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Transport of Acetic Acid in *Zygosaccharomyces bailii*: Effects of Ethanol and Their Implications on the Resistance of the Yeast to Acidic Environments

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Cells of *Zygosaccharomyces bailii* ISA 1307 grown in a medium with acetic acid, ethanol, or glycerol as the sole carbon and energy source transported acetic acid by a saturable transport system. This system accepted propionic and formic acids but not lactic, sorbic, and benzoic acids. When the carbon source was glucose or fructose, the cells displayed activity of a mediated transport system specific for acetic acid, apparently not being able to recognize other monocarboxylic acids. In both types of cells, ethanol inhibited the transport of labelled acetic acid. The inhibition was noncompetitive, and the dependence of the maximum transport rate on the ethanol concentration was found to be exponential. These results reinforced the belief that, under the referenced growth conditions, the acid entered the cells mainly through a transporter protein. The simple diffusion of the undissociated acid appeared to contribute, with a relatively low weight, to the overall acid uptake. It was concluded that in *Z. bailii*, ethanol plays a protective role against the possible negative effects of acetic acid by inhibiting its transport and accumulation. Thus, the intracellular concentration of the acid could be maintained at levels lower than those expected if the acid entered the cells only by simple diffusion.

Zygosaccharomyces bailii is a food spoilage yeast species. It is well known for its capacity to survive in stress environments and, in particular, in acid media with ethanol, such as in wine (8). An understanding of the mechanisms underlying the tolerance of the yeast to acids is necessary for the improvement of food and beverage preservation. This requires elucidation of the first step of their metabolism, that is, transport across the yeast plasma membrane. In the case of weak monocarboxylic acids, information on transport is restricted to a few yeast species, namely *Saccharomyces cerevisiae* (4), *Candida utilis* (3, 11), and *Torulaspora delbrueckii* (2). The available data on the uptake of short-chain monocarboxylic acids by these yeasts provide evidence for proton symports of their anionic forms. On the other hand, in *Kluyveromyces marxianus*, a uniport for monocarboxylates was suggested to be present (9). Although there are some studies pointing to the possible involvement of mediated transport systems for weak acid preservatives in *Z. bailii* (18, 19), no detailed characterization has been done.

In this paper we present a study on membrane transport of acetic acid and other weak organic acids in a strain of *Z. bailii* grown under different conditions. The aim of this work was to identify possible interactions of ethanol with the membrane transport of acetic acid and to investigate whether these could be correlated with the high resistance of the yeast during alcoholic fermentation. To this end, the effect of ethanol on the uptake of acetic acid was also studied.

MATERIALS AND METHODS

Microorganisms, growth conditions, and cell suspension preparation. The yeast *Zygosaccharomyces bailii* ISA 1307 was maintained in a medium containing glucose (2% [wt/vol]), peptone (1% [wt/vol]), yeast extract (0.5% [wt/vol]), and agar (2% [wt/vol]). For growth, a liquid mineral medium with vitamins (16) and 0.5% (wt/vol) of the desired carbon source and mechanical shaking at 25°C were used. The carbon sources tested were glucose, fructose, ethanol, and acetic, DL-lactic, propionic, formic, sorbic, and benzoic acids. The pH of the medium was adjusted to 5.0 by the addition of sodium hydroxide. At exponential growth phase, cells were harvested, centrifuged, washed twice with ice-cold distilled water, and suspended in distilled water to a final concentration of about 40 mg (dry weight) ml^{-1}.

Measurement of initial uptake rates. The initial uptake rates were measured by the use of [U-^{14}C]acetic acid, sodium salt, [1-^{14}C]propionic acid, sodium salt, or [7-^{14}C]benzoic acid. Yeast suspensions (10 μl) were mixed in 10-ml conical tubes with 30 μl of 0.1 M potassium phosphate buffer at the desired pH value. After 2 min of incubation in a water bath, at 25°C, the reaction was started by the addition of 10 μl of an aqueous solution of the labelled acid at the desired concentration and pH value and stopped by dilution with 5 ml of ice-cold distilled water. When the uptake of acid was measured in the presence of alkanol or uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), the cells were preincubated for 5 min with the compound, at the desired pH value, before the labelled acid was added. The concentration of CCCP was 50 μM, and the concentration of alkanol was as indicated in Results. Sampling times were 0, 5, and 10 s, times over which the uptakes of labelled acids were linear. After the reaction was stopped, the mixtures were immediately filtered through GF/C filters (Whatman, Inc., Clifton, N.J.), the filters were washed with 10 ml of ice-cold water, and the radioactivity was counted in the scintillation fluid OptiPhase HiSafe II (LKB Scintillation Products). The radioactivity was measured with a Packard Tri-Carb 2200 CA liquid scintillation counter, with correction for counting efficiency (Packard Instrument Co., Inc., Rockville, Md.). Nonspecific ^{14}C adsorption to the filters and/or the cells was determined by adding labelled acid after ice-cold water. The values estimated represented less than 5% of the total incorporated radioactivity.

The occurrence of proton movements associated with acid uptake was tested with a standard pH meter (PHM 82; Radiometer A/S, Copenhagen, Denmark) connected to a Perkin-Elmer (Norwalk, Conn.) model R 100 A recorder. The pH electrode was immersed in a water-jacketed chamber provided with magnetic stirring. To the chamber were added 4.5 ml of 10 mM potassium phosphate and 0.5 ml of yeast suspension. The pH was adjusted to the desired value, and a baseline was obtained. The desired amount of carboxylic acid (adjusted to the experimental pH value) was added, and the subsequent alkalization was monitored with the recorder. The initial rates of the proton uptake were calculated from the slope of the initial part of the pH trace. Calibration was performed with HCl.

Measurement of accumulation of labelled acetic and propionic acids. Suspensions of cells (20 μl) grown in the conditions referenced in Results and prepared as described above were added to 60 μl of 0.1 M potassium phosphate buffer (pH 5.0) and also to 60 μl of buffer containing ethanol, butanol, or CCCP and incubated for 5 min at 25°C with magnetic stirring. The reaction was started by the addition of 20 μl of [U-^{14}C]acetic acid at 0.1, 0.5 mM (about 20.0 $Bq \cdot nmol^{-1}$), and 7 mM (about 3 $Bq \cdot nmol^{-1}$) or [1-^{14}C]propionic acid at 0.5 mM (about 18.3 $Bq \cdot nmol^{-1}$). At appropriate times, 5 μl was taken from the reaction mixture, diluted with 5 ml of ice-cold water, and filtered immediately through Whatman GF/C filters. The filters were washed with 10 ml of ice-cold water, and the radioactivity was counted as indicated above.

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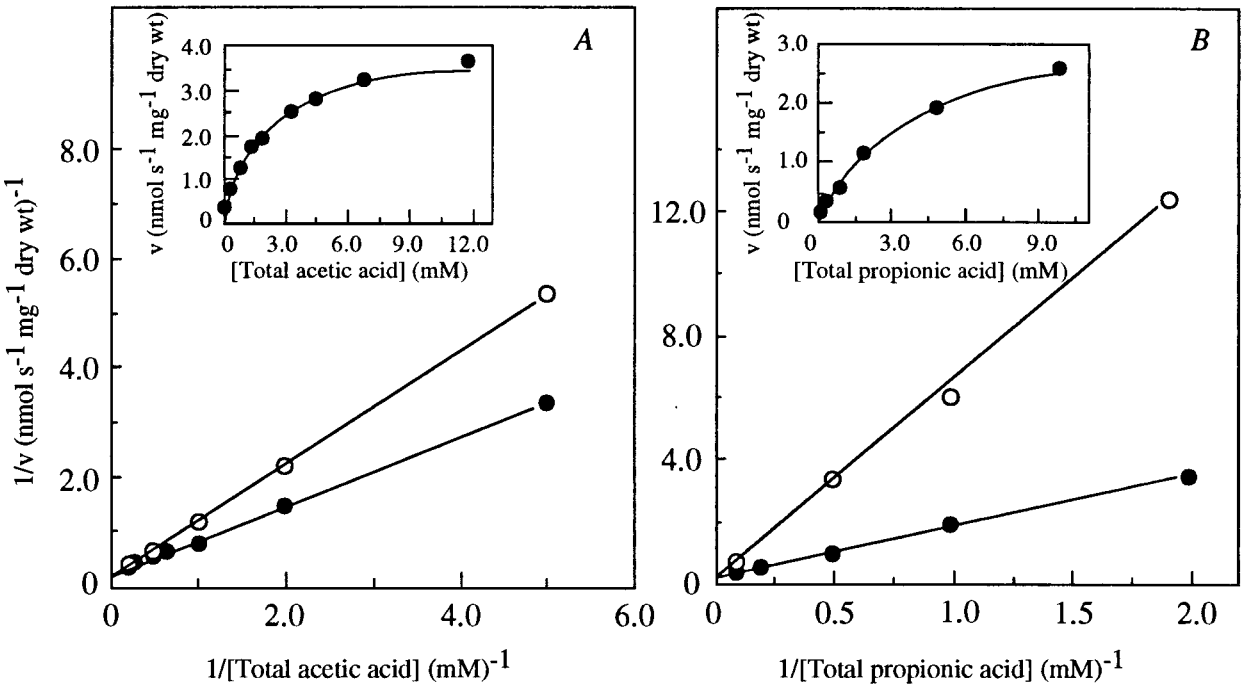

FIG. 1. Lineweaver-Burk plots of initial uptake rates of labelled acetic acid (A) or propionic acid (B) at pH 5.0 as a function of acid concentration by acetic acid-grown cells of *Z. bailii* ISA 1307 in the absence (●) and presence (○) of 20 mM cold propionic acid (A) and acetic acid (B). (Inserts) Initial uptake rates of labelled acetic acid (A) or propionic acid (B) as a function of acid concentration.

The intracellular concentration of acetic acid or propionic acid was calculated by using the values for the intracellular volume estimated as described below. The accumulation ratio, at each pH value, was estimated as the relationship between the intracellular and the extracellular acid concentrations expressed as the anionic form of the acid, assuming that under the experimental conditions used no significant intracellular acidification was induced.

Measurement of intracellular volume. The intracellular water volume was measured as previously described (6, 13). With cells grown in a medium with glucose or acetic acid, a value of 1.1 ± 0.19 or 1.3 ± 0.22 μl of intracellular water per mg (dry weight) of the yeast, respectively, was obtained.

Calculations of concentrations of carboxylic acid forms. The carboxylic acids under study in aqueous solution are incompletely dissociated, the equilibrium concentration forms of the acids being a function of the pH and of the total acid concentration. The following nomenclature was used for the different forms at equilibrium: undissociated acid (uncharged form), anion (charged form), and total carboxylic acid (combination of the two forms). Unless specified, the designation "acetic acid," or any other carboxylic acid, corresponds to total acid. Concentrations of undissociated acid and of its anionic form were calculated by the Henderson-Hasselbach equation with pK_a values of 4.76 for acetic acid and 4.87 for propionic acid (7).

Calculation of kinetic parameters for transport systems. The experimental datum points from the initial uptake experiments were analyzed as described previously (10) by a computer-assisted nonlinear regression analysis with the GraphPAD (San Diego, Calif.) computer program. By this method, the transport kinetics best fitting the experimental initial uptake rate were determined, and then estimates for the kinetics parameters were obtained.

Chemicals. The radioactively labelled [U-^{14}C]acetic acid and [1-^{14}C]propionic acid (Radiochemical Center, Amersham, Buckinghamshire, United Kingdom) used had specific activities of 2.18×10^9 and 2.52×10^8 Bq mmol^{-1}, respectively. The radioactively labelled [7-^{14}C]benzoic acid (NEN Products-Dupont, Boston, Mass.) used had a specific activity of 6.55×10^8 Bq mmol^{-1}. All other chemicals were reagent grade and were obtained from commercial sources.

Reproducibility of results. All the experiments were repeated at least three times, and the data reported here are the average values.

RESULTS

Transport of acetic acid and other weak acids in cells grown with different carbon sources. *Z. bailii* ISA 1307 was able to use acetic acid but not DL-lactic, propionic, formic, sorbic, and benzoic acids when added to the culture medium as sole carbon and energy sources. In cells grown in acetic acid medium, the transport of labelled acetic acid (from 0.2 to 12 mM) at pH 5.0 obeyed Michaelis-Menten kinetics. A Lineweaver-Burk plot showed monophasic kinetics over the entire acid concentration range (Fig. 1A). This agreed with the presence of a mediated transport system, with the kinetic parameters, V_{max} of 4.3 ± 0.4 nmol of total acetic acid s^{-1} mg (dry weight) of cells^{-1} and K_m of 2.6 ± 0.4 mM total acetic acid. Propionic acid (Fig. 1A) and formic acid (data not shown) were competitive inhibitors of acetic acid transport, suggesting that they probably shared the same carrier. Accordingly, acetic acid also competitively inhibited the transport of labelled propionic acid (Fig. 1B). Lactic acid had no significant effect on the transport of labelled acetic acid. Benzoic and sorbic acids acted as inhibitors but apparently not in a competitive way (data not shown), indicating that these acids were not recognized by the acetic acid carrier. Given the nature of the substrates under study, coexistence of a mediated transport system with a simple diffusion of the undissociated acid is conceivable. The relative concentration of undissociated acid increases with decreasing external pH. However, also at lower pH (3.0 and 4.0), kinetic analysis of acetic acid uptake revealed monophasic kinetics over the entire acid concentration range (P of <0.05; data not shown). Estimates of kinetic parameters for acetic acid transport were obtained from Lineweaver-Burk plots (Table 1). The variation of K_m with the pH (which ranged from 3.0 to 6.0) was greater when expressed as the concentration of undissociated acid (115-fold) than when expressed as the concentration of acetate (29-fold), suggesting that probably the anionic form of the acid rather than the undissociated one is the substrate for the transport.

Propionic acid, which, as illustrated above, behaved as a nonmetabolizable analog of acetic acid for the strain under study, was accumulated at pH 5.0. The protonophore CCCP, besides inducing a rapid efflux of about 90% of the free accumulated acid, prevented its accumulation when it was added

TABLE 1. Michaelis constant (K_m) of acetic acid transport system as a function of extracellular pH in *Z. bailii* ISA 1307 grown in a medium with acetic acid (0.5% [wt/vol]; pH 5.0) as the only carbon and energy source[a]

pH	K_m (mM) for:		
	Total acid	Undissociated acid	Anion
3.0	3.33	3.27	0.057
4.0	3.89	3.31	0.58
5.0	2.64	0.96	1.68
6.0	0.53	0.029	0.50

[a] The K_m values were obtained from the Lineweaver-Burk plots of the uptake of labelled acid and expressed as the concentration of undissociated acid or anionic form at the experimental pH, as described in Materials and Methods.

before the labelled acid (Fig. 2A). Furthermore, CCCP induced an inhibition of about 75% on the initial uptake rates of labelled acetic acid (from 0.2 to 12 mM) at pH 5.0.

When acetic acid was added to a suspension of cells in weak buffer (pH 5.0), a transient extracellular alkalization indicative of proton uptake was observed. The initial rates of proton disappearance from the medium obeyed Michaelis-Menten kinetics over the experimental range of 0.05 to 1 mM (data not shown). At higher acetic acid concentrations, it was not possible to estimate proton uptake rates, probably because of the buffering capacity of the acid itself. Similar results were obtained for propionic and formic acids.

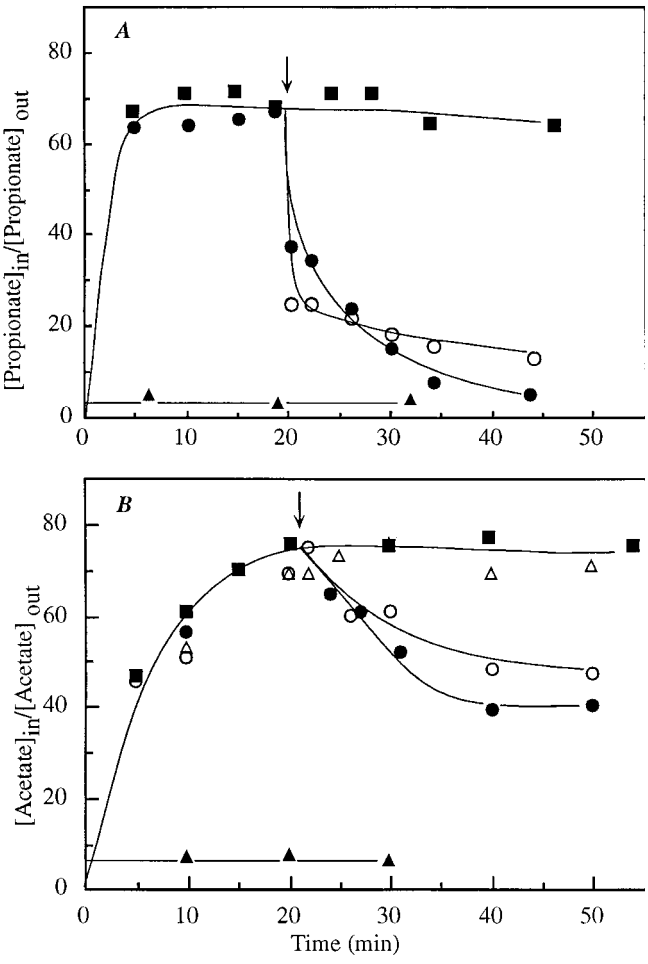

FIG. 2. Accumulation of labelled propionic acid (■) (A) and of labelled acetic acid (■) (B), at pH 5.0, by acetic acid-grown (A) and glucose-grown (B) cells of *Z. bailii* ISA 1307. At the times indicated by the arrows, 3.3 mM cold acetic acid (○), 3.3 mM cold proprionic acid (△), and 0.05 mM CCCP (●) were added, and 0.05 mM CCCP (▲) was added to the reaction mixture before the addition of labelled acid.

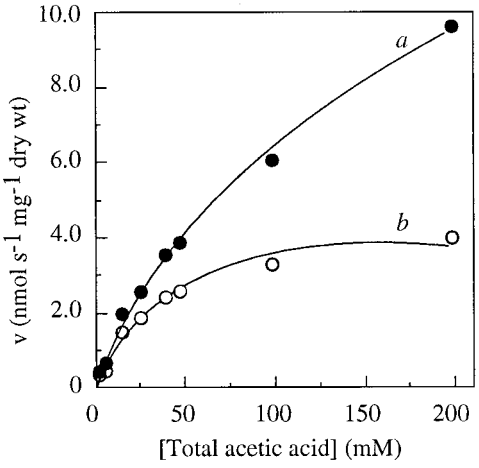

FIG. 3. Initial uptake rates of labelled acetic acid at pH 5.0 as a function of acid concentration by glucose-grown cells of *Z. bailii* ISA 1307. An experimental curve (*a*) and a theoretical curve (*b*) obtained for the mediated transport component without the contamination of the simple diffusion, by application of equation 1 are shown.

When cells were grown in a medium with ethanol or glycerol, activity for a mediated transport system similar to that observed for acetic acid-grown cells was found (data not shown).

Cells of *Z. bailii* ISA 1307 grown in glucose medium were also used to perform uptake studies of labelled acetic acid (Fig. 3). In this case, the experimental data were best fitted (significance level, P of <0.05) by one Michaelis-Menten term plus one first-order-kinetics term. The overall kinetics could be expressed by the following equation (15):

$$v = \frac{V_{max}S}{K_m + S} + k_dS \quad (1)$$

where S is the concentration of total acid, V_{max} is the maximum transport capacity of the mediated transport system, K_m is the respective Michaelis constant, and k_d is the rate constant of simple diffusion component. Assuming that the experimental curve shown in Fig. 3a is a combination of a first-order process with a carrier-mediated transport (equation 1), the following kinetic parameters were calculated: V_{max}, 4.8 ± 1.3 nmol of total acetic acid s^{-1} mg (dry weight) of cells^{-1}; K_m, 45 ± 8.0 mM total acetic acid; and k_d, 0.077 ± 0.004 μl s^{-1} mg (dry weight) of cells^{-1}. A theoretical curve for the mediated transport component without the contribution of the simple diffusion is shown in Fig. 3b. Similar results were obtained at pH 3.0 (data not shown), with the following kinetic parameter estimates: V_{max}, 4.8 ± 0.4 nmol of total acetic acid s^{-1} mg (dry weight) of cells^{-1}; K_m, 9.1 ± 1.9 mM total acetic acid; and k_d, 0.037 ± 0.005 μl s^{-1} mg (dry weight) of cells^{-1}. The initial uptake rates of labelled acetic acid measured, at pH 5.0, in the presence of cold acid showed competitive inhibition (data not shown), reinforcing the finding that a carrier was involved in the acid uptake. Propionic acid did not inhibit the transport of labelled acetic acid, suggesting that it was not recognized by the carrier and hence not behaving as a nonmetabolizable

analog of acetic acid. Therefore, the evaluation of the accumulation capacity of the transporter in glucose-grown cells was carried out by using labelled acetic acid. Despite the limitations associated with possible acid metabolism, the transport was accumulative at pH 5.0. After about 20 min, 50% of the accumulated radioactivity was probably nonmetabolized acetic acid since added cold acetic acid induced counterflow to this extent. The protonophore CCCP prevented accumulation and caused an efflux of accumulated nonmetabolized acid (Fig. 2B). Estimates of initial uptake rates of labelled acetic acid at different concentrations were also obtained after incubation with CCCP, pH 5.0, 65 and 20% inhibition being observed at 1 mM and 200 mM acetic acid, respectively. As in the case of acetic acid-grown cells, acetic acid added to suspensions of cells in a weak buffer at pH 5.0 induced proton uptake, with the initial rates of proton disappearance from the medium following Michaelis-Menten kinetics over the experimental range of 0.05 to 1 mM (data not shown). Lactic, formic, and propionic acids neither inhibited the acetic acid transport nor induced proton uptake. Furthermore, these acids, in contrast to acetic acid, did not induce any counterflow of the accumulated acid when added to preloaded labelled acetic acid cells. Figure 2B shows representative results for propionic acid. Under the same conditions, sorbic and benzoic acids inhibited transport of labelled acetic acid, at pH 5.0, but the type of inhibition was not clearly competitive (data not shown). The results suggested that all the referenced weak acids seem not to be recognized by the acetic acid carrier of glucose-grown cells. In addition, experimental evidence indicated that the uptake of labelled propionic or benzoic acid (from 0.2 to 11 mM), at pH 5.0, obeyed first-order kinetics (data not shown). Also, the uptake of labelled propionic or benzoic acid was not inhibited in the presence of cold propionic or benzoic acid, respectively. The results are consistent with the noninvolvement of a protein transporter in acid uptake. Rather it appeared that both acids entered the cells in the undissociated form by simple diffusion. Assuming this hypothesis, from the slopes of the respective linear plots, the estimated diffusion constants for propionic and benzoic acids were 0.116 and 1.10 $\mu l\ s^{-1}$ mg (dry weight) of $cells^{-1}$, respectively.

When the cells were grown in a medium with fructose (2% [wt/vol]), the transport of weak acids followed a pattern similar to that found in glucose-grown cells (data not shown).

Effect of ethanol on transport and accumulation of acetic acid by acetic acid- and glucose-grown cells. Uptake of labelled acetic acid by acetic acid-grown cells, determined under the conditions described above, was inhibited by ethanol. Ethanol inhibition increased with increasing alcohol concentration (Fig. 4). The affinity constant (K_m) for acetic acid was not significantly affected, the inhibitory effects being mainly on the transport capacity (V_{max}). V_{max}, estimated from double reciprocal plots, decreased exponentially with increasing ethanol concentration above a minimum inhibitory concentration (Fig. 5A), obeying equation 2 (17):

$$V^X_{max} = V^0_{max} e^{-k_i(X - X_{min})} \quad (2)$$

In equation 2, V^X_{max} and V^0_{max} are the maximum initial uptake rates at external concentrations X and 0 of ethanol, X_{min} is the minimum inhibitory concentration of ethanol and k_i is the exponential inhibition constant. At pH 3.0, the effects of ethanol on the initial uptake rates of labelled acetic acid were similar to those observed at pH 5.0 (Fig. 5A). From this figure and according to equation 2, the k_i values for ethanol, at pH 3.0 and 5.0, were 0.78 and 0.97 liter mol^{-1}, respectively.

In glucose or fructose-grown cells, ethanol also inhibited the transport capacity (data not shown). At concentrations below 2.43 M, inhibition was again noncompetitive and the effect on the V_{max} followed exponential kinetics (Fig. 5B). The k_i values at pH 3.0 and 5.0 were 0.55 and 0.67 liter mol^{-1}, respectively. At alcohol concentrations above 2.43 M, the inhibitory effects became less pronounced with the increase of the alcohol concentration. Uptake of propionic acid which, according to the kinetics analysis, entered glucose-grown cells by simple diffusion, was stimulated rather than inhibited by ethanol at concentrations up to 2.43 M (data not shown). Accordingly, the alcohol did not prevent accumulation of 0.5 mM of labelled propionic acid. Instead, an enhancement of its accumulation was observed at least during the first 20 min (Fig. 6C).

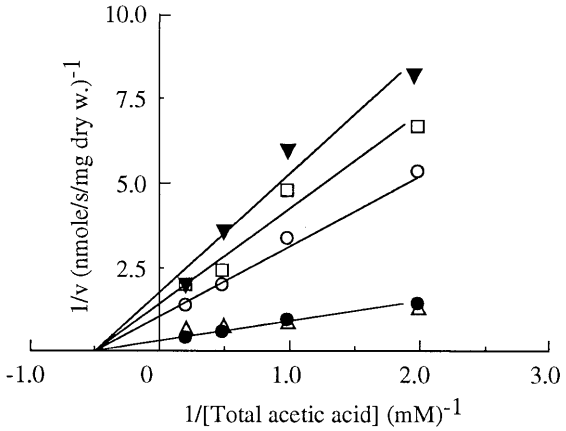

FIG. 4. Lineweaver-Burk plots of initial uptake rates of labelled acetic acid by acetic acid-grown cells of *Z. bailii* ISA 1307 in the absence (●) or presence of ethanol at 1.09 M (△), 1.74 M (○), 2.39 M (□), and 2.82 M (▼).

In both glucose- and acetic acid-grown cells, the accumulation of 0.1 or 7 mM labelled acetic acid, at pH 3.0 or 5.0, was significantly reduced in the presence of ethanol at concentrations above the value of X_{min}. Figure 6A and B show representative results of these experiments.

Butanol, a much more liposoluble alkanol than ethanol, was also tested for its effects on the acetic acid transport at pH 3.0, both in glucose- and acetic acid-grown cells. Again, an exponential, noncompetitive inhibition was observed (data not shown) in both types of cells. The exponential inhibition constants were 3.9 and 5.8 liters mol^{-1} for glucose-grown cells and acetic acid-grown cells, respectively.

DISCUSSION

Cells of *Z. bailii* ISA 1307 grown in different carbon sources transported acetic acid by a mediated transport system. As a transport mechanism, either facilitated diffusion of the undissociated form or an H^+ symport for the anion or undissociated form is conceivable. Given the nature of the substrate, neither the observation of a saturable extracellular alkalization accompanying the addition of the acids to the cells nor the accumulation of labelled acid allow differentiation of the mechanisms (3). Rather, one should take into account the effects induced by the protonophore CCCP which short-circuits the transmembrane proton-motive force. In acetic acid-grown cells, the rapid efflux of accumulated radioactive propionic acid that was observed after the addition of CCCP at the maximum accumulation (Fig. 2A) is a strong argument for a proton symport mechanism. While CCCP may eventually decrease the intracellular pH and thus induce efflux of propionic acid in the case of a facilitated diffusion system, this would be expected to be a

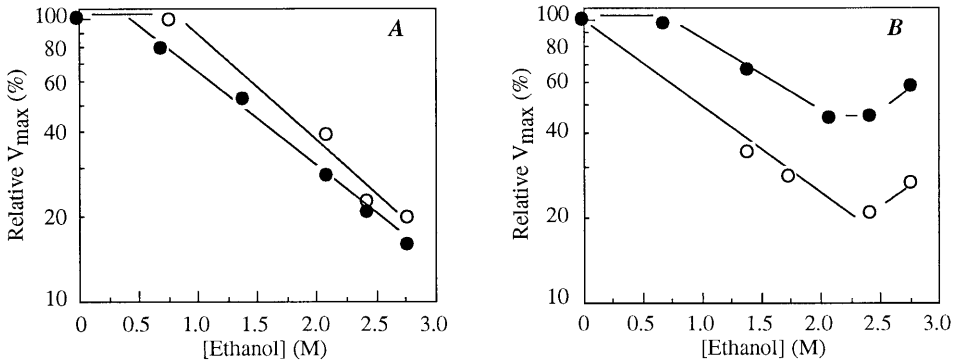

FIG. 5. Semilog plots of relative (%) maximum uptake rates of acetic acid by acetic acid-grown (A) or glucose-grown (B) cells of *Z. bailii* ISA 1307 as a function of the ethanol concentration. ●, pH 3.0; ○, pH 5.0.

much more delayed effect, if occurring at all, because of the buffering capacity of the cytosol (5). In glucose-grown cells such a rapid efflux by CCCP was not observed (Fig. 2A). However, in this case there was no available nonmetabolizable analog of acetic acid and the accumulation studies carried out with labelled acetic acid should be interpreted with the limitations associated with possible acid metabolism. In these cells, the main evidence supporting the involvement of the proton-motive force on the acid uptake was the effective inhibition induced by CCCP on the initial uptake rates of labelled acid at low concentrations. If undissociated acid was the transported form by facilitated diffusion, there is no obvious reason to expect such strong inhibitory effects. All these data are consistent with the involvement of the transmembrane proton-motive force on the transport and accumulation of acetic acid in both type of cells. Further studies will be necessary to elucidate the proton/substrate stoichiometry as well as which form of the acid is the substrate for the proton symporter.

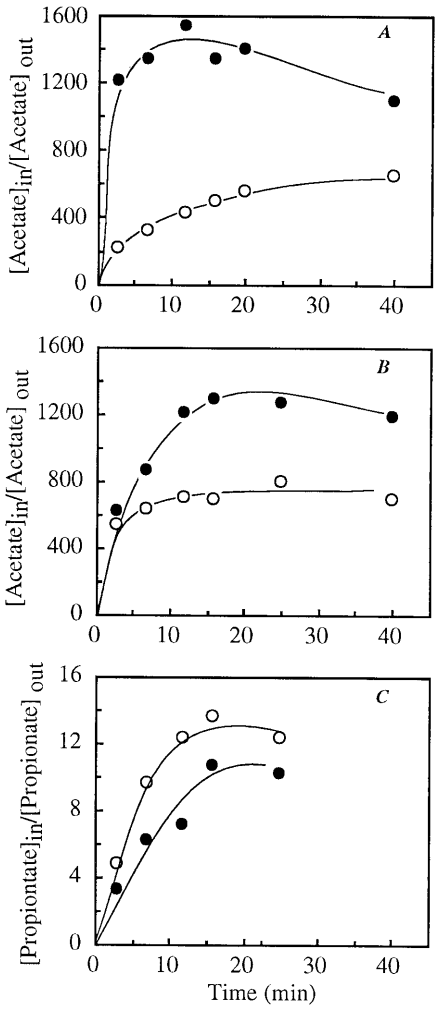

FIG. 6. Accumulation of labelled acetic acid at pH 3.0 by cells of *Z. bailii* ISA 1307 grown in a medium with acetic acid (A) or glucose (B) in the absence (●) or presence (○) of ethanol (2.43 M). (C) Accumulation of labelled propionic acid at pH 5.0 by glucose-grown cells of *Z. bailii* ISA 1307 in the absence (●) or presence (○) of 1.74 M ethanol.

Among the mechanisms that may underlie the high resistance of *Z. bailii*, possible interactions of ethanol with the membrane transport of toxic by-products of alcoholic fermentations could be considered. Data obtained for several other yeast species have provided evidence that when a passive diffusion mechanism is involved in the substrate uptake, ethanol, in general, enhances initial uptake velocities. In contrast, when the uptake occurs through a mediated transport system, ethanol negatively affects the transport capacity, the effect being most pronounced for active transport processes (17). The results described above showed that in both acetic acid- and glucose-grown cells of *Z. bailii*, ethanol inhibited the transport of acetic acid. In the former type of cells, inhibition was observed over the entire ethanol concentration range. In glucose-grown cells at ethanol concentrations above 2.43 M, the global inhibitory effect decreased. In these cells, the uptake of total acetic acid across the plasma membrane appears to be subject to opposite ethanol influences: a decrease due to the negative effects of the alcohol on the acetic acid carrier and an increase due to the enhancement of the simple diffusion of the undissociated acid. However, the net effect induced by the alcohol on the uptake of total labelled acid was inhibitory (Fig. 5). Accordingly, it appears that the simple diffusion of the undissociated acid as well as its enhancement by ethanol contribute, with a relatively low weight, to the global acid uptake. Under a fundamental point of view, the observed effects of ethanol reinforced that in *Z. bailii*, independently of the growth conditions, acetic acid enters the cell mainly through a transporter protein which in all likelihood behaves as a proton symporter. Comparison of the effects of ethanol and those of butanol showed that the exponential inhibition constants increased with the lipid solubility of the alkanol, suggesting that the hydrophobic membrane regions are the cell targets sensitive to alkanol interaction. These results are in accordance with the

data reported by others (14, 17) on the effects of lipophilic compounds on several membrane transport processes. In the case of propionic acid, the presence of extracellular ethanol neither inhibited the initial uptake rates of the labelled acid nor prevented its accumulation in glucose-grown cells (Fig. 6C). Instead, an enhancement of both was observed. This result reinforced the finding that this acid enters the cells in the undissociated form by simple diffusion.

In several yeast species described so far, the mediated transport systems present for the anionic form of the weak monocarboxylic acids are inducible (see Introduction). Additionally, either the transporters or the intracellular acid metabolism are subject to glucose catabolic repression. In glucose-grown cells of such yeast species, the acid enters the cell only by simple diffusion of the undissociated form, and if the extracellular pH is lower than the intracellular one, the acid then dissociates and eventually acidifies the cytosol and negative effects may occur. This appears to be the case for the yeast *S. cerevisiae* in which the toxic effects of acetic acid have been inputted to the repression of the transport and metabolism of the acid by glucose (1, 12, 17). The results presented herein show that in *Z. bailii* a mediated transport system for acetic acid is operating even in glucose-grown cells. In addition, preliminary results indicated that the intracellular metabolism of the acid was also operational in the presence of glucose (results not shown). This behavior, in association with the low permeability of the plasma membrane to the undissociated acid, could contribute to the control of the acid concentration inside the cell. The presence of ethanol in the medium appears to reinforce this control and plays a protective role for the yeast, inhibiting the acid uptake in such a way that its intracellular concentration could be maintained below toxic levels. Furthermore, our results show that such mechanisms may occur at concentrations of ethanol and acetic acid which are realistic during vinification and other alcoholic yeast fermentations. Hence, they could be considered as components of the global physiological strategy of *Z. bailii* to tolerate environments with those two fermentation end products, whereas other yeast species such as *S. cerevisiae* are unable to survive. Studies on the interactions of ethanol with the membrane transport of acetic acid in *S. cerevisiae* and how they could be related with the lower resistance of that yeast species to acid media with ethanol are now under development.

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