toxic anions and excess protons are removed from the cytosol while maintaining the balance of charge across the plasma membrane. The efflux of anions and protons in conjunction with a reduction in the diffusion coefficient of the membrane, which slows the reaccumulation of effluxed preservative (27), results in the maintenance of cell homeostasis such that the organism can survive and grow.

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