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Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*

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Abstract DNA microarrays were used to investigate the expression profile of yeast genes in response to ethanol. Up to 3.1% of the genes encoded in the yeast genome were up-regulated by at least a factor of three after 30 min ethanol stress (7% v/v). Concomitantly, 3.2% of the genes were down-regulated by a factor of three. Of the genes up-regulated in response to ethanol 49.4% belong to the environmental stress response and 14.2% belong to the stress gene family. Our data show that in addition to the previously identified ethanol-induced genes, a very large number of genes involved in ionic homeostasis, heat protection, trehalose synthesis and antioxidant defence also respond to ethanol stress. It appears that a large number of the up-regulated genes are involved in energy metabolism. Thus, 'management' of the energy pool (especially ATP) seems to constitute an ethanol stress response and to involve different mechanisms. © **2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: DNA microarray; Gene expression; Ethanol stress; Yeast

1. Introduction

Among environmental stresses that yeast cells undergo, ethanol constitutes the main stress factor during fermentation processes. From a physiological point of view, ethanol inhibits yeast growth and viability, affects different transport systems such as the general amino acid permease system and glucose uptake, and inhibits the activity of key glycolytic enzymes [1,2]. The main target of ethanol is the plasma membrane, the fluidity of which is altered during ethanol stress. This alteration results in changes in permeability to ionic species, especially protons [3]. Increased proton influx results in the rapid dissipation of the electrochemical gradient across the plasma membrane and subsequent intracellular acidification. Yeast cells have developed a panel of stress responses (transient) and adaptation mechanisms (long-term response) to cope with deleterious effects of ethanol. Heat shock proteins (HSPs), for example, are synthesised during ethanol stress [4]. The role of HSPs in ethanol stress is still not well understood. It remains to be determined whether they play a similar role during heat shock, i.e. a stabilising effect, preventing aggregation, and assisting the posterior refolding of proteins. Trehalose, which is considered to be a stress protectant, and HSPs are synthesised upon ethanol stress and have been reported to stabilise membranes and proteins, and to suppress protein aggregation [5].

Other physiological studies have shown that ethanol triggers an increase in plasma membrane ATPase activity, which counteracts the ethanol-induced proton influx [6,7]. Alterations in lipid composition of the membrane have been observed in response to ethanol stress and are thought to represent an adaptive mechanism towards ethanol-induced changes in plasma membrane fluidity [1,2].

From a molecular point of view, information concerning ethanol stress is rather patchy. No systematic studies have looked at the molecular process involved in the ethanol stress response. Although some genes, such as the HSP genes, have been shown to respond to ethanol stress [4], the pleiotropic effects of ethanol suggest that a large number of genes involved in this specific stress response are still to be discovered. Furthermore, although specific stress response pathways exist for osmotic, heat and oxidative stress, we still do not know how ethanol signalling occurs.

A few years ago it was difficult to carry out a global gene expression study to identify important genes regarding ethanol stress, however, today DNA microarrays allow gene regulation in response to ethanol to be assessed. Thus, we used microarrays to analyse changes in mRNA abundance during ethanol shock.

2. Materials and methods

2.1. Strain and growth conditions

The strain used was *Saccharomyces cerevisiae* S288C. Cells were grown in rich YPD medium at 28°C with agitation (250–300 rpm). During the early exponential phase ($OD_{660} = 0.8$) the culture was divided in two samples of 100 ml. Ethanol was added to one flask to a final volume of 7% (v/v). Cells were collected by centrifugation after 30 min for RNA extraction.

2.2. RNA extraction and mRNA purification

Both the control and ethanol-treated cells were harvested and washed with RNase-free water. Cells were broken using Trizol reagent (Gibco BRL, Life Technologies). After extraction, RNA was precipitated with isopropylic alcohol and the pellet was rinsed with 80% (v/v) ethanol and resuspended in RNase-free water. mRNA was isolated by use of the PolyAtract Isolation system (Promega, USA) according to the manufacturer's instructions.

2.3. Probe preparation and labelling

Fluorescently labelled cDNA was prepared from poly(A)$^+$ RNA as described by De Risi et al. [8]. Briefly, 2 μg mRNA was mixed with 5 μg oligo(dT), heated for 10 min at 70°C and chilled on ice. Reverse transcription was performed in the presence of 400 U SuperscriptII reverse transcriptase (Gibco BRL, Life Technologies), 25 mM dATP,

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dCTP, dGTP, 10 mM dTTP, 100 μM Cy3-dCTP or 100 μM Cy5-dCTP (Amersham Pharmacia Biotech). Probes were purified using the JETquick polymerase chain reaction kit (genomed).

2.4. Microarrays hybridisation and scannings

We used CMT™ yeast S288c gene array slides which contained 6138 encoding sequences (Corning, USA). We mixed 10 μl probe, 30 μl Digeasy buffer (Boehringer Mannheim, Germany) and 10 mg/ml salmon sperm DNA together. After 10 min at 95°C, the mixture was centrifuged at 12 000×*g* for 2 min. The solution was dropped onto the array and a coverslip placed on it. Hybridisation was conducted for 18 h in a CMT™ hybridisation chamber (Corning, USA). After hybridisation, the labelled microarray was washed and dried. The microarrays were scanned with a Gene Pix 4000 scanner (Axon instruments, Foster city, CA, USA) and the Gene Pix 4000 software package was used to locate spots in the microarray. To correct for the variations (artefact, background, different labelling efficiency, poor quality of some spots etc.) we normalised our data. This basically consists of applying a linear regression method to log data, as explained in detail on the web (http://afgc.stanford.edu/~finkel/talk.htm).

3. Results and discussion

We used cDNA microarrays for a genome-wide transcriptional analysis to measure the changes in the relative expression level of yeast mRNA during a short ethanol shock (30 min). As we were interested in the short-term response to ethanol stress and because of the large amount of data generated by microarray experiments, we limited the ethanol treatment to a single concentration and a single time point. The data were derived from three independent experiments. Genes were considered to be down- or up-regulated if the intensity ratio changed by at least a factor three in all three experiments after normalisation (all data are available on the INRA website, http://www.ensam.inra.fr/spo/yeastgenomic).

As predicted, ethanol stress altered the expression of a large number of the 6138 genes analysed in the yeast genome. Of these genes, 194 (3.1% of the genome) were up-regulated more than 3-fold, 85 (1.3% of the genome) by a factor of 5 and 18 genes were up-regulated by a factor of 10. Conversely, 201 genes were down-regulated by a factor of 3 after 30 min ethanol shock.

The distribution of genes, either up- or down-regulated upon ethanol exposure, in functional classes provides interesting information on the molecular mechanisms that allow the cell to survive ethanol stress (Fig. 1). However, it should be stressed that a large number of the genes, whose expression was altered, encoded proteins of unknown functions. Most of the down-regulated genes are involved in protein biosynthesis (34%), cell growth (4%), RNA metabolism (13%) and cellular biogenesis (3%) (Fig. 1). The down-regulation of these genes is thought to reflect growth arrest which occurs during different stress treatments and allows the cell to save energy and to adapt to new conditions [9].

Conversely, the genes that are up-regulated by ethanol are mainly involved in energetic metabolism, protein destination, ionic homeostasis, and the stress response (Fig. 1). Gasch et al. [9] recently depicted the environmental stress response (ESR) family genes. ESR corresponds to the cluster of all the genes that have similar expression profiles under various stress conditions. From the data available on the web (http://www-genome.stanford.edu/yeast_stress), we found that among the 300 genes up-regulated in the ESR, 73 were also up-regulated during ethanol stress.

Among them we found a group of highly responsive genes encoding HSPs. *HSP12*, *HSP26*, *HSP78*, *HSP104* were up-regulated during ethanol stress which is consistent with previous results [4] and reflects the reliability of the method. We report for the first time the ethanol-mediated induction of the *SSA1*, *SSA2*, *SSA3*, *SSA4*, *SSE1* genes, which encode HSPs from the HSP70 family. The induction of these genes supports the prediction that one of the main effects of ethanol is protein unfolding.

The regulation of the genes encoding HSPs is principally mediated via the HSE (heat shock element) promoter sequence [10], which is the binding site for the transcription factor, Hsf1p. However, Hsf1p is not the only factor involved in the induction of HSP genes, and several of them are controlled by the general stress response system in which two transcription factors, Msn2p and Msn4p, bind to a specific sequence called STRE for stress response element [11]. Ethanol induction through the STRE element is well recognised and we observed that 20 genes of 69 putative STRE-controlled genes reported by Moskvina et al. [12] were up-regulated during ethanol stress. Among them we found the genes involved in trehalose synthesis and their precursors including *TPS1*, *TLS1*, *TPS2*, *UGP1* and *PGM2* which are not STRE-regulated. These results were expected as trehalose has previously been reported to accumulate under ethanol stress and its protective role against ethanol has been established [13]. The co-induction of trehalose and the HSP genes during ethanol stress supports the existence of a tight link between these two protective agents, similar to that described for heat treatment. According to Winkler et al. [14], Tps1p is involved in HSP induction. More recently Singer and Lindquist [5] provided a model describing this interplay. In summary, in a first step, trehalose synthesis prevents the denaturation of proteins, subsequently HSPs stop protein aggregation, and finally the disaccharide is degraded because it impedes protein stabilisation by the HSPs.

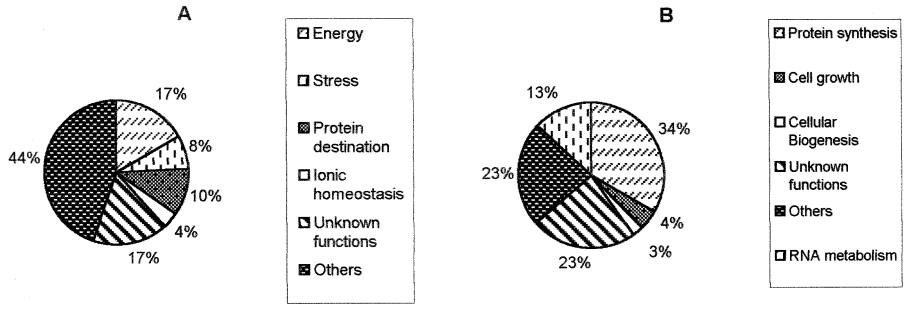

Fig. 1. Distribution of ethanol-induced genes in the most representative classes. A: Induced genes, B: down-regulated genes.

Table 1
Genes induced after 30 min ethanol shock (7% v/v)

Open reading frame code	Gene name	Induction fold	Gene description
Energy			
	glycolytic pathway		
YCL040W	GLK1	20.6	aldohexose-specific glucokinase
YFR053C	HXK1	7.3	hexokinase I
YDR516C		13.7	strong similarity to glucokinases
YIL107C	PFK26	4.2	6-phosphofructose-2-kinase
YJL052W	TDH1	6.4	glyceraldehyde-3-phosphate dehydrogenase 1
YDL021W	GPM2	6.9	phosphoglycerate mutase
YAL038W	CDC19	7.7	pyruvate kinase
YMR170C	ALD2	3.1	aldehyde dehydrogenase 2 (NAD^+)
YOR374W	ALD4	3.3	aldehyde dehydrogenase mitochondrial
	trehalose metabolism		
YBR126C	TPS1	6.8	α,α-trehalose-phosphate synthase, 56 kDa subunit
YML100W	TSL1	11.9	α,α-trehalose-phosphate synthase, 123 kDa subunit
YMR105C	PGM2	12.8	phosphoglucomutase, major isoform
YDR001C	NTH1	3.1	neutral trehalase (α,α-trehalase)
YDR074W	TPS2	4.2	α,α-trehalose-phosphate synthase, 102 kDa subunit
	glycogen metabolism		
YKR058W	GLG1	3.2	self glycosylating initiator of glycogen synthesis
YEL011W	GLC3	7.4	1,4-glucan branching enzyme
YFR015C	GSY1	5.5	UDP-glucose glucosyltransferase
YPR160W	GPH1	4.0	glycogen phosphorylase
YKL035W	UGP1	9.8	UTP-glucose-1-phosphate uridylyltransferase
	glycerol metabolism		
YDL022W	GPD1	4.2	glycerol-3-phosphate dehydrogenase (NAD^+), cytoplasmic
YER062C	HOR2	5.0	DL-glycerol phosphatase
YML070W	DAK1	4.3	dihydroxyacetone kinase, induced in high salt
	others		
YCR005C	CIT2	7.9	citrate (si)-synthase, peroxisomal
YDL130W-A	STF1	3.6	ATPase stabilising factor, 10 kDa
YER054C	GIP2	4.2	Glc7p-interacting protein
YGR008C	STF2	5.7	ATPase stabilising factor
YHR179W	OYE2	4.0	NADPH dehydrogenase (old yellow enzyme), isoform 1
YKL150W	MCR1	5.0	cytochrome *b*5 reductase
YNR001C	CIT1	3.7	citrate (si)-synthase, mitochondrial
YOL157C		3.1	strong similarity to α-glucosidases
YPL171C	OYE3	4.6	NADPH dehydrogenase (old yellow enzyme), isoform 3
YPR184W	GDB1	4.2	oligo-1,4-1,4-glucantransferase/amylo-1,6-glucosidase
YDL124W		8.3	similarity to aldose reductase
YPL240C	HSP82	4.5	HSP
YGL062W	PYC1	4.0	pyruvate carboxylase 1
Stress			
YBL075C	SSA3	3.8	HSP of HSP70 family, cytosolic
YBR072W	HSP26	12.0	HSP
YDR258C	HSP78	7.6	HSP of clpb family of ATP-dependent proteases, mitochondrial
YER103W	SSA4	21.5	HSP of HSP70 family, cytosolic
YFL014W	HSP12	11.2	HSP
YHL046C		4.3	strong similarity to members of the Srp1p/Tip1p family
YHR104W	GRE3	5.6	aldose reductase
YLL026W	HSP104	11.5	HSP
YNL160W	YGP1	5.9	secreted glycoprotein
YPL240C	HSP82	4.5	HSP
YPL106C	SSE1	3.4	HSP of HSP70 family
YLL024C	SSA2	11.5	HSP of HSP70 family, cytosolic
YAL005C	SSA1	7.9	HSP of HSP70 family, cytosolic
YGR088W	CTT1	11.6	catalase T, cytosolic
YLR109	AHP1	5.3	alkylhydroperoxide reductase
YMR251W-A	HOR7	7.9	hyperosmolarity-responsive protein
Protein destination			
YBL078C	AUT7	6.9	essential for autophagy
YBR139W		4.1	strong similarity to carboxypeptidase
YCL043C	PDI1	5.0	protein disulfide isomerase precursor
YDL020C	RPN4	4.0	26S proteasome subunit
YDR171W	HSP42	18.7	weak similarity to *Streptomyces* HSP18 protein
YDR258C	HSP78	7.6	HSP of clpb family of ATP-dependent proteases, mitochondrial
YDR518W	EUG1	4.9	protein disulfide isomerase
YEL012W	UBC8	4.4	E2 ubiquitin-conjugating enzyme
YKL073W	LHS1	3.5	chaperone of the endoplasmic reticulum (ER) lumen
YLR120C	YPS1	6.2	aspergillopepsin
YLR121C	YPS3	6.2	GPI-anchored aspartyl protease 3 (yapsin 3)

Table 1 (*continued*)

Open reading frame code	Gene name	Induction fold	Gene description
YLR216C	CPR6	4.8	member of the cyclophilin family
YML130C	ERO1	9.8	required for protein disulfide bond formation in the ER
YMR018W		4.2	similarity to tetratricopeptide-repeat protein PAS10
YNL077W		3.0	similarity to dnaJ protein homologue YDJ1
YEL060C	PRB1	4.8	protease B, vacuolar
YNL015W	PBI2	5.3	proteinase B inhibitor 2
Ionic homeostasis			
YOR137C		3.0	similarity to YLR270W
YCR021C	HSP30	7.6	HSP
YDR270W	CCC2	3.0	probable copper-transporting ATPase
YEL031W	SPF1	3.0	P-type ATPase
YER053C		17.8	strong similarity to mitochondrial phosphate carrier protein
YHR175W	CTR2	6.4	copper transport protein
YJL094C	KHA1	5.9	K^+/H^+ exchanger
YCR024C-A	PMP1	4.1	H^+-ATPase subunit, plasma membrane

It is noteworthy that in addition to the genes involved in trehalose synthesis, *NTH1*, which encodes the neutral trehalase, was also up-regulated. The fact that ethanol stress induces genes involved in both trehalose synthesis and degradation may allow the yeast to adjust its trehalose content rapidly to assist the HSPs in protein folding. However, this may be over-simplified and trehalose metabolism may be one of several futile energetic cycles as suggested by the detailed analysis of the energy genes. We observed that the mRNA levels of genes involved in glycerol synthesis (*GPD1*, *HOR2*) and glycerol catabolism (*DAK1*) also increased. Similarly, several genes involved in both glycogen synthesis and degradation, such as *UGP1* encoding UDP-glucose pyrophosphorylase responsible for UDP-glucose formation, *GSY1* the minor isoform of glycogen synthase, the branching enzyme *GLC3*, *GLG1* an initiator of glycogen synthesis and *GPH1* involved in glycogen degradation, all responded to ethanol stress. Trehalose is known to accumulate during ethanol stress and to protect the cells, however, the intracellular concentrations of glycerol and glycogen have not been reported to increase under such conditions. Furthermore, although glycerol protects the cells under certain adverse conditions, the role of glycogen in stress protection is still unknown.

Although contradictory, the induction of genes involved in the synthesis and catabolism of trehalose, glycerol and glycogen is consistent with previous studies on other stress responses [15,16]. These three metabolic pathways are energy consuming, which may help to control the energy balance of the cell. Blomberg [17] recently proposed the following hypothesis to explain the existence of these futile cycles during salt stress: in summary, salt stress decreases the growth rate of cells which leads to lower activity of the synthesis machinery and consequently a lower ATP demand. In these conditions Pi is rapidly depleted by a mechanism called 'turbo design pathway' [18] in which hexose monophosphate and fructose-6-phosphate accumulate and lead to the depletion of Pi, which causes cell death. To prevent such an effect, the futile glycerol, trehalose and glycogen cycle increases ATP consumption.

Although both the down-regulated genes involved in protein biosynthesis and growth curve (data not shown) provide evidence for cell growth arrest, this model is not completely satisfactory.

The global gene expression during ethanol stress suggests the existence of three futile cycles that only seem to constitute one aspect of energy control as indicated by the up-regulation of other groups of genes involved in ATP generation or utilisation. Indeed, most of the genes involved in glycolysis were up-regulated (Table 1, Fig. 2) during ethanol stress. These include two genes encoding sugar kinase, *HXK1* and *GLK1*, which were up-regulated by 7.2-fold and 20-fold respectively. Moreover, *YDR516C*, which is very similar to glucokinase, was induced 13.7-fold. Induction of these genes might be necessary to provide glucose-6-phosphate for the synthesis of trehalose. On the other hand, induction of the genes involved in the lower part of the glycolysis may lead to the generation of ATP. This is consistent with the increased demand for ATP during ethanol stress. The plasma membrane ATPase, responsible for the creation of the electrochemical gradient, is activated by ethanol stress and probably counteracts the ethanol-induced proton influx [6,7]. Although we did not observe any induction of the H^+-ATPase gene (*PMA1*), a set of genes involved in ATPase regulation were up-regulated. It is noteworthy that two up-regulated genes (*PTK2*, which encodes a protein kinase, and *YOR137C*) are both implicated in the H^+-ATPase activation mechanism [19,20]. In contrast, the gene encoding a negative regulator of the H^+-ATPase, *HSP30* [21], was up-regulated 7.6-fold. All of these observations are consistent with the fine-tuning of the H^+-ATPase activity that is necessary during ethanol stress. Consequently, it appears that following ethanol stress, two contradictory events take place; the genes that lead to energy consumption are induced and as are those that result in generation of ATP. In fact, these apparently contradictory mechanisms might allow a fine-tuning of the energy pool, which enables the cell to cope with rapid transitions in energy demand. Furthermore, this fine regulation may be completed by the tight control of the enzymes at a post-translational level.

The maintenance of the electrochemical proton gradient by the H^+-ATPase is vital during ethanol stress, both for pH homeostasis and ionic homeostasis. Ethanol also affects the translocation of ions such as Ca^{2+} and Mg^{2+}, which may alter the ionic homeostasis [22,23]. Our data emphasised that other mechanisms, as well as the activation of H^+-ATPase, might contribute to the maintenance of ionic homeostasis. Thus, the observed induction of *CCC2* and *CTR2*, which encode a copper transporter and *KHA1*, a K^+/H^+ exchanger, might be implicated in the maintenance of ionic homeostasis. The K^+/H^+ exchanger may also compensate for the ethanol-induced proton influx.

Finally, *CTT1* and *AHP1*, which encode the oxidative stress

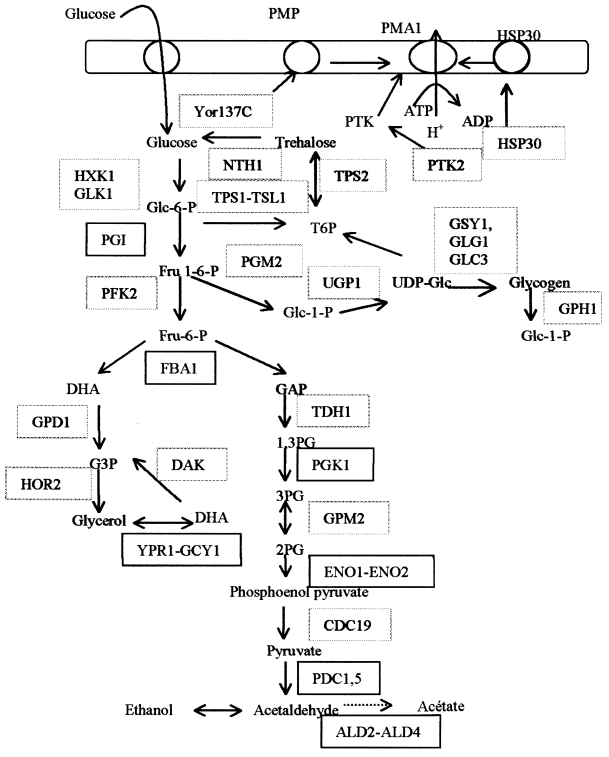

Fig. 2. Effect of ethanol on the mRNA levels of genes involved in glycolysis, trehalose, glycogen and glycerol metabolism, and ATPase regulation. Gene names are boxed, induced genes are highlighted with dashed boxes.

proteins catalase T and alkyl hydroperoxide reductase respectively, were also induced and thus help the cell to avoid the damaging effects of reactive oxygen species that are generated during ethanol stress [24]. Consistent with our results, a recent study by Costa et al. [25] showed that superoxide dismutase (*SOD*) genes are not induced after 30 min ethanol shock (8% v/v), which agrees with our results. However, the cellular content of *SOD2* mRNA increased after 1 h ethanol shock. This may be because changes in mRNA levels during stress are either time-dependent or transient [9,26].

Another interesting aspect is the obvious implication of different signal transduction pathways involved in ethanol stress response. According to the different gene families whose expression increases during ethanol stress, the cells probably detect different primary signals that are transmitted via different signalling pathways.

It is clear from our study that one of the signal transduction pathways used by the cell to respond to ethanol stress is the general stress response pathway, because 20 of the 69 putative STRE-regulated genes were up-regulated upon exposure to ethanol. Given the large number of up-regulated HSP genes, the HSE-mediated signal transduction pathway is probably involved in the ethanol stress response. Another potential transduction pathway involved in ethanol response is the HOG pathway because *GPD1*, *HOR2*, *GRE3*, *DAK1*, *HOR7*, which were up-regulated during ethanol exposure, are all dependent on the HOG pathway [16]. These results were surprising because Tamas et al. [27] found that ethanol does not stimulate the HOG pathway. In their study they did not detect any increase in *GPD1* expression after ethanol exposure. Conversely, Ogawa et al. [28] compared different ethanol tolerant strains and found that *GPD1*, *SPI1* and *HOR7*, which are up-regulated by osmotic shock, are expressed at a higher level in the ethanol tolerant strain. Our study could not determine whether ethanol-mediated induction is due to the HOG. It is obvious from previous studies that other uncharacterised signal transduction pathways exist and are involved in ethanol stress signalling [16,29].

Finally, the global gene expression of yeast, when challenged by ethanol stress, detected genes of unknown function, which probably have important roles in the ethanol stress response. For example, *YER053C* encodes a protein similar to mitochondrial phosphate carrier protein that is induced 17.8-fold.

It is also striking to note that some isogenes, such as *GLK1* (a putative glucokinase), *YDR516C* (an uncharacterised glucokinase homologue), *GPM2*, *ALD2*, *GSY1*, *TDH1* and *YDL124W* (similar to aldose reductase), are up-regulated by ethanol stress. Similar results have been observed for osmotic shock and other stresses [9,16]. We hypothesise that the way these genes are regulated differs from the major isoforms and, therefore, these genes may be up-regulated in situations in which the mRNA level of the major isogenes either does not change or decreases.

4. Conclusion

As predicted by previous studies [26,30,31], the use of DNA microarray for the investigation of the global changes in gene expression after ethanol shock is a powerful tool. Our data contribute to the understanding of the ethanol adaptation mechanisms underlying ethanol stress. We show that the number of genes that respond to ethanol stress is at least 10-fold greater than the number previously reported.

Although our study is only the first step in the investigation of the ethanol stress response, it provided a more detailed picture of the previously identified ethanol adaptation mechanisms. For example, we have shown that most of the genes involved in trehalose synthesis are up-regulated during ethanol stress. Similarly, we demonstrated that a whole set of HSP genes are induced. Furthermore, there is evidence for other ethanol adaptation mechanisms, such as ionic homeostasis and oxidation defence, although the importance of these mechanisms in ethanol adaptation remains to be clarified.

The most interesting aspect highlighted by our study is the tight regulation of the energy pool that probably occurs at the level of the glycolysis pathway, and trehalose, glycogen, glycerol metabolism and ATPase regulation. A major task now is to elucidate the mechanism by which these changes contribute to ethanol adaptation. However, as discussed above the 'management' of the energy pool is probably a priority under stress conditions, which might explain the existence of complex processes to fulfil this requirement.

It must be emphasised, however, that a large number of the changes in transcript abundance observed after 30 min ethanol shock are probably transient. Indeed, Gasch et al. [9] demonstrated that apart from starvation all other challenging conditions tested lead to a transient response before a steady state transcript level is reached.

Thus, it is noteworthy that we did not detect the induction of any genes involved in lipid metabolism or cell wall biogenesis, which are known targets of the ethanol stress re-

sponse. It is likely that these changes constitute long-term adaptation processes.

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