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Quantitative Analysis of the Modes of Growth Inhibition by Weak Organic Acids in *Saccharomyces cerevisiae*

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Weak organic acids are naturally occurring compounds that are commercially used as preservatives in the food and beverage industries. They extend the shelf life of food products by inhibiting microbial growth. There are a number of theories that explain the antifungal properties of these weak acids, but the exact mechanism is still unknown. We set out to quantitatively determine the contributions of various mechanisms of antifungal activity of these weak acids, as well as the mechanisms that yeast uses to counteract their effects. We analyzed the effects of four weak organic acids differing in lipophilicity (sorbic, benzoic, propionic, and acetic acids) on growth and intracellular pH (pH_i) in *Saccharomyces cerevisiae*. Although lipophilicity of the acids correlated with the rate of acidification of the cytosol, our data confirmed that not initial acidification, but rather the cell's ability to restore pH_i, was a determinant for growth inhibition. This pH_i recovery in turn depended on the nature of the organic anion. We identified long-term acidification as the major cause of growth inhibition under acetic acid stress. Restoration of pH_i, and consequently growth rate, in the presence of this weak acid required the full activity of the plasma membrane ATPase Pma1p. Surprisingly, the proposed anion export pump Pdr12p was shown to play an important role in the ability of yeast cells to restore the pH_i upon lipophilic (sorbic and benzoic) acid stress, probably through a charge interaction of anion and proton transport.

Almost 40% of the food produced for human consumption is spoiled by microbes, an important issue because of the considerable loss of resources and economic impacts (17). Because of consumer safety, only a few preservatives are approved for foods (36, 43). In recent years, consumer demands have shifted toward fewer and reduced amounts of food additives, creating a need for better mechanistic understanding of traditional preservatives in order to find alternative preventive measures or improve the efficacies of existing ones. A major group of traditional preservatives that are allowed are weak organic acids (23). Weak acid preservatives, such as sorbic, benzoic, propionic, and acetic acids, are widely used antimicrobial compounds in the food and beverage industry to prevent microbial spoilage (3). The specific inhibitory effect of these preservatives depends on pH, causing them to be effective mostly in foods with low pH (10, 12).

At low extracellular pH, weak acids are protonated and uncharged. With a rate that depends on the membrane solubility of the molecule, the protonated form of the acids can diffuse over the plasma membrane and enter the cell. At the near-to-neutral cytosolic pH during exponential growth on glucose (33), the acid dissociates to generate protons and anions, which are charged and therefore cannot simply diffuse back out. This causes intracellular acidification and anion accumulation, both of which impede metabolic function (23, 34, 35). Indirect consequences of weak acid stress are oxidative damage (41) and an inferred perturbation of the plasma membrane (47, 50).

Yeasts can overcome many of the hurdles put up to prevent the microbial spoilage associated with their growth. *Saccharomyces cerevisiae*, commonly known as baker's yeast or brewer's yeast, is an ordinary spoilage yeast that is able to withstand the presence of many weak acids in the concentrations maximally allowed in food (48). During weak acid stress, *S. cerevisiae* uses the plasma membrane H^+-ATPase Pma1p to export protons and induces the ATP-binding cassette (ABC) anion efflux pump Pdr12p, which exports the remaining anions out of the cell. Cells with reduced Pma1p and Pdr12p membrane proteins or activity show weak acid hypersensitivity (19, 20, 39). However, induction of Pdr12p alone is not sufficient to acquire generic weak acid resistance (36), and other pumps may contribute to anion efflux (11).

Another major cause of growth inhibition by weak acids could be energy depletion. Krebs et al. concluded that weak acids likely inhibit glycolysis by acidification, because a key enzyme of glycolysis, phosphofructokinase, is sensitive to low pH *in vitro* (23). Inhibition of glycolysis should eventually lead to ATP depletion. However, while depletion of ATP during weak acid stress was observed, this appeared not to be due to an inhibition of ATP-generating capacity. Rather, the energy demand of intracellular pH (pH_i) restoration and anion export was proposed as the major cause of the depletion of the intracellular ATP pool (20). Toxicity of weak acid preservatives also depends on their structure; acetic acid and sorbic acid have different inhibitory effects, despite their identical pK_a values. The toxicity of weak acids increases with their carbon chain length, affecting lipophilicity. Lipophilicity of a weak acid is reflected by its partitioning between an organic solvent and water. Toxicity of weak acids strongly correlates with such partition coefficients, suggesting that the weak acids' plasma membrane-mediated entry into the yeast cell also is an important determinant of the efficacy of growth inhibition. Therefore, compared to the lipophilic sorbic acid, a much higher concentration of the hydrophilic acetic acid is required to cause similar growth inhibition (47, 51).

Received 5 July 2012 **Accepted** 17 September 2012
Published ahead of print 21 September 2012
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Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.02126-12

TABLE 1 Yeast strains used in this study

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
pma1-007	BY4741 *ygl007w*::*kanMX4*	EUROSCARF
*pdr12*Δ	BY4741 *ypl058c*::*kanMX4*	EUROSCARF
*haa1*Δ	BY4741 *ypr008w*::*kanMX4*	EUROSCARF
*tpo2*Δ	BY4741 *ygr138c*::*kanMX4*	EUROSCARF
AK001	BY4741 + pYES-ACT-pHluorin (*URA3*)	This study
AK002	*pma1-007* + pYES-ACT-pHluorin (*URA3*)	This study
AK003	*pdr12*Δ + pYES-ACT-pHluorin (*URA3*)	This study
AK004	*haa1*Δ + pYES-ACT-pHluorin (*URA3*)	This study
AK005	*tpo2*Δ + pYES-ACT-pHluorin (*URA3*)	This study

At present, the exact mechanisms that yeast uses to adapt to weak acids are still unknown. In order to optimize the use of these well-known safe preservatives, it is important to prevent such adaptation. Interfering with adaptation is best done with knowledge-based means, so that new, optimal preservation strategies can be formulated while improving robustness and maintaining the required safety. In order to disentangle the quantitative contributions of the various inhibitory mechanisms of weak acid preservatives, we compared the effects of four weak acids, sorbic acid (HS), acetic acid (HA), benzoic acid (HB), and propionic acid (HP), on pH_i and growth of *S. cerevisiae*. By using mutants with reduced proton- or anion-excreting capacities, we systematically disrupted specific aspects of the mechanisms counteracting the stress. We established that acidification is the main cause of growth inhibition by acetic acid, while specific anions contribute much to growth inhibition by lipophilic acids. Our data clearly show that acid and anion expulsion are interdependent.

MATERIALS AND METHODS

Yeast strains and culture condition. Strains used in this study are listed in Table 1. *Saccharomyces cerevisiae* BY4741 and three isogenic derivatives, *pma1-007*, *pdr12*Δ, and *haa1*Δ, were transformed with the plasmid pYES-ACT-pHluorin (33).

Strains were cultivated in defined mineral medium (53) with 2% (wt/vol) glucose as the only carbon source. This medium contains ammonium sulfate as a nitrogen source and full supplements of vitamins, minerals, and trace elements, but it is low in fluorescence. Precultures were generated in Erlenmeyer flaks on a rotary shaker at 200 rpm at 30°C. To buffer external pH, potassium citrate was added to a final concentration of 25 mM. All experiments were done at an external pH of 5.0. For pH-controlled growth experiments, strains were cultivated in 500-ml batch fermentors with a steady airflow (500 ml/min) and stirring rate (600 rpm). The pH was controlled at the values indicated by automatic titration with 0.2 M KOH, using an ADI 1030 controller (Applikon, Schiedam, The Netherlands). Cultures were inoculated to an optical density at 600 nm (OD_{600}) of approximately 0.2 in batch fermentors and grown to an OD_{600} of 0.8, corresponding to $\sim 2.4 \times 10^7$ cells/ml. For microplate assays, cells were transferred to Cellstar black polystyrene clear-bottom 96-well microtiter plates (BMG Labtechnologies, Germany) for pH_i and growth measurements.

Measurement of pH_i. pH_i was registered using the pH-sensitive green fluorescent protein (GFP) ratiometric pHluorin as described before (25, 33). Briefly, cultures expressing cytosolic pHluorin were excited with 390- or 470-nm light. Emission was registered at 510 nm in a FLUOstar Optima apparatus (BMG Labtechnologies). Background fluorescence of untransformed cultures was subtracted from both signals independently, before the ratio of the two signals was determined. The pH_i signal was calibrated using cultures of the same yeast strain grown in defined mineral medium with glucose. The cells were permeabilized with digitonin (100 μg/ml in phosphate-buffered saline) for 10 min at room temperature. Cells were collected by centrifugation (5 min at 4,000 rpm), and culture aliquots were resuspended in phosphate-citrate buffers with a range of pH values between 4.5 and 8.5. The emission ratios at 510 nm upon excitation at 390 and 470 nm were determined. GFP ratiometric pHluorin fluorescence ratios can be accurately transformed to pHi values between 5.1 and 8.1 ($r^2 = 0.99$).

Weak acid stress. Stock solutions of all four weak acids were prepared in water (Sigma-Aldrich, Germany). The pH of the stock solutions was adjusted to a value of 5.0 with potassium hydroxide or hydrochloric acid. Stress experiments were initiated after registration of the baseline pH_i. In short-term experiments, such as acidification rate assays, the pH_i was registered in seconds after the acid pulse. For these experiments, cells were grown in batch fermentors to an OD_{600} of 1.0 and then transferred to microtiter plates. For long-term assays in the plate reader, exponentially growing cells were used and the medium was buffered at pH 5.0 with 25 mM potassium citrate. In these experiments growth (OD_{600}) and fluorescence were measured every 10 min for 16 h.

Propidium iodide staining. The effect of weak acid stress on the membrane integrity of yeast cells was assessed using staining with propidium iodide (PI; Sigma-Aldrich, Germany). Growing cultures of wild-type *S. cerevisiae* were exposed to concentrations of weak acids and ethanol (EtOH) that inhibited growth by 50% (1.0 mM HS, 42 mM HA, 0.6 mM HB, 10 mM HP, 12.3% EtOH). Cells were harvested 4 and 24 h after exposure to the stress by centrifugation (5,000 rpm, 5 min) and washed in H_2O. The pellet was incubated with 10 μg/ml PI in H_2O for 10 min at room temperature in the dark. After staining, cells were washed twice to remove excess dye (2). Stained cells were visualized with a fluorescence microscope (Axiovert 40 CFL; Carl Zeiss) using appropriate filters. Image acquisition was done with a Canon Power Shot A640 camera using the Canon remote capture software (v. 2.7.5.27) and analyzed with ImageJ software (v. 1.45s; National Institutes of Health). For each sample, approximately 500 cells from 5 random fields of a slide were counted, and the percentage of PI-positive cells was calculated.

Data analysis. (i) Calculation of growth inhibition. Raw data were exported from the Optima data analysis software to Microsoft Excel 2010 for analysis. We corrected the OD_{600} data to fix the nonlinearity of OD and cell density as described earlier (55). Background was subtracted, and nonlinearity was corrected by measuring OD_{600} values of samples with a range of ODs in the plate reader and adequately diluted for a spectrophotometer (Pharmacia LKB Biochrom). This resulted in the following calibration formula: $[1.1252(OD_{600})^2 + 0.6808(OD_{600}) - 0.0002]$. The corrected OD_{600} was normalized to time zero and log transformed (52). Maximum specific growth rates (μ_{max}) were calculated from transformed growth curves as described previously (22). Briefly, the slope of the growth curve was determined by linear regression of seven consecutive time points (corresponding to 1 h of growth), using a sliding window over the entire growth curve. The maximum two values were removed, and the three highest values were averaged over at least 3 biological replicates. Growth inhibition was calculated as the percentage of the maximal specific growth rate of nonstressed control cultures: growth inhibition = $100 \times [1 - (\mu_{max}\ \text{stress})/(\mu_{max}\ \text{control})]$. In all experiments, nonstressed control cultures were analyzed simultaneously.

(ii) pH_i analysis. The initial acidification was considered the minimum pH_i reached within 2 min upon exposure to the acids, while the recovered pH_i represented the pH_i that cells reached when growing at the maximum specific growth rate after acid exposure. Weak acid entry rates were determined by approximation of the measured acidification in 1-s intervals upon exposure to the acid. As acid-base equilibria settle almost instantaneously, measurement of acidification is an accurate approximation of measurement of entry of a protonated acid. For analysis, only fluorescence ratios corresponding to pH_i values higher than 5.0 were used.

(iii) Calculation of undissociated fractions of weak acids. The concentrations of the undissociated weak organic acids at pH 5.0 were calculated using the Henderson-Hasselbalch equation, $pH = pK_a + \log([A^-]/$

[HA]), using pK_a values of 4.76, 4.21, 4.88, and 4.76 for sorbic, benzoic, propionic, and acetic acids, respectively (27).

(iv) Statistics. All comparative analyses were analyzed for significance using Student's *t* test with a *P* value cutoff of 0.05, unless mentioned otherwise in the text. The correlation of variables was assessed based on the Pearson correlation coefficient (*R*), and the significance of the relation was expressed as *P* values.

RESULTS

The growth-inhibitory effect of weak acids relates to the extent of pH_i restoration after stress. We quantitatively investigated the inhibitory effects of a wide range of concentrations of four commonly used weak acids (acetic, propionic, sorbic, and benzoic acids) on the growth of *S. cerevisiae* BY4741. Weak acids were added to exponentially growing cells of strain BY4741 at pH 5.0. Growth (OD_{600}) was recorded for 16 h after acid addition, during which time control strains went from lag phase through exponential growth to the post-diauxic phase, and we determined the maximum growth rate compared to that of unstressed cells (Fig. 1A and B; see also Fig. S1 in the supplemental material). The effects of four different weak acids on growth are presented as a function of the concentration of the undissociated form of the acid, which is known to be the main contributor to the growth-inhibitory effect (10, 31, 49).

As observed before (51), HB and HS caused growth inhibition at concentrations 1 to 2 orders of magnitude lower than HP and HA, with 50% growth inhibition at 0.6 mM, 1.0 mM, 10 mM, and 42 mM, respectively. We hypothesized that the main cause of growth inhibition is intracellular acidification. Therefore, we recorded pH_i along with growth during 16 h following addition of a range of concentrations of the four weak acids (Fig. 1C to F). The initial decrease in pH_i is the combined outcome of dissociation of the newly entered acid, protons absorbed by cellular buffers, and protons extruded by the activities present in the unstressed cells. Specific anion extrusion might affect the acid-base equilibrium, but anion extrusion activity is negligible before prior exposure to the acids (40). The pH_i that is eventually reached after an adaptation phase depends on these same aspects, but it may additionally be affected by newly expressed or activated mechanisms that assist in recovery, such as anion extrusion pumps, an increased proton extrusion capacity, or changes in cellular buffering. The initial decrease in pH_i caused by weak acid addition (measured after 1 min) varied strongly between the acids, where concentrations of HS that completely inhibited growth caused the pH_i to drop to only just below 6, while concentrations of HA leading to a much stronger pH reduction did not cause any growth inhibition (Fig. 1C to F, white bars; see also Fig. S2 in the supplemental material).

Since in nonstressed cells the buffering and proton extrusion activities present are a given, and not dependent on the acid to which the cells are exposed, the differences in initial acidification between different acids at identical dosages must be caused by differences in entry rates. Since dissociation is extremely rapid, different entry rates also lead to different rates of acidification of the cytosol. We recorded the pH_i every second for the first minute after acid exposure (see Fig. S3 in the supplemental material). The initial slope of these curves represented the rate of acidification (Fig. 2) and reflected the rate of acid entry (zero *trans*-influx). Indeed, the rate of acidification was highest for benzoic and sorbic acids (Fig. 2A) and much lower for propionic and acetic acids (Fig. 2B; note the difference in the acid concentration ranges). Interestingly, the relation between weak acid concentration and acidification rate was linear for HB, HS, and HP ($R^2 > 0.99$; $P < 10^{-4}$), which substantiated an entry mode of simple diffusion. Entry of HA was not linear with acid concentration, suggesting a combination of entry modes with different kinetics. The curve could, however, not be fit with known mechanisms, such as the combination of diffusion and a Michaelis-Menten term.

To assess whether the drop in pH_i as a consequence of acid entry is likely to be the cause of growth inhibition (34), we assessed the correlation of the two parameters. While initial acidification correlated relatively well with growth inhibition for each individual acid (Fig. 3A), marked differences were observed between the hydrophilic HA and the more lipophilic HB, HS, and HP. Overall, growth inhibition correlated better with the pH_i that the cells were able to restore after an adaptation period (the overall correlation of growth inhibition with pH_i for all acids increased from an *r* of -0.79 to -0.92, mostly because acetic acid now acted more similarly to the other three acids [Fig. 3B]). We therefore decided to investigate the role of molecular mechanisms for proton and acid extrusion.

Role of proton efflux pumping in weak acid stress. Since the pH_i correlated with growth, we tested the role of proton pumping during weak acid stress. First, we showed that the pH_i that is reached upon acid exposure indeed depends on a combination of intracellular buffering capacity and an energy-consuming process. We showed in the previous part that identical dosages of different acids caused a different extent of intracellular acidification. In Fig. 1C and D, we found, for instance, that a 2 mM concentration of HS caused the pH_i to drop to 5.4, while an identical concentration of HA caused no apparent drop in pH_i, even although the acids have an identical pK_a. We assessed the effect of depletion of cellular energy by starving the cells for glucose for 30 min, a time which is not sufficient for glucose derepression (13, 32). In these glucose-starved cells, proton extrusion activity was abolished (see Fig. S4 in the supplemental material). In the absence of the proton pumping activity, a 1 mM concentration of either HA or HS caused an identical decrease in the pH_i after 1 min (Fig. 4). This suggested that the pH_i that is observed in the presence of glucose depends on the balance of acid entry and proton pumping. Pma1p is responsible for this activity in *S. cerevisiae* (4, 20), pumping protons at the expense of ATP. In the absence of glucose, ATP cannot be replenished. Pma1p is the most abundant plasma membrane protein of yeast, and *PMA1* is an essential gene. However, a disruption of an open reading frame (ORF; *ygl007w*) in the promoter region results in a reduction of *PMA1* mRNA, protein, and activity (44, 56) to approximately 50%. We used this hypomorphic allele of *PMA1* (*pma1-007*) to assess the contribution of proton pumping activity to growth inhibition. We analyzed the resistance of *pma1-007* toward the same four acids and found that the reduction of Pma1p did not enhance the growth-inhibitory effects of HS, HB, or HP (and, in fact, the mutant was less sensitive to HP). It did cause a severe increase in the growth inhibition caused by HA, reducing the 50% inhibitory concentration from 42 mM to 24 mM (Fig. 5A to D). Contrary to expectations, this reduction of plasma membrane proton pumping capacity did not lead to a larger drop in the pH_i upon acid exposure compared to the wild type, even in the case of acetic acid (Fig. 6, white bars). Also, the rate of acidification measured in the first seconds after acid exposure was not significantly affected for any of the acids compared to the wild type (see Fig. S5 in the supple-

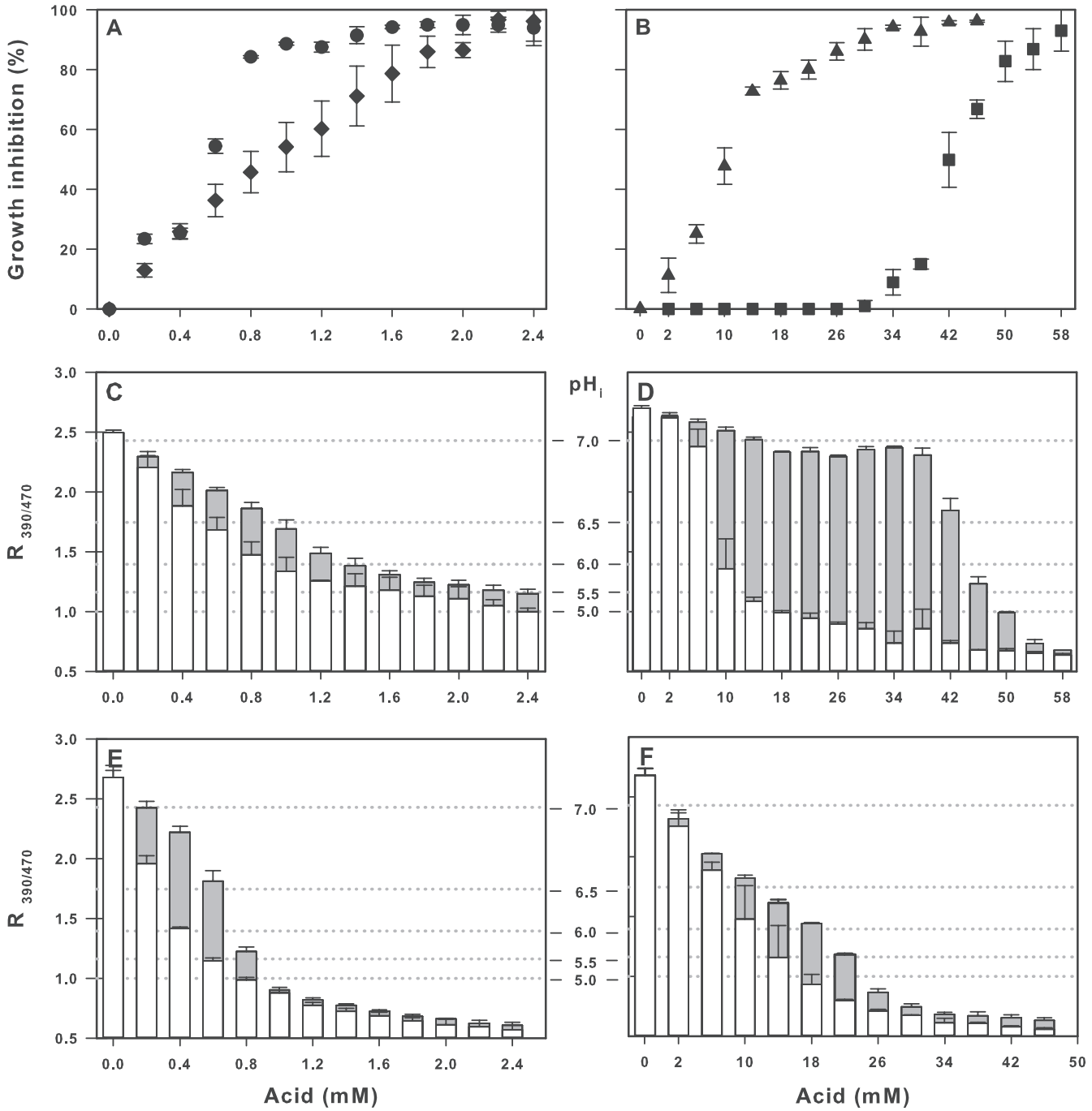

FIG 1 The effects of weak acid preservatives on growth and pH_i of *Saccharomyces cerevisiae* BY4741. Exponentially growing cultures were stressed with increasing concentrations of sorbic acid (◆ [A and C]), acetic acid (■ [B and D]), benzoic acid (● [A and E]), or propionic acid (▲ [B and F]). Addition of weak acid stress caused growth inhibition (A and B) and reduced pH_i (C to F). (A and B) Growth inhibition was determined as described in Materials and Methods. (C to F) Open bars represent the minimum pH_i reached within 1 min after acid exposure, and gray bars represent recovered pH_i after adaptation to stress. The i pH is presented as the fluorescence ratio ($R_{390/470}$), after subtracting the background fluorescence, and dotted lines represent the corresponding pH_i values. Data represent means ± standard deviations of at least three biological replicates.

mental material). This suggests that the initial pH_i is primarily determined by the intrinsic cellular buffer capacity rather than the by direct ATP-coupled extrusion of protons. However, the restoration of pH_i after HA exposure was impaired in the *pma1-007* hypomorph (Fig. 6B) compared with the wild type (Fig. 1D). This suggests that for the three more-lipophilic acids, Pma1p activity, as well as the ATP required by the pump, is well in excess of the amount required for proton pumping to maintain the pH_i. Additionally, comparison of the relation of growth inhibition with recovered pH_i (Fig. 3B) indeed showed that growth in the presence of these three acids was more inhibited than can be explained by pH_i alone: HS, HB, and HP caused more growth inhibition at any

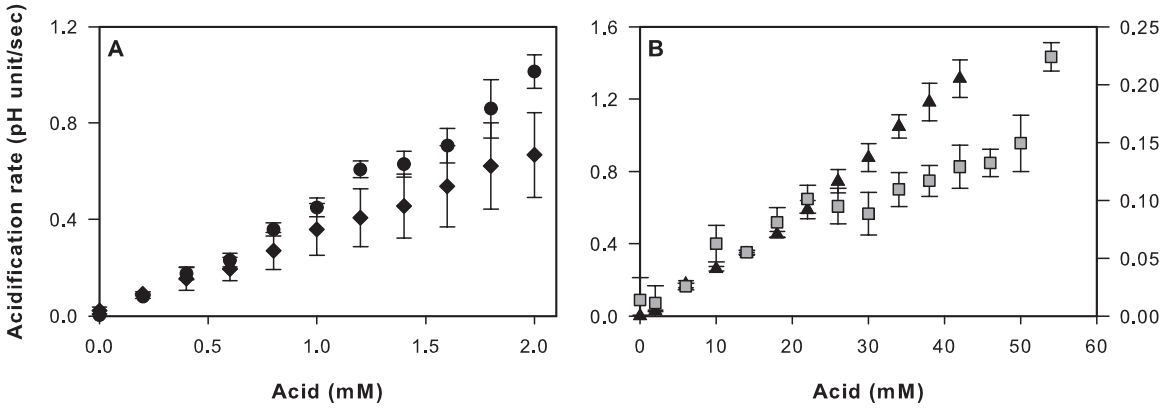

FIG 2 Acidification rates caused by weak acids. After the addition of increasing concentrations of sorbic (◆) or benzoic acid (●) (A) or propionic (▲) or acetic acid (■, secondary axis) (B), the pH_i was measured in 1-s intervals. The initial rate of acidification is plotted against the weak acid concentration. Notice that the *x* axes in panels A and B have different scales.

pH_i than did HA. Therefore, there must be additional growth-inhibitory activities of these acids. This confirms that acidification is not the only cause, or even major cause, of growth inhibition for HS, HB, and HP. Growth-inhibitory concentrations of HA appeared to lead to a net release of protons in the range of the efflux capacity of Pma1p, and a reduction of Pma1p activity affected the balance between influx and efflux, causing a larger pH_i drop and stronger growth inhibition.

Anion efflux affects pH_i homeostatic capacity. If pH_i homeostasis is not the major cause of growth inhibition for lipophilic acids, then what could be the reason for this growth inhibition? Although an effect of the lipophilic acids on membrane integrity has been suggested before (50, 51), we found no strong increase in PI permeability of cells exposed to 50% growth-inhibitory concentrations of the four acids for 4 or even 24 h (Table 2). A concentration of 12.3% ethanol, also leading to 50% inhibition of growth, did result in a significant destabilization of the plasma membrane in this assay. A contribution of the weak acid anions to growth inhibition has also been suggested, and we used a *pdr12*Δ mutant to test this.

Pdr12p is a plasma membrane ABC efflux pump that is believed to have a major role in the resistance of *S. cerevisiae* to lipophilic acids, likely because it catalyzes the export of the anions. Deletion of *PDR12* has been shown to lead to hypersensitivity to weak organic acids (16, 19, 40), although only for the more lipophilic acids. Its affinity appears highest for HB and HS, which also lead to induction of expression of the protein (19).

We investigated the role of the Pdr12p transporter on growth and pH_i recovery during weak acid stress. Indeed, deletion of *PDR12* increased sensitivity toward the more lipophilic acids HB, HS, and also HP, while it did not sensitize toward HA (Fig. 5). Benzoic and propionic acids became more potent growth inhibitors than HS in the absence of the ABC transporter, suggesting that Pdr12p is important for eliminating HB and HP from the cell interior. As with *pma1-007*, deletion of *PDR12* did not lead to a

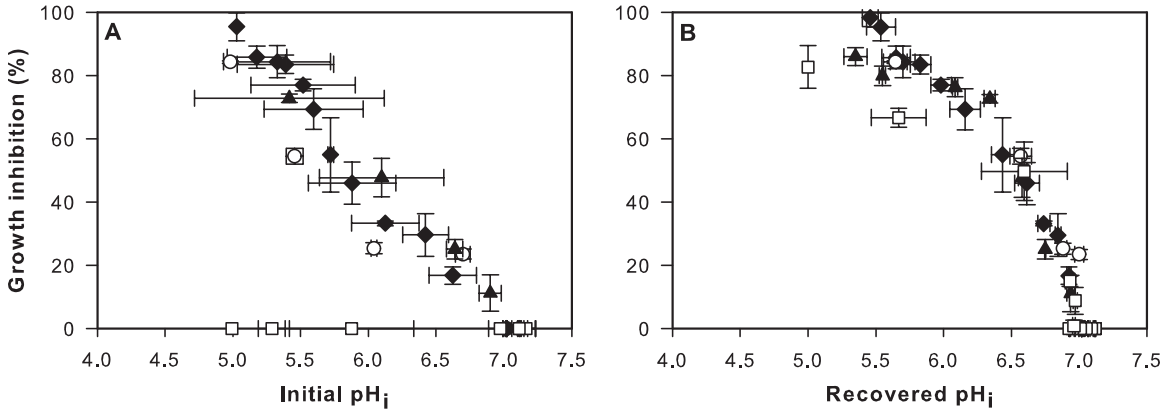

FIG 3 Correlation of growth and pH_i upon weak acid exposure. Growth inhibition data for the wild type were compared with initial acidification (A) and recovered pH_i (B). Data represent the means ± standard deviations of at least three biological replicates. The relation of growth inhibition with initial and recovered pH_i was calculated as described in Materials and Methods. The initial acidification correlated significantly with the growth inhibition caused by lipophilic weak acids (correlation coefficients [*r*] of −0.94, −0.97, and −0.99 [$P < 10^{-7}$] for HB (○), HS (◆), and HP (▲), respectively), while for HA (□) no significant correlation could be observed. The stable pH_i that the cells were able to restore after adaptation to the initial stress (see Fig. 1C to F) correlated better overall with the growth behavior (correlation coefficients of −0.97, −0.98, −0.99, and −0.94, for HB, HS, HP, and HA, respectively; *P* values were $\ll 0.005$ for all acids).

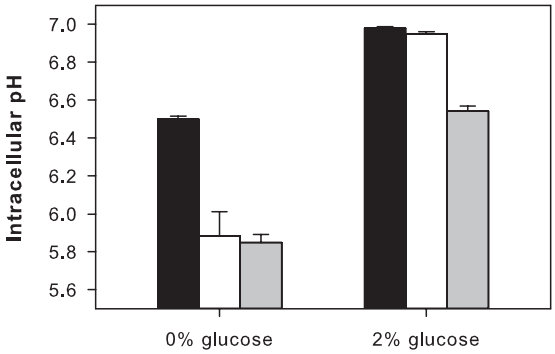

FIG 4 Maintenance of a high pH_i upon acetic acid stress depends on an energy-consuming process. Exponentially growing cells of *S. cerevisiae* wild type were harvested and starved for 30 min in mineral medium without glucose. Cultures with and without glucose were exposed to medium (black bars), sorbic acid (gray bars), or acetic acid (white bars), and intracellular acidification was measured after 1 min. Data represent means ± standard deviations of at least three biological replicates.

bigger initial drop in the pH_i (Fig. 7). In the case of acetic acid stress, the cells restored their pH_i similar to the parental strain, and the *pdr12*Δ mutant showed no acetic acid hypersensitivity. However, in the cases of HP, HS, and HB, the recovery of the pH_i was strongly impaired.

We conclude that in *Saccharomyces cerevisiae* Pdr12p is involved in HP, HS, and HB stress resistance but not in HA stress resistance, as observed earlier (16). Remarkably, it appears that Pdr12p-mediated anion efflux affects proton homeostasis. To see if indeed anion efflux in general is important for pH_i restoration, we decided to test whether acetate efflux also interacted with pH_i homeostasis. To do so, we analyzed pH_i responses of a *haa1*Δ mutant. Haa1p is a transcriptional activator of multidrug transporters Tpo2p and Tpo3p, which are known to be required for acetate efflux. Cells lacking the Haa1p transcription factor accumulate higher concentrations of acetate than the parental strain (11, 26). Growth analysis of the *haa1*Δ strain in the presence of HA (Fig. 8A) was consistent with previous findings where *haa1*Δ showed enhanced sensitivity to acetic acid (26). Interestingly, we found that the strain lacking Haa1p displayed significantly reduced recovery of pH_i (Fig. 8B; see also Fig. S6 in the supplemental material), which likely reflects its transcriptional activation of Tpo2p, as a *tpo2*Δ strain also had a reduced recovery of pH_i (see Fig. S7 in the supplemental material). This showed that acetate efflux, like benzoate, sorbate, and propionate efflux, is directly relevant for proton extrusion and therefore pH_i homeostasis.

DISCUSSION

A number of theories have been presented to explain the antifungal properties of weak acids, including acidification of the cytoplasm, anion accumulation, membrane perturbation, and ATP depletion (4, 29, 50). To what extent these various mechanisms contribute to the antifungal activities of weak acids is still unresolved. We addressed this issue by systematic quantitative analysis

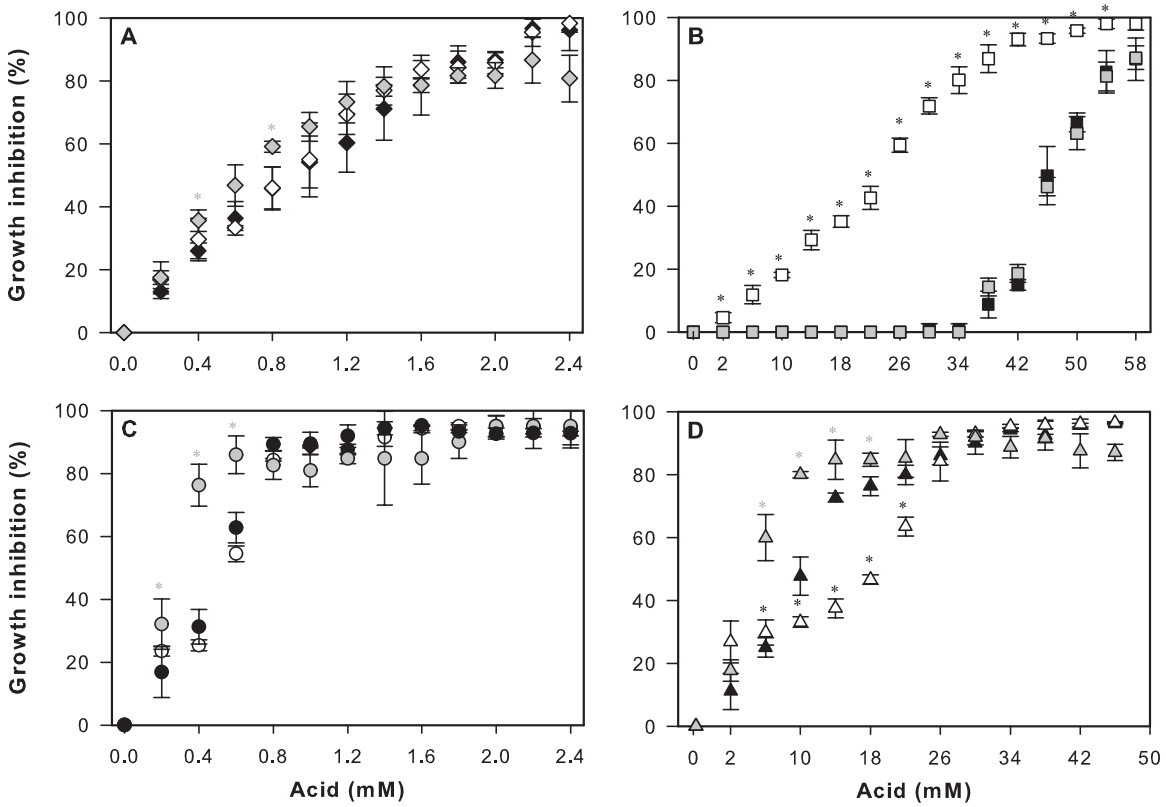

FIG 5 Effects of weak acids on growth of *S. cerevisiae*. The wild type (black symbols) and *pma1-007* (open symbols) and *pdr12*Δ (gray symbols) mutants were stressed with increasing concentrations of the weak acids sorbic acid (A), acetic acid (B), benzoic acid (C), or propionic acid (D). Growth inhibition was determined as described in Materials and Methods. Data represent means ± standard deviations of at least three biological replicates. *, $P < 0.05$ for *pdr12*Δ versus wild type and for *pma1-007* versus wild type.

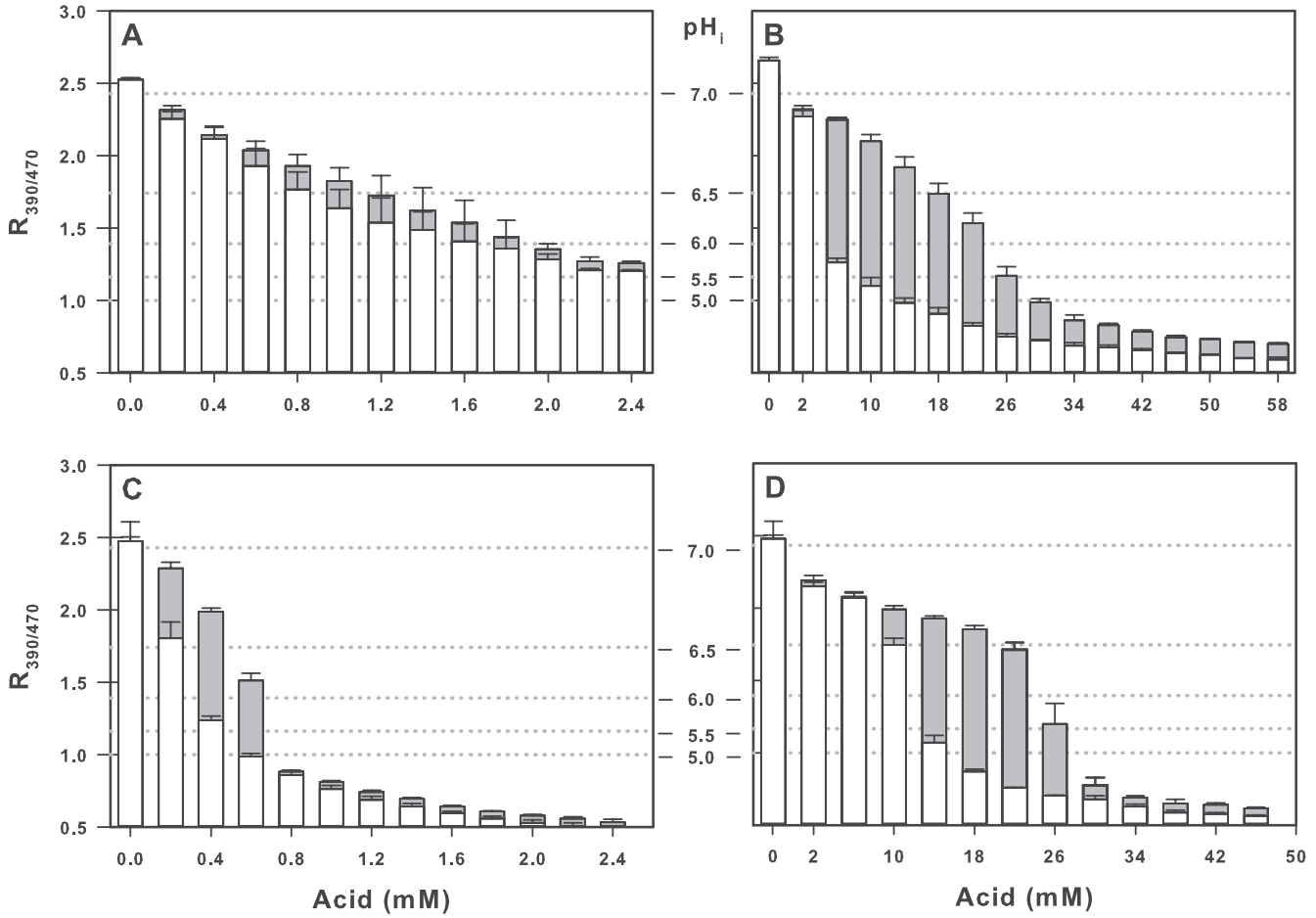

FIG 6 Plasma membrane ATPase Pma1p is crucial for pH_i recovery under acetic acid stress. Growing cultures of strain *pma1-007* were stressed with increasing concentrations of sorbic acid (A), acetic acid (B), benzoic acid (C), or propionic acid (D) to assess the role of Pma1p during stress. Open bars represent the acidification after a 2-min acid exposure, and gray bars represent the recovered pH_i after adaptation to stress. Intracellular pH is presented as the fluorescence ratio ($R_{390/470}$), after subtracting background fluorescence, and dotted lines represent the corresponding pH_i values. Data represent means ± standard deviations of at least three biological replicates. To allow direct comparisons of these results with the wild type (Fig. 1C to F), all experiments were performed under identical conditions.

of growth and pH_i dynamics of *Saccharomyces cerevisiae*. In wild-type yeast, growth inhibition correlated with the lipophilicity of weak acids, fitting the notion that the acids need to diffuse over the membrane to cause growth inhibition (1, 51). Indeed, for the three lipophilic acids, diffusion appeared to be the main mode of entry. Acetic acid appeared to enter by a (combination of) mechanism(s) that did not fit with diffusion. Since the cells were growing on glucose, it is unlikely that the glucose-repressed H^+-acetate symporter (Ady2p) contributes to the entry (7). For example, entry through the Fps1p aquaglyceroporin (28) cannot be excluded, but this should theoretically appear as part of the diffusion kinetics and therefore does not satisfactorily explain the profile observed.

TABLE 2 Membrane integrity is not significantly affected by weak organic acids[a]

Stress	% PI-positive cells at:	
	4 h	24 h
Control (no stress)	0.63	0.68
Ethanol	18.33	40.64
Sorbic acid	1.18	1.21
Acetic acid	0.62	1.01
Benzoic acid	0.92	1.44
Propionic acid	0.97	0.75

[a] Growing cultures of yeast were exposed to 12.3% EtOH, 1.0 mM HS, 42 mM HA, 0.6 mM HB, or 10 mM HP, all leading to 50% growth inhibition. Percentages of propidium iodide-positive cells were determined after 4 and 24 h of continuous exposure.

Acidification as a mode of growth inhibition. Our first approach was to determine whether the intracellular acidification caused by the four weak acids could be considered the main mechanism of growth inhibition, as the pH_i was shown to be in control of growth rate in nonstressed cells (34). Addition of weak acids to growing cells instantly acidified the cells, after which the pH_i recovered to various extents. Both initial and recovered intracellular pH data correlated with the growth inhibition for each acid individually. However, different acids at concentrations causing the same level of acidification led to a different extent of growth inhibition: at any specific pH_i, lipophilic acids caused stronger inhibition than hydrophobic acids. A direct comparison of pH_i and growth inhibition indeed showed that initial acidification cannot be the only or major growth-inhibitory mechanism of the lipo-

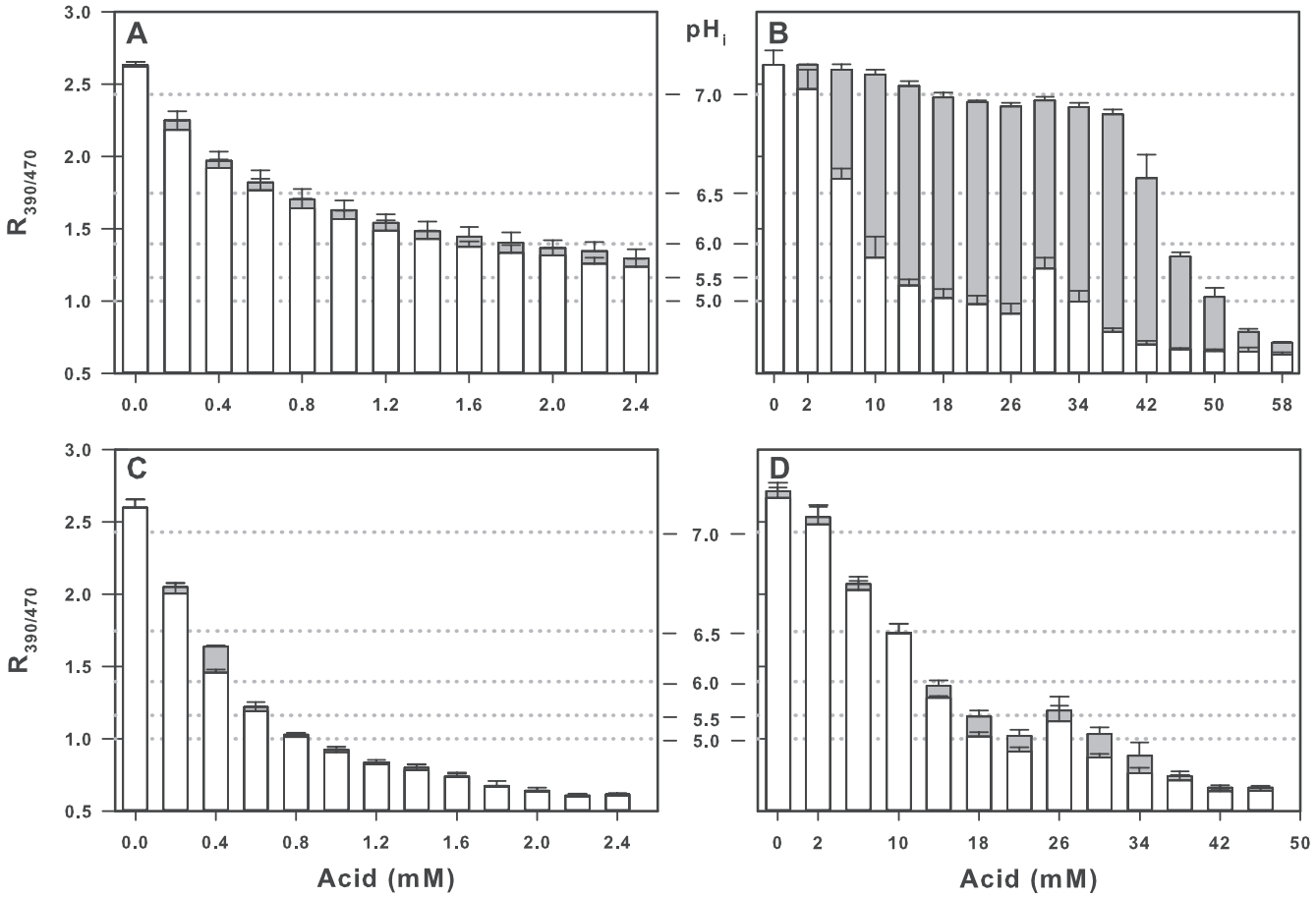

FIG 7 The Pdr12p ABC transporter is essential for pH_i recovery upon hydrophobic acid stress. Growing cultures of the *pdr12*Δ mutant were stressed with increasing concentrations of sorbic acid (A), acetic acid (B), benzoic acid (C), or propionic acid (D). Open bars show the pH_i, after 2 min of stress, while gray bars represent the recovered pH_i after adaptation to stress. The intracellular pH is presented as a fluorescence ratio ($R_{390/470}$), after subtracting background fluorescence, and dotted lines represent the corresponding pH_i values. Data represent means ± standard deviations of at least three biological replicates. To allow direct comparisons of these results with the wild type (Fig. 1C to F), all experiments were performed under identical conditions.

philic weak acids. Pma1p activity is a key determinant of acetic acid resistance. This strongly contrasts with the other three acids, for which the decrease in Pma1p content did not affect growth inhibition. This finding strongly suggests that the capacity of Pma1p to deacidify the cytosol is required fully for pH_i recovery in the case of acetic acid and that acidification is the major cause of growth inhibition by this acid. Conversely, we can conclude that the other mechanisms do not contribute much, and therefore that HA does not cause membrane damage and the anion is not (very) toxic, even at high concentrations. The latter does make sense, as yeast cells can produce and metabolize acetate at relatively high concentrations (14, 21), and therefore metabolism must not be strongly negatively affected by the compound. Benzoic and propionic acids had a very comparable relation between pH_i and growth inhibition, while sorbic acid more effectively inhibited growth at the same pH_i, suggesting the involvement of growth-inhibitory mechanisms other than acidification. Lowering the capacity of Pma1p affected the rate of recovery of the pH_i under sorbic or benzoic acid stress, but the pH_i that was reached was similar to the wild type, and the effect on growth was negligible. The combined data for these acids suggest that acidification does not contribute strongly to the growth-inhibitory effect of lipophilic acids and that the other proposed antifungal mechanisms of lipophilic acids are the primary cause of growth inhibition. As we did not observe an apparent effect on membrane integrity, we first explored the hypotheses that energy limitation and anion toxicity are important contributors to the growth-inhibitory potential of weak acids.

Energy limitation as a cause of growth reduction. The Pma1p ATPase activity for recover of the pH_i requires a lot of ATP (53) and already consumes some 20% of the ATP generated during normal growth on glucose (30). At the same time, acidification decreases ATP production through the inhibition of glycolysis (23). Together, this is thought to lead to depletion of the cellular ATP pool, which may well contribute to growth inhibition. Indeed, to conserve energy, Pma1p activity can be downregulated by Hsp30p (42). While deletion of Hsp30 did not lead to increased sensitivity to different stresses but rather extended the adaptation period, this is still an indication of the need for energy conservation during adaptation (42). Also, upon exposure to glucose, Pma1p is phosphorylated with activating phosphates (24), leading to increased substrate affinity and activity (45). One reason for the apparent resistance to propionic acid and lack of sensitivity to HB and HS of the *pma1-007* mutant could be a lower use of ATP for

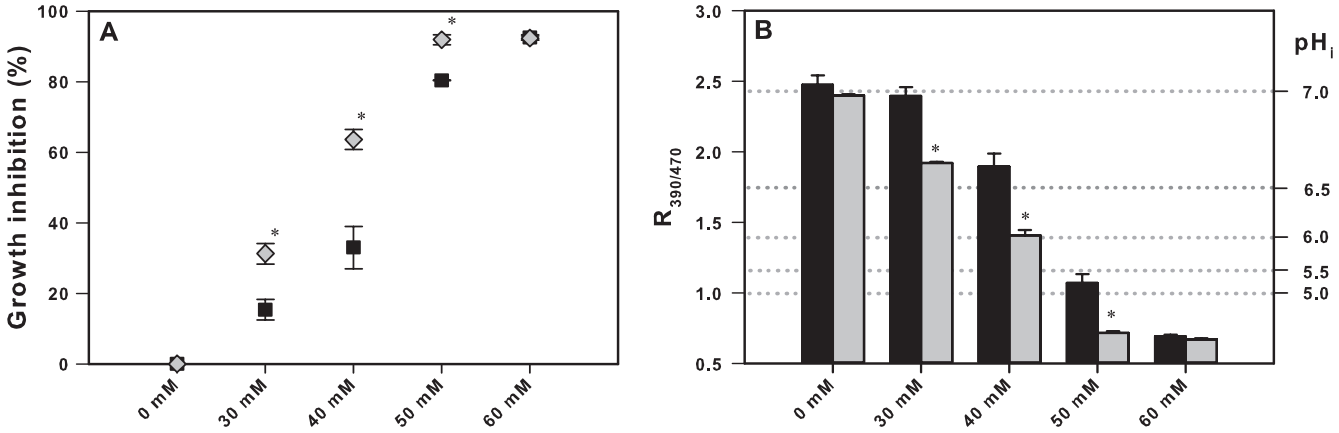

FIG 8 Anion accumulation perturbs pH_i recovery. Effect of increasing concentrations of acetic acid on the growth (A) and pH_i (B) of growing cultures of *S. cerevisiae* wild type (black) and *haa1*Δ mutant (gray) cells. The pH_i data represent the recovered pH_i after stress. The intracellular pH is presented as the fluorescence ratio ($R_{390/470}$), after subtracting background fluorescence, and dotted lines represent the corresponding pH_i values. Data represent means ± standard deviations of at least three biological replicates. *, $P < 0.05$ versus wild type.

proton pumping in this mutant, saving energy for an improved adaptive response. Interestingly, in the absence of glucose, sorbic acid and acetic acid caused similar acidification levels (at the same concentration of acid), while in the presence of glucose, acetic acid reduced the pH_i much less than did sorbic acid at the same concentration. This showed that the high pH_i maintained during HA stress is dependent on an ATP-driven process. However, even though the Pma1p-mediated proton extrusion is responsible for this process, in the cases of both the hydrophilic and the lipophilic acids (54), we showed that in the wild type the Pma1p capacity, depending on both expression and ATP availability, was still far from limiting with the lipophilic acids. Wild-type cells exposed to high dosages of weak organic acids leading to complete growth arrest did not deplete the glucose in the medium during the time course of our experiments (unpublished data). This suggests that the futile cycle of energy-consuming proton and anion export, followed by extracellular reassociation and influx, is not sustained by glucose consumption. Whether the cells cannot sustain the cycle or whether mechanisms are in place that prevent the futile cycle remains to be established.

The contribution of the anion to growth inhibition. The third contribution of weak acid stress to growth inhibition may be the specific anion itself. Both the dosages and the nature of the anion are quite different for the four different acids, as they vary in carbon chain length and structure. Unlike some (spoilage) yeasts, *S. cerevisiae* does not metabolize these anions (29) in the presence of glucose (6). To counteract anion stress, *S. cerevisiae* induces the Pdr12p ABC transporter, which exports anions at the expense of energy (5, 40). Indeed, while a *pdr12*Δ mutant did not display increased sensitivity to acetic acid, it was hypersensitive to benzoic and propionic acids (16, 29, 40). Cells deleted for the Pdr12 pump (*pdr12*Δ) showed little sensitivity to sorbic acid, and the induction of this pump was previously shown to be insufficient for the acquisition of resistance against sorbic acid (36). Others have indeed shown a stronger sensitivity of the mutant toward benzoic acid than sorbic acid (19), and the exact extent of sensitivity may well depend on, for instance, the growth medium used and the pH, as rich medium at low pH results in some weak acid stress because of weak organic acid components of the medium itself (33).

We have argued that acidification is not the major antifungal mechanism of the lipophilic acids, and that anions might also play a role. We cannot measure the anion concentrations, but with our data it is possible to make estimations. Weak acids acidify the cytosol in seconds, depending on the lipophilicity of the acid. If we assume that the concentrations of undissociated acid in and outside the plasma membrane reach equilibrium during this time (50), we can estimate the intracellular anion concentration by using our pH_i determinations and the Henderson-Hasselbalch equation. Interestingly, such an analysis would suggest that the anion concentration in the *pdr12*Δ mutant was lower than that in the wild-type strain upon HS exposure (data not shown). This is not consistent with the observation that the *pdr12*Δ mutant is hypersensitive to HS. We believe, therefore, that the assumption of equilibrium for internal and external sorbic acid levels is incorrect, which implies that the proton and possibly also anion export fluxes are high with respect to the influx, so that such a simple equilibrium cannot be established.

Interestingly, we revealed an interaction between anion extrusion capacity and the ability to restore pH_i; our pH_i data showed that upon acetic acid stress, the *pdr12*Δ mutant behaves like the wild type and can restore its pH_i. However, in the case of sorbic, benzoic, and propionic acids, we clearly observed that the *pdr12*Δ mutant could not efficiently restore the pH_i. While previous reports showed that Pdr12p exports different kinds of molecules, such as water-soluble carboxylate anions, fluorescein (19), caffeine (37), and fusel acids (18), no data have been reported that suggest how Pdr12p might be involved in pH_i regulation itself. It is apparent from our data that the Pdr12p protein does not interfere with pH_i regulation directly, as the mutant did not show defects in pH_i recovery under HA stress. This suggests a relation with its function as an anion exporter. It could be that the protons are cotransported with anions, but since Pma1p activity is easily high enough, there is no reason to assume that the absence of such symport would cause a major defect in deacidification. Another possible explanation could be that these specific anions hamper the cells' ability to generate ATP from glucose (38). However, the fact that a *haa1*Δ mutant, which cannot express the acetate transporters Tpo2p and Tpo3p and was shown to accumulate acetate,

had a similar defect in pH_i restoration but not upon acetic acid stress suggests that rather than an anion-specific effect, there may be an aspecific effect of anion accumulation on proton pumping. This might, for instance, be due to an effect on the membrane potential, through a defect in charge balancing, which in turn would affect Pma1p or other proton translocation activities (15, 46), but this cannot be confirmed yet. Formally, we cannot exclude that, rather than being a consequence, the defect in pH_i restoration is the primary effect and that this defect is a cause of the increased anion accumulation in *pdr12*Δ and *haa1*Δ strains (11, 26). Simply the low pH_i values do not explain this, as at equilibrium, a low pH_i should lead to a decrease in the anion concentration. However, such a reversal of cause and consequence would imply a different type of function for these anion pumps, for which currently no evidence is at hand.

It is important to note that recovery of the pH_i to neutral in the absence of mechanisms to prevent acid entry would only lead to more acid influx, more intracellular dissociation of the acid, and eventually higher accumulation of the anion. Additionally, since the ratio of undissociated and dissociated acid in the cell depends on the pH_i, it is quite obvious that increasing the pH_i without a matching export of anions would lead to a tremendous accumulation of anions in the cell, to within the molar range. It might be that a low pH_i is preferable to the accumulation of high concentrations of organic anions, and/or energy loss for the cell. Therefore, it might be beneficial for the cell not to export protons if anions are abundantly present and, thus, prevent an energy-consuming futile cycle and anion accumulation. Pdr12p-dependent anion extrusion reduces the negative charge present in the form of weak acid anions, which should facilitate Pma1p-mediated extrusion of positively charged protons, as electrostatic charge across the plasma is required for Pma1p activity (9, 40). Also, it is well possible that a mechanism has evolved that regulates Pma1p activity in response to the intracellular concentrations of weak acid anions. The failure of the *pdr12*Δ mutant to restore the pH_i upon lipophilic acid exposure, and of the *haa1*Δ mutant, which is defective in expression of acetate exporters, to do so upon acetic acid stress fits with the existence of such a mechanism. Taken together, our data strongly suggest that proton extrusion is repressed by anion accumulation.

ACKNOWLEDGMENTS

We are grateful to HEC, Pakistan, for financial support (A.U.).

We thank many of our lab members for fruitful discussions and laboratory assistance.

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