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Regular paper

Pdr12p-dependent and -independent fluorescein extrusion from baker's yeast cells

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Fluorescein efflux from *S. cerevisiae* cells was measured to study the peculiarities of fluorescein transport system, which is important for yeast resistance to certain drugs and weak organic acid preservatives. Glucose-independent and glucose-stimulated fluorescein effluxes were characterized using iodoacetate, cyanide and orthovanadate, inhibitors of glycolysis, electron transport chain, and ATPases, respectively. It is supposed that in glucose-free medium fluorescein extrusion is ATP-dependent and the energy for this efflux is mainly provided by respiration. In glucose-containing medium, glycolysis plays a critical role for extrusion of fluorescein. The results indicate that acetic acid inhibits the fluorescein efflux from yeast cells. The inhibition constant of glucose-stimulated fluorescein efflux is significantly lower in parental strain than in two mutants defective in *PDR12* (ABC-transporter Pdr12p) or *WAR1* (transcription factor of Pdr12p). It can be suggested that the membrane protein Pdr12 is involved in fluorescein extrusion from the yeast cells, but component(s) other than Pdr12p is (are) also important.

Keywords: *Saccharomyces cerevisiae*, fluorescein, glucose, inhibitors, acetic acid

INTRODUCTION

In *Saccharomyces cerevisiae*, several anion efflux transporters can be induced by different compounds (Kolaczkowska *et al.,* 2008). ATP-binding cassette (ABC) efflux pumps are involved in the transport of a wide variety of substrates across the plasma membrane. Among others the ABC-transporter Pdr12p is believed to be one of the important factors preventing intracellular accumulation of acid anions in the yeast cell (Piper *et al.,* 1998; 2001; Nobel *et al.,* 2001; Schüller *et al.,* 2004; Jungwirth & Küchler, 2006; Mollapour *et al.,* 2008). Yeast lacking Pdr12p were found to be hypersensitive to weak organic acids commonly using to preserve foods and beverages (Holyoak *et al.,* 1999; Hatzixanthis *et al.,* 2003). Pdr12p provides energy-dependent efflux of catabolic products derived from amino-acid catabolism, multicyclic compounds, C_3-C_7 monocarbonic acids as well as small aromatic monocarbonic acids such as fluorescein (Piper *et al.,* 1998; Holyoak *et al.,* 1999; Parsons *et al.,* 2004; Hazelwood *et al.,* 2006; Papadimitriou *et al.,* 2007). Expression of the *PDR12* gene is under control of the transcriptional factor War1p named for weak acid resistance (Kren *et al.,* 2003).

The role of the pump in acetate efflux is still discussed. Several years ago it was reported that previous experiments, in which Pdr12p had been identified as the main system responsible for acetic acid extrusion from yeast cells, were an experimental artifact (Bauer *et al.,* 2003). Knocking out of the *PDR12* gene in tryptophan-auxotrophic strains de-

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Abbreviations: ABC, ATP-binding cassette; FDA, fluorescein diacetate.

creased their facility to import external tryptophan. Increased external tryptophan concentrations suppressed the *Δpdr12* phenotype. Therefore it was concluded that actually Pdr12p was not responsible for the apparent increased sensitivity of *Δpdr12* to acetic acids. At the same time, the expression of the *PDR12* gene was not changed by either high levels of external tryptophan or overexpression of Tat2p (amino acid permease) (Bauer *et al.*, 2003).

Here we measured fluorescein efflux from yeast cells under different experimental conditions with the aim to study the peculiarities of transport systems in *S. cerevisiae*. Two components of fluorescein exrusion were studied: the glucose-independent one and that stimulated by glucose. It was supposed that in glucose-free medium fluorescein extrusion was ATP-dependent and the energy for this process was mainly provided by respiration. In glucose-containing medium, glycolysis played a critical role. Exogenously added acetic acid inhibited the fluorescein efflux from the yeast cells. Pdr12p increased the sensitivity of the fluorescein transport system in the yeast to acetic acid in the presence of glucose. Therefore we can conclude that the membrane protein Pdr12 is involved in fluorescein extrusion from yeast cells, but also component(s) independent of Pdr12p have a role.

MATERIALS AND METHODS

Yeast strains and chemicals. *Saccharomyces cerevisiae* strains used in this study were W303-1A (wild type, *MAT*a *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*) and its isogenic derivatives YBB14 (W303-1A *pdr12Δ::hisG-URA3-hisG*) and YAK120 (W303-1A *war1Δ::HIS3*). The strains were kindly provided by Professor K. Küchler (Vienna, Austria). Chemicals were obtained from Sigma-Aldrich Chemical Co. (USA), Reanal (Hungary), Fluka (Germany).

Growth conditions. Yeast were grown at 30°C with shaking at 175 r.p.m. to early stationary phase (24 h) in a liquid medium containing 1% glucose, 2% bactopeptone, 1% yeast extract (YPD). For experiments requiring induction of Pdr12, 50 mM acetic acid was added to medium (Holyoak *et al.*, 1999).

Cell loading with fluorescein diacetate. Cells were harvested by centrifugation at 3000×*g* for 1.5 min, washed with 50 mM Hepes/NaOH (pH 7.0) and resuspended in 50 mM Hepes/NaOH containing 5 mM 2-deoxy-D-glucose and 50 μM fluorescein diacetate (FDA) (FDA from a 5 mM stock solution in dimethyl sulfoxide). Resulting cell suspensions (10^8 cells/ml) were incubated at 30°C with shaking at 175 r.p.m. for 15–180 min (time dependence) or 20 min (all other experiments).

Fluorescein distribution and efflux from whole cells. FDA diffuses freely into intact cells (Breeuwer *et al.*, 1995). Inside the cells, FDA is hydrolyzed to the polar fluorescent dye fluorescein by intracellular esterases (Holyoak *et al.*, 1999). After loading with FDA the yeast cells were: (*i*) pelleted and cell-free supernatant was used for measurement of extracellular fluorescein fluorescence, (*ii*) vortexed in the incubation medium for measurement of total fluorescence of intra- and extracellular fluorescein, or (*iii*) harvested, washed and vortexed for intracellular fluorescence measurement in cell-free extract. For measurement of intracellular fluorescence, cells were washed with 50 mM K-phosphate buffer (pH 7.0), then resuspended in the same buffer. The cell suspensions were vortexed for 15 cycles of 1 min of vortexing with 1 volume of glass beads (450–500 μm) followed by 1 min of cooling on ice. Cell debris were removed by centrifugation for 1.5 min at 15000×*g*. The resulting supernatants were used to assay the intensity of fluorescence.

For measurement of fluorescein efflux, aliquots of dye-loaded cells were harvested, washed with 50 mM Hepes/NaOH (pH 7.0) and resuspended in the same buffer (pH 7.0 or 4.5). Fluorescein efflux was measured as non-stimulated or stimulated by 10 mM glucose (Holyoak *et al.*, 1999). Samples of 250 μl were taken at set intervals over a period of 4.5 min, and cells were removed by centrifugation for 1.5 min at 15000×*g*. The resulting supernatants were used to assay the intensity of fluorescence. All measurements were done with an excitation wavelenght of 435 nm and an emission wavelenght of 525 nm.

Fluorescein efflux inhibition. Inhibitors, such as 1 mM sodium orthovanadate (Holyoak *et al.*, 1999) or 1 mM potassium cyanide (Kwast *et al.*, 1999) were added to cell suspensions 5 min prior to the measurement of fluorescence, while 1 mM iodoacetic acid (Newcomb *et al.*, 2003) was added to cell suspensions 60 min prior to the yeast loading with FDA. Increased concentrations of acetic acid (25–400 mM) were added to cell suspensions 5 min prior to the measurement of the fluorescence (Holyoak *et al.*, 1999).

Calculation and statistics. Experimental data are expressed as the mean value of 3–6 independent experiments±standard error of the mean (SEM), and statistical testing used Student's *t*-test. Inhibition constants were calculated using a computer kinetics program (Brooks, 1992).

RESULTS AND DISCUSSION

The ABC-family transporters, including Pdr12p, play a critical role in *S. cerevisiae* resistance to a wide range of compounds, particularly to weak or-

ganic acids (Jungwirth & Küchler, 2006). It has been shown that under certain conditions weak organic acids induce the expression of the *PDR12* gene, thus increasing the yeast capacity to extrude acid anions from the cells (Piper *et al.,* 1998; Papadimitriou *et al.,* 2007; Mollapour *et al.,* 2008). This process is under control of the transcriptional factor War1p (Kren *et al.,* 2003; Mollapour *et al.,* 2008). Pdr12p was shown to have broad substrate specificity, being responsible for yeast resistance to monocarboxylic acids with chain lengths from C_3 to C_7 (Piper *et al.,* 1998; 2001). Pdr12p also carries out active extrusion of multicyclic compounds such as caffeine and catabolic products derived from amino acids (Parsons *et al.,* 2004; Hazelwood *et al.,* 2006). The pump also provides energy-dependent efflux of fluorescein (Breeuwer *et al.,* 1995; Holyoak *et al.,* 1999).

In this study, yeast cells were loaded with fluorescein diacetate (FDA), molecules of which freely cross the cellular membrane. In the cytosol, FDA is cleaved by intracellular esterases to the hydrophilic fluorophore fluorescein. We measured fluorescein efflux from the cells with the aim to study the peculiarities of fluorescein transport system in *S. cerevisiae*, which is believed to be important for yeast resistance to certain drugs and weak organic acid preservatives.

First of all, we optimized the yeast cell's loading by FDA and investigated the dynamics of intra- and extracellular fluorescein distribution. Figure 1 shows that yeast incubation with FDA for different periods of time resulted in a peak of intracellular fluorescein accumulation at 20–30 min followed by a decrease and reaching the lowest values after 60-min incubation, whereas the amount of extracellular fluorophore was continuously increased in a hyperbolic manner. Figure 1 also shows that the total fluorescence of intra- and extracellular fluorescein increased, demonstrating a pattern similar to that of the extracellular fluorophore pool.

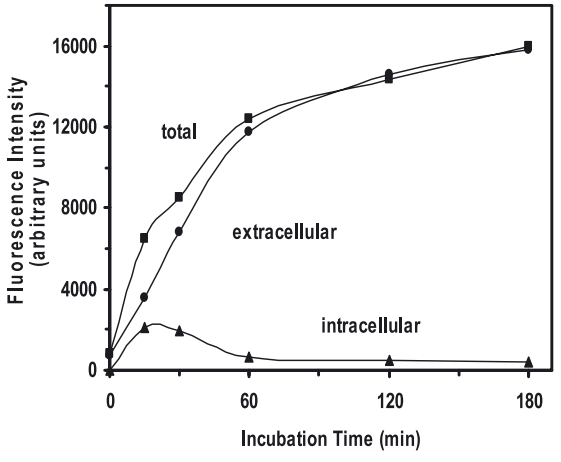

Figure 1. Time-course of intracellular and extracellular fluorescein distribution in *S. cerevisiae* W303-1A (wild type).
Cells were grown in YPD (pH 6.75) to early stationary phase (24 h). Aliquots of experimental culture were incubated with FDA for 15–180 min followed by the measurement of total, intracellular and extracellular fluorescence intensity. Data are from representative experiment.

Under conditions used in this study the fluorescence intensity depended on at least three main processes: (*i*) diffusion of FDA molecules into the cell, (*ii*) hydrolytic cleavage of FDA by intracellular esterases, and (*iii*) fluorescein efflux. Therefore the results shown in Fig. 1 may have the following physiological meaning. The pool of extracellular fluorescein represents FDA molecules which entered yeast cells, were cleaved by esterases and extruded from the cells (Breeuwer *et al.,* 1995). Intracellular fluorescence corresponds to the level of fluorescein molecules which did not leave the cells at the moment of meausurement. During the first 20 min of exposure to FDA, the accumulation of fluorescein in the cells increased in parallel to its extrusion. Then the equilibrium between the two processes shifts and the intracellular accumulation of fluorescein *versus* its efflux changes in a time-dependent manner. The level of extracellular fluorescein exceeds its intracellular content. During the whole period of yeast incubation with FDA the total fluorescense grew. It should be noted that it corresponded well to the sum of the two pools, the intra- and extracellular ones. The intracellular concentration of fluorescein reached the highest value at 20–30-min incubation (Fig. 1). In order to have the most reproducible and sensitive system to study fluorescein efflux from yeast cells, further experiments were performed using 20-min incubation with FDA. After exposure the cells were quickly harvested by centrifugation and fluorescence intensity in the extracellular medium was measured.

Next we investigated the dynamics of fluorescein efflux from the yeast cells. Figure 2 shows that this process in fact was linear at least during the first 4.5 min of measurement. That let us evaluate the initial velocity of the process. The angle of the trendline represents the relative velocity of fluorescein efflux from the yeast cells. As seen in Fig 2, fluorescein extrusion took place without glucose as an energy source in the medium. These data are not in accord with those by others (Holyoak *et al.,* 1999), which showed that yeast were unable to extrude fluorescein in the absence of glucose. Therefore it was suggested that this process was energy-dependent.

In order to clarify the situation we also added glucose and observed an almost doubled fluorescein efflux speed. This fact fits well to the earlier-found energy-dependent fluorescein extrusion from yeast cells (Holyoak *et al.,* 1999). Further we will analyse independently two components of fluorescein efflux:

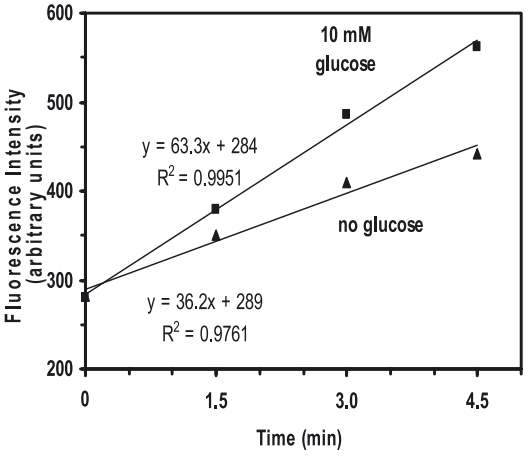

Figure 2. Time-course of glucose-stimulated and non-stimulated fluorescein efflux from *S. cerevisiae* W303-1A (wild type).
Cells were grown in YPD (pH 6.75) to early stationary phase (24 h). Aliquots of experimental culture were incubated with FDA for 20 min followed by the measurement of extracellular fluorescence intensity. Glucose, 10 mM, added 5 min prior to fluorescence measurement. Data are from representative experiment.

non-stimulated and stimulated by glucose. The latter one was calculated by subtraction of the non-stimulated speed efflux from the total one. We assumed that the non-stimulated fluorescein efflux was either a passive flux or an energy-dependent one, powered by internal energy sources. To check if energy was needed to extrude fluorescein in glucose-free medium, we used a set of inhibitors (Fig. 3). Orthovanadate, an inhibitor of ATPases, reduced fluorescein efflux by 34% in glucose-free medium, showing the possible involvement of ATPases in fluorescein extrusion (Fig. 3A). Iodoacetate, an inhibitor of glycolysis, did not affect the fluorescein efflux (Fig. 3A). At the same time, cyanide, an inhibitor of oxidative phosphorylation, reduced the process by 75% in glucose-free medium (Fig. 3A). Therefore it may be suggested that: (*i*) fluorescein extrusion is ATP-dependent, and (*ii*) the energy for this process is mainly provided by oxidative phosphorylation. Figure 3A demonstrates also that the glucose-stimulated component of fluorescein efflux was totally suppressed by iodoacetate, while orthovanadate and cyanide did not affect it (Fig. 3A). This may show that glucose added prior to the measurement suppresses respiration, and glycolysis plays a critical role in this case.

Further we studied fluorescein efflux from mutant cells: *Δpdr12* (YBB14 strain) and *Δwar1* (YAK120 strain). Comparing Figs. 3A–C, one may note that, surprisingly, fluorescein was extruded from the isogenic *Δpdr12* and *Δwar1* mutant cells about 2.3-fold faster than from wild-type cells (W303-1A strain). In the two mutants, all three inhibitors used, orthovanadate, iodoacetate and cyanide, decreased fluorescein efflux in glucose-free medium. In the *Δpdr12* strain, orthovanadate, iodoacetate and cyanide inhibited fluorescein efflux by 48%, 55% and 95% respectively, while in *Δwar1* it was 38%, 70% and 92%, respectively. These results show that the deficiency in Pdr12p (in the *Δwar1* strain the *PDR12* gene is not

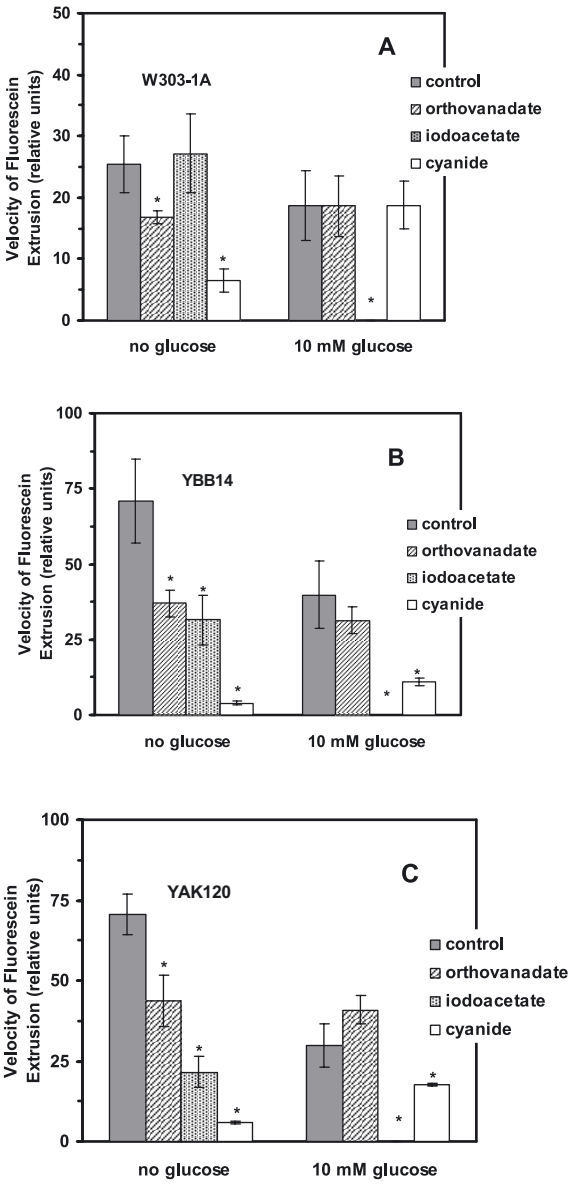

Figure 3. Glucose-stimulated and non-stimulated initial velocity of fluorescein extrusion from *S. cerevisiae* in the presence of different inhibitors.
Strains used: wild type (A), *Δpdr12* (B) and *Δwar1* (C). Yeast were grown in YPD (pH 6.75) to early stationary phase (24 h). Aliquots of experimental culture were incubated with FDA for 20 min followed by the measurement of extracellular fluorescence intensity. Sodium orthovanadate or potassium cyanide were added 5 min prior to fluorescence measurement, iodoacetic acid was added 60 min prior to FDA loading. *Significantly different from the respective values for control cells (non-treated by inhibitors) with $P<0.05$. Data are mean±SEM (n=3–4).

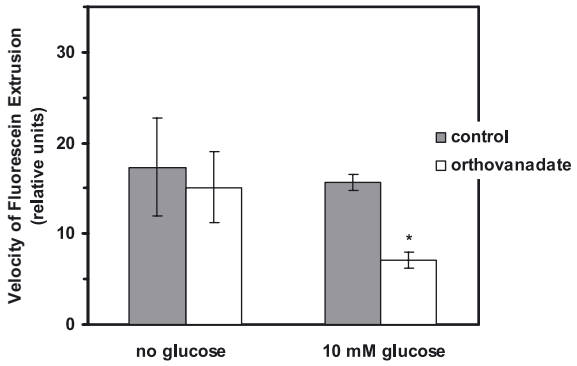

Figure 4. Glucose-stimulated and non-stimulated initial velocity of fluorescein extrusion from *S. cerevisiae* W303-1A (wild type) in the presence of sodium orthovanadate. Yeast were grown in YPD (pH 6.75) to early stationary phase (24 h). Aliquots of experimental culture were incubated at pH 3.0 in the presence of 200 mM acetic acid for 120 min prior to FDA loading. Sodium orthovanadate was added 5 min prior to fluorescence measurement. *Significantly different from the respective values for control cells (non-treated by orthovanadate) with $P<0.05$. Data are mean±SEM (n=3–4).

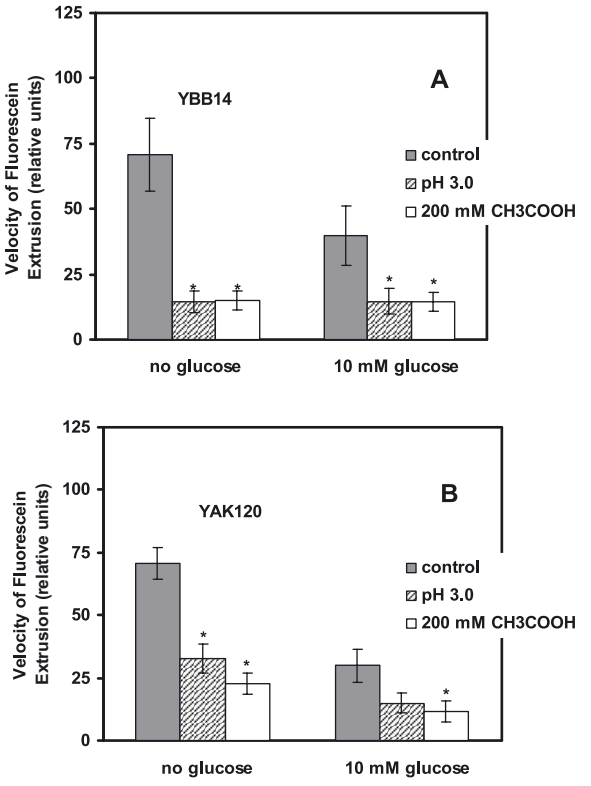

Figure 5. Glucose-stimulated and non-stimulated initial velocity of fluorescein extrusion from *S. cerevisiae* in the presence of acetic acid.
Strains used: *Δpdr12* (A) and *Δwar1* (B). Yeast were grown in YPD (pH 6.75) to early stationary phase (24 h). Aliquots of experimental culture were incubated at pH 3.0 in the presence of 200 mM acetic acid or its absence for 120 min prior to FDA loading. *Significantly different from the respective values for control cells (grown at pH 6.75 without acetic acid) with $P<0.05$. Data are mean±SEM (n=3–4).

expressed either (Kren *et al.,* 2003)) makes the system more sensitive to the mentioned inhibitors, and suggest an involvement of glycolysis and oxidative phosphorylation in providing energy for fluorescein extrusion from the defective cells.

In the defective strains, similarly to the wild type, the glucose-stimulated efflux was not sensitive to orthovanadate, whereas iodoacetate totally inhibited fluorescein extrusion in glucose-supplemented medium. However, in both mutants, unlike in the parental one, cyanide significantly decreased the rate of fluorescein efflux from yeast cells. Thus, the obtained results clearly show that Pdr12p and War1p are in some way involved in fluorescein extrusion. At the same time, there is an energy-dependent Pdr12-independent component of fluorescein efflux.

Our data do not correspond to an erlier report by Holyoak and colleagues (1999), who did not observe Pdr12p-independent fluorescein transport. They found that Pdr12p was the only protein responsible for the energy-dependent fluorescein extrusion from yeast cells. The apparent disagreement between the data of two laboratories may be connected with experimental conditions. In the abovementioned work, fluorescein efflux from cells was studied in yeast grown in the presence of subinhibitory concentrations of sorbic acid. Low doses of sorbate were found to increase the activity of Pdr12p (Holyoak *et al.,* 1999; Papadimitriou *et al.,* 2007). It is possible that under conditions used in our study, where we grew yeast on glucose, the Pdr12p activity was relatively low and we therefore were able to observe one more component responsible for fluorescein extrusion in an energy-dependent manner.

It is widely believed that Pdr12p is essential for the yeast adaptation to weak acid stress, but recent work reported that *PDR12* expression to highest levels by mild sorbic acid stress did not lead to acquired resistance (Papadimitriou *et al.,* 2007). Earlier it was found that *Δpdr12* mutants were hypersensitive to sorbic, benzoic and propionic acids as well as high acetate levels (Piper *et al.,* 1998; Schüller *et al.,* 2004). However, several years ago the same team reported that the acetate hypersensitivity phenotype previously observed for yeast strain deficient in *PDR12* (Piper *et al.,* 1998) was an experimental artefact and Pdr12p did not confer resistance to acetic acid stress (Bauer *et al.,* 2003). The inability of the mutant lacking Pdr12p to grow in the presence of acetate was observed only in yeast cells auxotrophic for tryptophan. The hypersensitivity to acetic acid was almost totally suppressed by high levels of exogenous tryptophan. Thus it was concluded that weak organic acid stress inhibited the uptake of tryptophan from the medium (Bauer *et al.,* 2003). However, tryptophan supplementation

and overexpression of Tat2p (amino acid permease) did not change the expression of the *PDR12* gene. It should be noted that the role of Pdr12p in acetate efflux from yeast cells is still under discussion.

Next we studied the possible influence of acetic acid on the fluorescein transport system in yeast cells. First of all, we checked if medium acidification in either presence or absence of acetic acid might affect fluorescein efflux. Two approaches were used: growth at low pH and incubation in acidified medium. In the first case, both non-stimulated and glucose-stimulated components of fluorescein efflux were similar to the ones in cells grown at pH 6.75 (not shown). In the second case, wild-type cells were grown at pH 6.75 as described above, then yeast were incubated in medium HCl-adjusted to pH 3.0, either supplemented with acetic acid or not. In these experiments, we also found that neither incubation at pH 3.0 without acetate nor in the presence of 200 mM acetic acid changed the efficiency of fluorescein extrusion in wild-type cells (not shown). But due to some reasons, in cells exposed to low pH in glucose-containing medium orthovanadate inhibited fluorescein efflux (Fig. 4) in contrast to yeast which were not treated with low pH (Fig. 3A). Thus, incubation at low pH redistributed the ratio of components of the fluorescein extrusion system sensitive and insensitive to orthovanadate, demonstrating the appearance of its new properties.

In order to investigate if Pdr12p-independent fluorescein transport (see Fig. 3B and C) is regulated by low pH, we used *Δpdr12* and *Δwar1* mutants. Unlike for the parental strain, the incubation at pH 3.0 without acetate or in the presence of 200 mM acetic acid reduced 3–5-fold the velocity of Pdr12p-independent efflux of fluorescein from cells of the two mutants (Fig. 5A and B). It can be suggested again that there are at least two components responsible for fluorescein extrusion from the yeast cells. One of them is related to Pdr12p, but the other Pdr12p-independent component can not be identified yet.

In preliminary experiments we found that 200 mM acetic acid inhibited fluorescein efflux in wild-type cells grown in media (pH 4.5) containing 50 mM acetic acid. Therefore, we used these conditions to study the character of acetic acid inhibition of the transport process in all three strains investigated. In this experiment, different concentrations of acetic acid were added 5 min prior to fluorescence measurement. The inhibition constants of fluorescein efflux by exogenic acetic acid in wild-type cells and in both mutants in glucose-free medium were virtually the same (Table 1). However, the inhibition constants of glucose-stimulated fluorescein efflux from the cells of the parental strain (W303-1A) constituted only about 35% of those found in the two defective strains, *Δpdr12* (YBB14) and *Δwar1* (YAK120). And in contrast to the wild type, in the *Δpdr12* and *Δwar1* mutants the inhibition constants of glucose-stimulated fluorescein efflux were found to be very similar to the glucose-independent ones. Thus, Pdr12p increased the sensitivity of fluorescein transport system in the yeast to acetic acid in the presence of 10 mM glucose.

Table 1. Constants of inhibition (mM) of glucose-stimulated and non-stimulated fluorescein efflux from *S. cerevisiae* in the presence of different concentrations of acetic acid

Strain	No glucose	10 mM glucose
W303-1A (wild type)	62.5±12.0	18.6±4.0*
YBB14 (*Δpdr12*)	75.6±11.0	53.9±2.8
YAK120 (*Δwar1*)	51.8±9.5	55.0±2.8

*Significantly different from the respective values for non-stimulated cells (no glucose) with $P<0.05$. Data are mean±SEM (n=4).

In summary, it can be suggested that yeast have several systems responsible for fluorescein extrusion: (*i*) ATP-dependent and ATP-independent, (*ii*) glucose-stimulated and non-stimulated by glucose, and (*iii*) the membrane protein Pdr12 is involved in fluorescein extrusion from yeast cells, but there is (are) also component(s) independent of Pdr12p.

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