Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*

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Abstract

To identify new genes involved in acetate uptake in *Saccharomyces cerevisiae*, an analysis of the gene expression profiles of cells shifted from glucose to acetic acid was performed. The gene expression reprogramming of yeast adapting to a poor non-fermentable carbon source was observed, including dramatic metabolic changes, global activation of translation machinery, mitochondria biogenesis and the induction of known or putative transporters. Among them, the gene *ADY2/YCR010c* was identified as a new key element for acetate transport, being homologous to the *Yarrowia lipolytica GPR1* gene, which has a role in acetic acid sensitivity. Disruption of *ADY2* in *S. cerevisiae* abolished the active transport of acetate. Microarray analyses of *ady2* strains showed that this gene is not a critical regulator of acetate response and that its role is directly connected to acetate transport. Ady2p is predicted to be a membrane protein and is a valuable acetate transporter candidate.

Keywords: monocarboxylate transport; yeast; acetate; gene expression; *GPR1*

Introduction

Several yeast species, including *Saccharomyces cerevisiae*, are able to utilize acetic acid as sole carbon and energy source under aerobic conditions (Barnett *et al*., 1990), acetate being a normal and frequent end-product of fermentation (Flores *et al*., 2000). When cells of *S. cerevisiae* are grown on respiratory substrates, activity for at least two monocarboxylate proton symporters are found, with differences in their mechanisms of regulation and specificity (Casal *et al*., 1996). A lactate–pyruvate–acetate–propionate transporter, induced in lactic or pyruvic acid-grown cells, is encoded by the gene *JEN1* (Casal *et al*., 1999). *JEN1* is the first and sole gene known to encode for a monocarboxylate plasma membrane permease in fungal cells. In addition to Jen1p, another permease, which accepts acetate, propionate or formate, is present in cells grown in non-fermentable carbon sources (Casal *et al*., 1996; Makuc *et al*., 2001). Throughout this work this permease will be referred to as 'the acetate transporter'. The gene coding for this protein has not yet been identified. Several strategies have been employed to identify the genes coding for other monocarboxylate transporters in *S. cerevisiae*. Mutants affected in the acetate–formate–propionate permease activity were obtained by classical mutagenesis; however, the attempts to clone the genes associated with this transport system have not been successful (Paiva *et al*., 1999). On the other hand, a group of five putative genes coding for monocarboxylate permeases were identified by *in silico* analyses (Paulsen *et al*., 1998; Nelissen *et al*., 1997). However, recent studies revealed that neither these proteins nor the protein Yhl008p, which had been proposed to be an acetate proton symporter (Paulsen *et al*., 1998), were involved in monocarboxylate transport under all tested conditions (Makuc *et al*., 2001). The capacity to transport monocarboxylates by a mediated mechanism is not present when cells are grown

on glucose (Casal *et al*., 1996). We thus assumed that the expression of the as-yet unknown acetate transporter gene(s) should be repressed in cells grown in glucose and induced when cells are transferred to a medium containing acetic acid, as the sole source of carbon and energy. Therefore, the aim of this work was to use DNA microarray analyses of the transcriptome of yeast cells shifted from glucose to acetic acid, in order to screen for the potential acetate–propionate–formate transporter in *S. cerevisiae*. This strategy allowed us to identify the membrane protein Ady2p as an essential component of the acetate active transport in these conditions and a valuable candidate as a new acetate transporter in yeast.

Materials and methods

Yeast strains and growth conditions

The strains of *S. cerevisiae* used in this study are listed in Table 1. Yeasts were grown in 0.7% (w/v) Difco yeast nitrogen base (YNB), supplemented with adequate quantities of nutrients required for prototrophic growth. Carbon sources were glucose (2%, w/v) or acetic acid (0.5%, v/v, pH 6.0). Growth was carried out with shaking (200 r.p.m.) at 28 °C. For growth under repression conditions the yeast cells were collected during the exponen- tial phase of growth in YNB medium with glu- cose. For derepression conditions glucose-grown cell suspensions were centrifuged, washed twice in ice-cold deionized water and inoculated into fresh YNB medium with acetic acid.

Transport assays

Uptake rates of labelled acetic acid [(1-^{14}C)acetic acid, sodium salt, Amersham; 3000 dpm/nmol, pH 5.0] were estimated as described by Casal and Leao (1995). Cells incubated under derepression conditions were harvested by centrifugation, washed twice in ice-cold deionized water and resuspended in ice-cold deionized water to a final concentration of about 25–35 mg dry weight/ml. A computer-assisted non-linear regression analysis program (GraphPAD software, San Diego, CA, USA) was used in order to determine the best-fitting transport kinetics to the experimental data and to estimate the kinetic parameters. All the experiments were performed in triplicate, and the data represent average values.

YCR010c knock-out

The *S. cerevisiae* Y03490 strain deleted in *ADY2/YCR010c* was obtained from the EUROSCARF collection. Two primers, A-YCR010c (AGA CTG CAT TTT CTT ACA GC TTT TT) and D-YCR010c (AGA CAA GTA AAG GAG TCA GCA AAA A), were used to amplify by PCR the YCR010c::KanMX4 allele in Y03490 and the PCR product was used to transform the strain W303-1A. Successful integration of the *YCR010c::KanMX4* cassette was scored by presence of the *YCR010c::KanMX4* band (2088bp) and absence of the *YCR010c* wild-type band (1478 bp) following analytical PCR on whole cells using the same primers. This strain was named Ace 718.

Microarray analyses

Detailed protocols are at **www.biologie.ens.fr/fr/genetiqu/puces/protocoles puces.html**. mRNA were prepared using the Micro Fast track mRNA purification kit (Invitrogen). Home-made microar- rays containing probes for most of the yeast open reading frames (about 6000 oligonucleotides) were used, deposited in duplicate. The oligonucleotides

Table 1. *S. cerevisiae* strains used in this work

Strain	Genotype	Source or reference
W303-1A	*MAT*a *ade2 leu2 his3 trp1 ura3*	[Thomas *et al*., 1989]
BLC203	W303-1A; *jen1 ::HIS3*	[Casal *et al*., 1999]
Y10000	BY4742; *MATα his3 leu2 lys2 ura3*	Euroscarf
Y03490	BY4742; *MATα his3 leu2 lys2 ura3 ycr010c::KanMX4*	Euroscarf
Y15377	BY4742; *MATα his3 leu2 lys2 ura3 ynr002c::KanMX4*	Euroscarf
Y14220	BY4742; *MATα his3 leu2 lys2 ura3 ydr384c::KanMX4*	Euroscarf
Ace718	W303-1A; *ycr010c::KanMX4*	This work

were supplied by MWG Biotech (Yeast oligo set). The arrays were analysed using Genepix 3.0 software (Axon). Each microarray result presented here is an average of at least two measurements. Artefactual spots, saturated spots and low-signal spots were excluded. On average, 85% of the spots on each microarray used in this study were quantified (minimum 74%, maximum 91%). The Cy3:Cy5 ratios were normalized by use of the median of all the ratios using Arrayplot (Marc and Jacq, 2002). The data of Figure 4 have been represented using Treeview (Eisen *et al*., 1998).

Results

Transport of acetate in yeast cells shifted from glucose to acetic acid

To set up the best conditions for microarray experiments, initial uptake rates of labelled acetic acid were measured over time in cells of the strains *S. cerevisiae* W303-1A and BLC203 (*jen1*), taken at different times after the shift from a medium containing glucose to fresh medium containing acetic acid as sole carbon and energy source. In both strains, after 4 h in the induction conditions, the transport of acetic acid still obeyed to a simple diffusion mechanism, whereas a mediated transport system was found for the labelled acetic acid, after 6 h of induction (Figure 1).

The magnitude of the initial uptake rates was of the same order in both strains. These results showed that the activity of the acetate permease was present only after 6 h of induction. Moreover, the deletion of *JEN1*, the sole known gene coding for a monocarboxylate permease, did not affect the ability of the cells to transport acetic acid by a mediated mechanism.

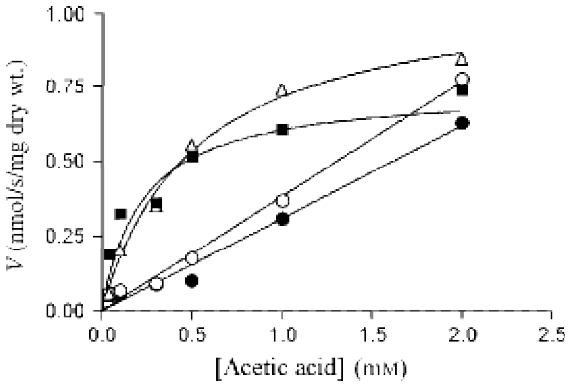

Figure 1. Initial uptake rates of labelled acetic acid, pH 5.0, by acetic acid-derepressed cells of *S. cerevisiae* strains W303-1A and BLC203 (*jen1*), as a function of the acid concentration. °, W303-1A after 4 h induction; Ž, BLC203 after 4 h induction; , W303-1A after 6 h induction; , BLC203 after 6 h induction

Genome-wide analyses of yeast adaptation to acetic acid

Considering the results presented above, RNA was extracted from cells of *S. cerevisiae* W303-1A shifted from glucose to acetic acid for 0, 4 and 6 h. The transcriptomes of times 0 h and 4 h, on one hand, and of times 4 h and 6 h on the other hand were then compared using DNA microarrays. Few significant changes were found in gene expression between times 4 h and 6 h (data not shown). It was assumed that if the acetate permease is active only after 6 h as a protein (Figure 1), the expression of the corresponding gene might be induced earlier, by around 4 h. We then focused on the time 0 h vs. time 4 h comparison. The adaptation of the cells to the shift from glucose to acetic acid was accompanied by extensive changes in gene expression. About 600 genes had their expression changed by more than two-fold (430 up, 170 down; see Supplementary Table I).

More than 50% of the changes in expression concerned genes encoding proteins involved in metabolism (Figure 2 and Supplementary Table I). The metabolic pathways of glucose and a poor carbon source such as acetic acid are completely different (Flores *et al*., 2000). Glucose is metabolized through glycolysis to produce energy and metabolites, such as pyruvate, that can be fermented to produce more energy or that can be used to produce other complex macromolecules. Moreover, glucose represses the expression of the genes involved in gluconeogenesis and in the metabolism of other carbon sources (Gancedo, 1998). Acetic acid is a non-fermentative substrate that is metabolized to acetyl-CoA, which enters the TCA and glyoxylate cycles to fulfil the needs in energy and biosynthetic metabolites (Flores *et al*., 2000). In our experiments, the effect of both glucose exhaustion and the presence of acetic acid as a sole carbon source was clearly observed (Figure 2). A dramatic increase was detected in the expression of the genes involved in gluconeogenesis (*PCK1, FBP1*), in acetate conversion to acetyl-CoA (*ACS1*

and *ACH1)*, together with the genes encoding the enzymes of the glyoxylate shunt and TCA cycle. The genes involved in the production of acetyl-CoA from fatty acid degradation in the peroxisomes, or lactate and propionate metabolism in mitochondria, were also overexpressed (Figure 2). By contrast, expression of the genes encoding fermenting and glycolytic enzymes was decreased. The amino acid biosynthetic pathways were also downregulated (see Supplementary Table I). This last effect, also observed in *Escherichia coli* (Oh *et al*., 2002), suggests that in poor carbon sources microorganisms tend to limit energy and metabolite expenses.

Yeast cells in acetate shift to a respiratory metabolism. As expected, the genes encoding the components of the oxydative phosphorylation chain and heme biosynthesis were overexpressed (Figure 2). Moreover, a global activation of genes involved in mitochondrial biogenesis was observed (Supplementary Table I). This included genes encoding proteins required for the assembly and stability of the oxidative complexes and proteins of the import machinery, molecular chaperons and maturation enzymes involved in the translocation and processing of nuclear-encoded mitochondrial proteins.

Quick adaptation to a shift between two very different growth conditions implies large changes in the protein composition of the cells. We observed a general increase in the expression of the genes encoding ribosomal proteins and translation-promoting factors in cytoplasm and in mitochondria (Supplementary Table I). An activation of the expression of some genes encoding the enzymes involved in the processing of ribosomal RNAs was also detected (Supplementary Table I).

Most of the known regulators of the catabolite derepression were activated in our experiments (Supplementary Figure 1). The transcription factor *CAT8* (2.9-fold activation) and *SIP4* (four-fold induction) are the major activators of the genes encoding the enzymes of the gluconeogenesis and glyoxylate cycle (Haurie *et al*., 2001). *HAP4* (9.8-fold induction) and *ADR1* (10-fold induction) positively regulate the TCA cycle, respiration complex and heme biosynthesis and peroxysomal activity, respectively (Gancedo, 1998). A negative feedback control of these regulations was found: *ROX1*, the transcriptional repressor of genes encoding the components of the respiratory complex IV, was activated, probably in response to the activation of the heme biosynthesis pathway (Gancedo, 1998).

ADY2/YCR010c is essential for the mediated transport of acetic acid

Many genes encoding transporters are regulated during the shift from glucose to acetic acid (Table 2). The mitochondrial carriers involved in the transport of ATP/ADP, inorganic phosphate and carboxylic acids intermediates of the TCA and glyoxylate cycles were overexpressed, as well as the genes encoding plasma membrane hexose transporters and the lactate permease *JEN1*. It is noteworthy that the five members of the MCP family were downregulated in these conditions (Table 2), which strengthens our previous findings that they do not transport acetate *in vivo* (Makuc *et al*., 2001).

Actually, the only genesencoding as-yet unknown membrane proteins to be clearly activated in our experiments were *YCR010c/ADY2*, *YNR002c/FUN34* and *YDR384c/ATO3*, which are homologous to the *GPR1* gene of *Yarrowia lipolytica*. Interestingly, mutations in the *GPR1* gene of *Y. lipolytica* causes alterations of the sensitivity of the cells to acetic acid (Tzschoppe *et al*., 1999). The *S. cerevisiae* Gpr1p-homologous proteins are predicted to have five to seven transmembrane domains and are of unknown function. Their expression profiles in the yMGV database (Le Crom *et al*., 2002) show that these genes are highly expressed during growth in stationary phase, ethanol and sporulation media and are repressed in rich carbon sources, which fits with the activity profile of the unknown acetate permease (Casal *et al*., 1996). We checked by Northern blots that *ADY2* is also induced in lactic acid, pyruvic acid and glycerol (data not shown), all conditions in which the acetate permease is active (Casal *et al*., 1996). All these data indicated the three members of the S. cerevisiae *GPR1* family as good candidates for encoding proteins involved in acetate transport. Acetate uptake was measured in deletion mutants for each of the *GPR1* homologues (*ADY2, FUN34, ATO3*) to assess their role in acetate transport 6 h after the shift from glucose to acetic acid. The assays were performed in *S. cerevisiae* BY4742 mutant strains purchased from the EUROSCARF strain collection (Table 1). Only a mutant deleted

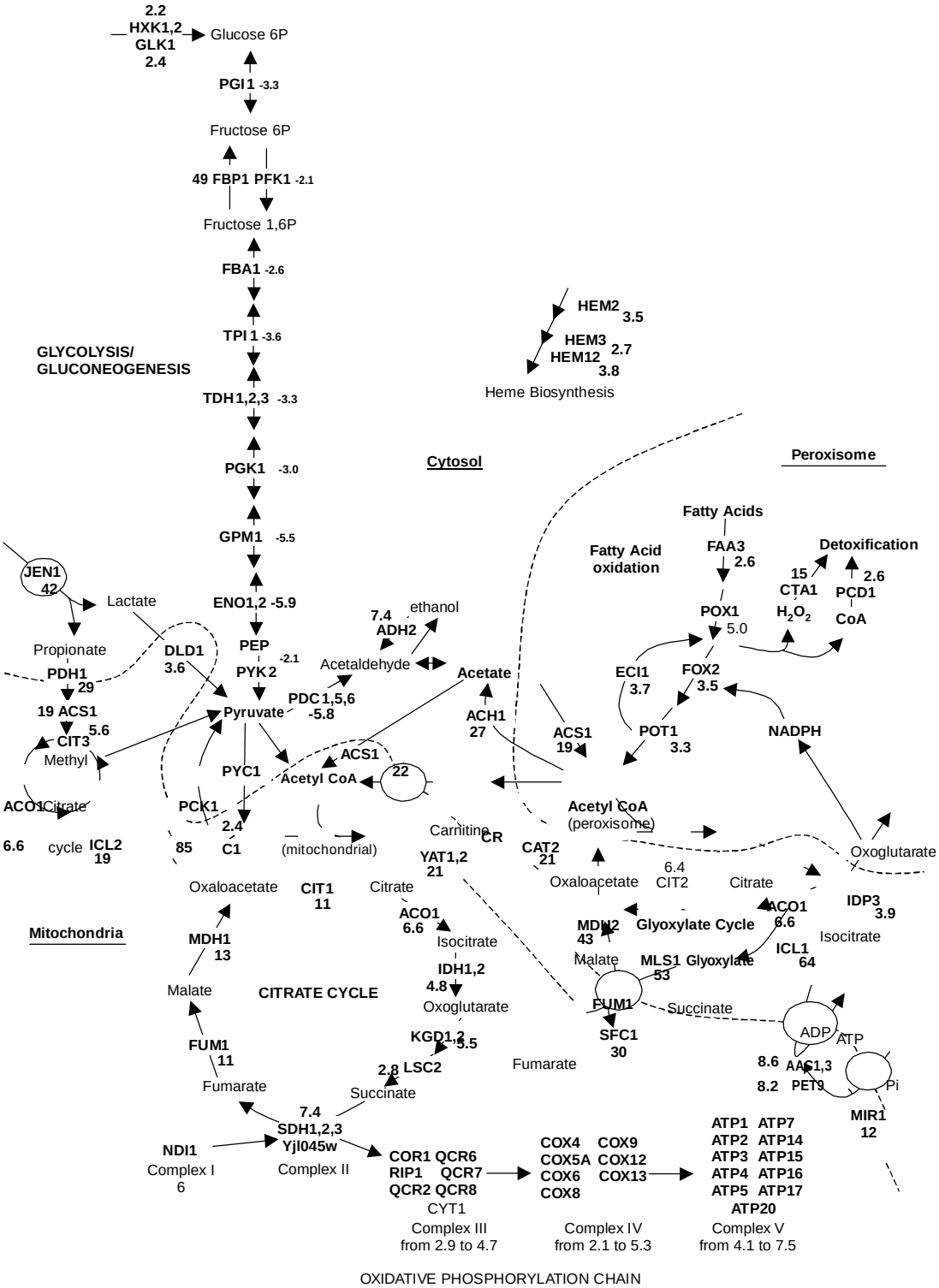

Figure 2. Gene expression changes following the shift from glucose to acetate: reprogramming of basal metabolism. The dashed lines symbolize the intracellular location of the enzymes (from left to right: mitochondria, cytosol, peroxisome). The proteins within circles are transporters, the others are enzymes. The numbers close to the gene names represent the corresponding fold induction or repression, as follows: X = X-fold induction, −X = X-fold repression. The genes repressed are in italic, the genes activated in bold

Table 2. Looking for the unknown acetate permease in yeast. Expression changes in gene encoding known or putative transporters. The fold induction or repression are indicated as follows: X = X-fold induction, −X = X-fold repression. The fold change and description for individual genes are available in the supplemental Table I

	Fold induction after 4 h in acetate	Substrate
Mitochondrial transporters		
TCA cycle intermediates		
SFC1	30	Succinate/fumarate antiport
CRC1	22	Carnitine
ODC1	9.3	Oxoglutarate
OAC1	−5.1	Oxaloacetate
Oxydative phosphorylation products		
AAC1, AAC3, PET9	8.2 to 8.6	ATP/ADP antiport
MIR1	12	Inorganic phosphate
Others		
ACP1	3.0	Fatty acids precursors
DIC1	−3.0	Dicarboxylates
Plasma membrane transporters		
Carboxylic acids		
JEN1	42	Lactate, propionate, acetate
MCH3, MCH4, MCH5	−2.2 to −3.7	Unknown
Amino acids and carnitine		
AGP1	2.3	Asparagine, glutamine, cysteine, etc.
TAT1	8.1	Valine, leucine, tyrosine, etc.
MUP1	3.4	Methionine
AGP2	5.7	Carnitine
Hexoses		
HXT1, HXT2, HXT3, HXT4, HXT5, HXT6, HXT7, HXT8	2 to 6.3	Hexoses
Inorganic ions		
SMF2	2.5	Manganese
CTR1	4.2	Copper
FET3, FTR1	2.1/3.1	Iron
ENA1, ENA2, ENA5	2.3	Sodium, lithium
Unknown membrane proteins		
ADY2	27	Unknown
FUN34	9.8	Unknown
ATO3	2.6	Unknown

in *ADY2* was strongly affected in the ability to transport acetate, whereas the deletion of the two other *GPR1*-like genes had no significant effect compared with the wild-type strain (data not shown). Since the BY4742 strain exhibits a poor growth in acetic acid, we decided to disrupt *ADY2* in a W303-1A background, which grows more efficiently on this carbon source. The *ADY2*-deleted strain, Ace718, exhibited a dramatic decrease in the activity of the acetate permease (Figure 3). In this strain, the acetate crossed the plasma membrane by a simple diffusion mechanism.

A *Y. lipolytica* strain deleted for *GPR1* has been shown to have a delay in adaptation to acetic acid (Augstein *et al*., 2003). Therefore, the defect

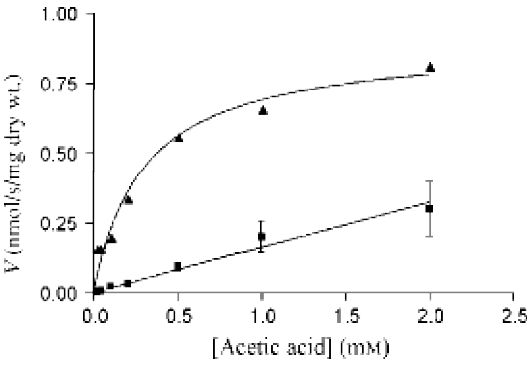

Figure 3. Initial uptake rates of labelled acetic acid by YNB acetic acid-derepressed cells of *S. cerevisiae* , W303-1A and , Ace 715 (*ady2*) strains, as a function of the acid concentration

in acetic acid transport in *ady2* cells that we observed after 6 h in acetate could be due to a delay in the activation of the acetate permease. To test this hypothesis, we measured the ability of W303-1A and Ace 718 (W303-1A, *ady2*) strains to adapt to YNB media containing acetic acid. No significant differences could be found between the wild-type and the mutant strain (Figure 4). From these experiments, we conclude that the acetate transport defect of *ady2* strain is actually due to a defect in acetate permease activity and not to a difference in adaptation or growth rates.

Comparison of gene expression profiles between W303-1A and *ady2* strains

trans-Dominant mutations of *GPR1* have been shown to alter the activity and expression of the enzymes of the glyoxylate pathway in *Yarrowia lipolytica* (Tzschoppe *et al*., 1999). We wondered if Ady2p could have such regulatory effect. We performed microarray analyses of strains deleted for *ADY2* in glucose or 4 h after having been shifted to acetate (maximum of transcriptional response). As expected, the deletion of *ADY2* has no obvious effect on gene expression in glucose, as compared with the wild-type (data not shown). After 4 h in acetate, the *ady2* strain does not have a dramatic difference in the global transcriptional response compared to the wild-type (Figure 5 and Supplementary Table II). This result is in agreement with the normal growth of *ady2* cells in acetate

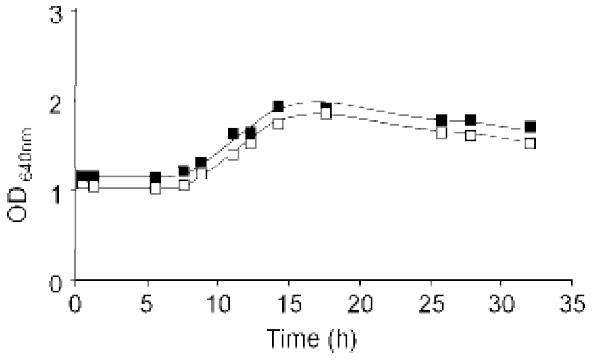

Figure 4. Adaptation of *S. cerevisiae* , W303-1A and , Ace 715 (*ady2*) strains to acetic acid (0.5%, pH 6.0). Cells were cultivated as described in Materials and methods for repression and derepression conditions. At time 0, glucose-grown cells were centrifuged, washed twice in ice-cold deionized water and transferred to fresh YNB medium with acetic acid (0.5%, pH 6.0)

(Figure 4). It suggests that *ADY2* is not a key regulator of the transcriptional response to acetic acid. However, we observed a slight increase (less than three-fold) in the expression of genes involved in the use of acetate (*ACS1, ACH1*) and in the methylisocitrate and glyoxylate cycle genes (*ICL1, PDH1, ICL2, PDH1, MDH2, MLS1*) compared to the wild-type. These differences are weak, considering that all these genes are highly induced (ratio between 6 and 80 times) in the wild-type under these conditions (Figure 5). They can be interpreted as being more like an attempt of the cells to adapt to a change in the intracellular concentration of acetate, connected to the defect in acetate transport, than like a direct regulatory effect of Ady2p on the expression of these genes. This interpretation is supported by the induction in *ady2* cells of *ACS2*, which encodes the second yeast Acetyl CoA synthetase (conversion of acetate to acetyl CoA) and which is normally repressed in acetate (Supplementary Table II).

One possible explanation for the transport defect observed in the *ADY2*-deleted strain would be that the inactivation of this gene negatively affects the expression of the acetate permease. We found few negative effects in the *ady2* strain compared to the wild-type (Figure 5). In acetic acid *ady2* cells have a significant defect only in the expression of some *HXT* genes, which encode hexose transporters (three- to five-fold decrease). These *HXT* genes are not induced after transfer of cells to a sporulation medium containing acetate as a sole carbon source (Chu *et al*., 1998) and are repressed in ethanol (Gasch *et al*., 2000), whereas it has been shown that an acetate permease activity does exist under these conditions (Casal *et al*., 1996; Makuc *et al*., 2001). Therefore, these *HXT* genes are unlikely to be involved in acetate permease activity. From all these results, we propose a direct the role of Ady2p in acetate transport.

Discussion

We used DNA microarrays to characterize the transcriptional adaptation of yeast cells to a shift from glucose to non-toxic conditions of acetic acid. Such metabolic adaptation is a critical challenge for microorganisms, which have evolved efficient mechanisms to reprogram the protein composition of the cell according to the nutrients available.

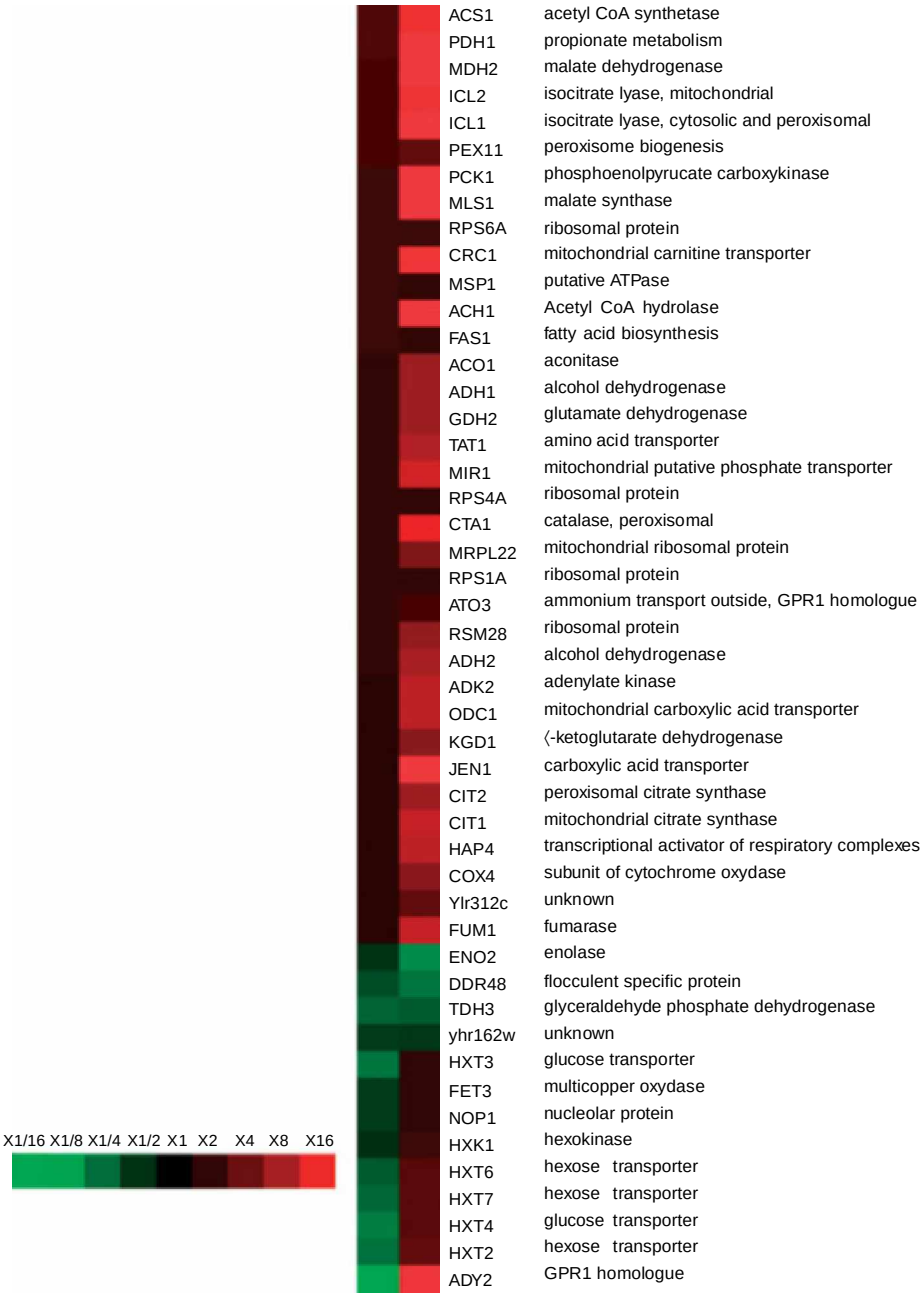

Figure 5. *ADY2* deletion moderately affects the acetate response. Treeview software (Eisen *et al*., 1999) was used to represent the ratios of expression (see colour scale) of genes of the acetate response that differed between *ady2* and wild-type (W303A) after a shift of 4 h from glucose to acetate (left column). For comparison, the ratios of expression of these genes in the wild-type between glucose and after shift of 4 h in acetate are indicated (right column). The role of these enzymes and transporters in metabolic pathways can be found in Figure 2. Complete results and fold changes for the *ady2* vs. WT comparison in acetate can be found in Supplementary Tables I and II

As was previously shown for the diauxic shift (DeRisi *et al*., 1997), these mechanisms originate mainly at the transcriptional level and microarray analyses are adapted, powerful tools to reveal the physiological events in these cases. In this study we present a complete picture of the metabolic reprogramming, the activation of mitochondrial biogenesis and the mobilization of the translation machinery, which are the main features of yeast adaptation to poor carbon sources. These features make these conditions of metabolic shift very powerful for functional assignment of unknown proteins and the study of mechanisms such as translation regulation or mitochondrial biogenesis. We fulfilled our initial goal by identifying the *ADY2* gene as a new essential component of acetate uptake in yeast cells. *ADY2* is predicted to encode a membrane protein and it is homologous to the *GPR1* gene of *Y. lipolytica*. *GPR1* has a role in the sensitivity of the cells to acetic acid and ethanol (Tzschoppe *et al*., 1999). However, the function of these two homologous genes might be different, since the expression of *GPR1* is only moderately repressed by glucose in *Y. lipolytica* and deletion of *GPR1* slows down adaptation of cells to acetic acid (Augstein *et al*., 2003), whereas *ADY2* is under glucose repression and its deletion has no effect on acetate adaptation in *S. cerevisiae* (this study). *ADY2* is known to be under the control of the transcription factor *CAT8*, which regulates catabolite derepression (Haurie *et al*., 2001). As a matter of fact, *ADY2* is induced in conditions of poor carbon source, e.g. in late stationary phase, sporulation medium, growth in ethanol (see yMGV database: Le Crom *et al*., 2002), lactic and pyruvic acids (our unpublished results), all conditions in which the putative acetate permease is active. Interestingly, *ADY2* was first characterized as a gene required for proper ascus formation on a sporulation medium in which acetate is the main carbon source (Rabitsch *et al*., 2001). Transport and microarray analyses of a *ADY2*-deleted strain showed that this gene is critical for active acetate transport but not for the global transcriptional response of the cells to the presence of acetic acid. We thus propose that Ady2p plays a direct role in acetate transport. This is supported by the recent demonstration that Ady2p is located to the plasma membrane (Z. Palkova, personal communication). Additionally, it is not excluded that *ADY2* plays other roles in regulating yeast membrane transport, not exclusively related to acetate transport. Indeed, *ADY2* was recently shown to be important for the periodic ammonium export from *S. cerevisiae* colonies during their late development (Palkova *et al*., 2002).

Supplementary material

The supplementary tables and figures for this paper can be found at **http://www3.interscience.wiley.com/cgi-bin/jabout/3895/OtherResources.html**.

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