RESEARCH ARTICLE

Proteomic evolution of a wine yeast during the first hours of fermentation

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Abstract

The inoculation of active dry wine yeast (ADWY) is one of the most common practices in winemaking. This inoculation exposes the yeast cells to strong osmotic, acidic and thermal stresses, and adaptation to the new medium is crucial for successful fermentation. We have analysed the changes that occur in the ADWY protein profile in the first hours after inoculation under enological-like conditions at a low temperature. Protein changes mainly included enzymes of the nitrogen and carbon metabolism and proteins related to the cellular stress response. Most of the enzymes of the lower part of the glycolysis showed an increase in their concentration 4 and 24 h after inoculation, indicating an increase in glycolytic flux and in ATP production. However, the shift from respiration to fermentation was not immediate in the inoculation because some mitochondrial proteins involved in oxidative metabolism were induced in the first hours after inoculation. Inoculation in this fresh medium also reduced the cellular concentration of stress proteins produced during industrial production of the ADWY. The only exception was Cys3p, which might be involved in glutathione synthesis as a response to oxidative stress. A better understanding of the yeast stress response to rehydration and inoculation will lead to improvements in the handling efficiency of ADWY in winemaking and presumably to better control of fermentation startup.

Introduction

The use of active dry wine yeast (ADWY) is a widespread practice in wine technology. It replaces spontaneous fermentations in order to obtain more reproducible wines by better control of alcoholic fermentation (Ribereau-Gayon *et al.*, 2000). ADWY is obtained from selected natural wine yeast, which is propagated in molasses and then desiccated. The molecular response of yeast to propagation and desiccation has been studied (Perez-Torrado *et al.*, 2005; Singh *et al.*, 2005), and it is well established that this desiccation process causes a water deficit that leads to the arrest of cellular functions. A rehydration period is, therefore, required for these cellular functions to resume and for the membrane functionality to recover fully (Boulton *et al.*, 1995). The current process is to rehydrate the dried yeast by incubating it in water at 37 °C for a short period of time, followed by seeding the rehydrated cells into large fermentation tanks. One of the main characteristics of optimal wine fermentation is the set of physiological and metabolic changes that occur immediately on inoculating yeast cells into the musts (Bauer & Pretorius, 2000).

When ADWY is inoculated into the must, the yeast cells are subjected to different stress situations as a consequence of the high sugar concentration or the low pH of the medium. Moreover, some oenological practices increase these stressful conditions for ADWY. This is the case with low-temperature fermentations. Fermentation at low temperatures improves taste by restructuring flavour profiles with potential oenological applications (Feuillat *et al.*, 1997; Charoenchai *et al.*, 1998; Torija *et al.*, 2003). However, lowering the fermentation temperature to 13 °C or even lower has some disadvantages, including increasing the length of the process and the risk of stuck or sluggish fermentations (Meurgues, 1996; Bisson, 1999). Thus, although low-temperature fermentation can be useful for

the enological industry, it also has an adverse effect on cell growth, increasing yeast stress during wine production.

Successful ADWY adaptation involves a metabolic reorganization in order to maintain cell activity (vitality). There remains much we do not know about many aspects of this molecular response to early adaptation to musts. These mechanisms could be analysed by the whole array of 'omics' currently available. In our group, we used DNA microarrays to analyse the transcriptional response of wine yeast after rehydration and inoculation in different media (Novo *et al.*, 2007). The main responses after inoculation in a fermentable medium were the activation of some genes of the fermentation pathway and of the nonoxidative branch of the pentose pathway, and the induction of a huge cluster of genes related to ribosomal biogenesis and protein synthesis. In a similar study, Rossignol *et al.* (2006) analysed the transcriptomic changes of wine yeast throughout different points of the lag phase, detecting a substantial transcriptional remodelling during this period. In addition to these global transcriptional analyses, other studies have addressed the protein modifications during the lag phase and growth initiation in *Saccharomyces cerevisiae* (Brejning & Jespersen, 2002; Brejning *et al.*, 2005). The changes that occur during the lag phase are characterized by an overall change in protein synthesis and reflect the physiological condition of the yeast, which affects its fermentative capacity and fermentation performance (Quain, 1988; Blomberg, 1997).

In this study, we have focused on the changes that occur in the ADWY protein profile after inoculation in a synthetic grape must. These changes should reflect the yeast cells' adaptation to the new medium. This adaptation is especially dramatic in fermentations at a low temperature when yeasts have longer lag phases, thus increasing the length of fermentation and the risk of growing nondesirable microorganisms.

Materials and methods

Yeast strain and growth conditions

The commercial wine yeast *S. cerevisiae* QA23 (Lallemand SA, Canada) was cultivated in a synthetic must (SM) prepared according to Riou *et al.* (1997), but with 200 g L^{-1} of reduced sugars (100 g L^{-1} glucose and 100 g L^{-1} fructose) and without anaerobic factors. The yeast assimilable nitrogen (YAN) content was 300 mg N L^{-1}: ammoniacal nitrogen (NH_4Cl) 120 mg N L^{-1} and amino acids 180 mg N L^{-1}. This ADWY was rehydrated in water using the manufacturer's recommendations (30 min at 37 °C). After counting under the microscope, the appropriate dilution of the rehydrated QA23 was transferred to SM to obtain an initial cell concentration of 2×10^6 cells mL^{-1}. The cell suspension was incubated at 13 °C with a slight agitation to obtain a homogeneous nutrient distribution in laboratory-scale fermenters: 2-L bottles filled with 1.8 L medium and fitted with closures that enabled the carbon dioxide to escape and the samples to be removed. Samples were collected at time 0 (rehydrated yeast) and after 1, 4 and 24 h. The number of viable cells was monitored by plating in yeast peptone dextrose medium.

Protein extraction and two-dimensional electrophoresis (2DE)

Protein extracts were prepared as described in Blomberg (2002). Briefly, this was as follows: cell suspension was vortexed for 4×30 s with glass beads containing phenylmethylsulphonyl fluoride as a protease inhibitor (with placement on ice between vortexing) and subsequently boiled for 5 min with sodium dodecyl sulphate (SDS)/mercaptoethanol buffer. Following nuclease treatment of the cells, protein contents of the extract were estimated using a 2-D quant kit (Amersham Pharmacia Biotech). Soluble proteins were run in the first dimension using a commercial horizontal electrophoresis system (Multiphor II; Amersham Pharmacia Biotech). Forty-five micrograms of protein from whole-cell lysates were mixed with immobilized polyacrylamide gel (IPG) rehydration buffer (8 M urea/2% NP-40/10 mM dithiothreitol; final volume of 500 μL) and loaded onto polyacrylamide strips. IPG strips with a nonlinear pH gradient 3–10 were allowed to rehydrate overnight. Samples were run at 50 μA per strip, in the first step, voltage was ramped to 500 V over a period of 5 h, maintained at 500 V for 5 h more, ramped again to 3500 V over a period of 9.5 h and finally maintained at 3500 V for 5 h. After the first dimension, IPG strips were then equilibrated twice for 15 min in equilibration solution (0.05 M Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, and 2% w/v SDS), first with 65 mM dithiothreitol (reduction step) and finally with 135 mM iodoacetamide (alkylation). The second dimension was carried out in a vertical electrophoresis system (Ettan DALTsix; Amersham Pharmacia Biotech) in a 12.5% (26 cm × 20 cm × 1 mm) polyacrylamide gel where proteins were separated based on molecular size. Electrophoresis conditions were 1 W per gel until the dye front reached the bottom of the gel. Sets of three gels were used for each sampling time.

Silver staining protocol and image analysis

The staining protocol was performed as described by Blomberg (2002). Gels were scanned using an Image Scanner UMAX, Amersham (300 dpi, 12-bit image), that allowed us to obtain spot intensities in pixel units. The images were analyzed using PDQUEST software (Bio-Rad). Normalization was performed by the mentioned software based in the total in gel density to compensate image differences caused by

variations in experimental conditions (e.g. protein loading or staining). Spot detection was implemented using PDQUEST automated spot detection algorithm. The gel image showing the highest number of spots and the best protein pattern was chosen as a reference template of the image analysis, and spots in the standard gel were then matched across all gels. 2DE gels were divided into four different groups corresponding to the already described sampling points: rehydrated yeast and 1, 4 and 24 h after inoculation in the media. Matching features of the software were used to relate and compare the sets of gels. Finally, to achieve maximum reliability and robustness of the results, Student's *t*-test was performed allowing us to identify those sets of proteins that showed a statistically significant difference with a confidence level set at 95%.

In-gel digestion of proteins

Protein spots were excised manually and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany) according to Schevchenko *et al.* (1996). Minor variations of the method were introduced. Gel plugs were reduced with 10 mM dithiothreitol (Amersham Biosciences, Uppsala, Sweden) in 50 mM ammonium bicarbonate (99.5% purity; Sigma, St Louis, MO) and alkylated with 55 mM iodoacetamide (Sigma Chemical) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison, WI) at a final concentration of 13 ng μL^{-1} in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion proceeded at 37 °C for 6 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Sigma Chemical) was added for peptide extraction.

Matrix-assisted laser desorption ionization (MALDI)-MS(/MS) and database searching

An aliquot of the above digestion solution was mixed with an aliquot of α-cyano-4-hydroxycinnamic acid (Bruker-Daltonics) in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid. This mixture was deposited onto a 600 μm AnchorChip MALDI probe (Bruker-Daltonics) and allowed to dry at room temperature. MALDI-MS(/MS) data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonics) equipped with a LIFT-MS/MS device (Suckau *et al.*, 2003). Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100–1500 individual spectra were averaged. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Ionized fragments generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analysed after passing the ion reflector. Measurements were in part performed using post-LIFT metastable suppression, which allowed the removal of the precursor and metastable ion signals produced after extraction from the second ion source. Peptide mass mapping data were analysed in detail using FLEXANALYSIS software (Bruker-Daltonics). MALDI-TOF mass spectra were calibrated internally using two trypsin autolysis ions with $m/z = 842.510$ and $m/z = 2211.105$; MALDI-MS/MS, spectra were calibrated with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800–3200 m/z^1 region. MALDI-MS and MS/MS data were combined through the MS BIOTOOLS program (Bruker-Daltonics) to search the NCBInr database using MASCOT software (Matrix Science, London, UK) (Perkins *et al.*, 1999).

Results

Experimental design

Commercial ADWY *S. cerevisiae* strain QA23 was used to ferment at a low temperature (13 °C) for 24 h. We monitored the cell population during this time (Fig. 1). No growth was detected in the two first sampling times (1 and 4 h) and the population had barely duplicated after 24 h. Therefore, we considered that the cells were found in the lag phase in the 1- and 4-h samples and in the early exponential phase in the 24-h sample. The total cell protein profile was analysed at these times of fermentation. Rehydrated cells (time 0) were used as the control for this proteomic study.

A total of 336 spots were detected on 2-D gel in rehydrated cells (control) (Table 1). After 1 h in SM, 61% of detected proteins were matched to proteins on the control gel, whereas for the samples taken at 4 and 24 h after inoculation, the total matches were 68% and 52%, respectively (Table 1). Qualitative and quantitative differences were detected between matched spots from the corresponding gels of the different collection points. We

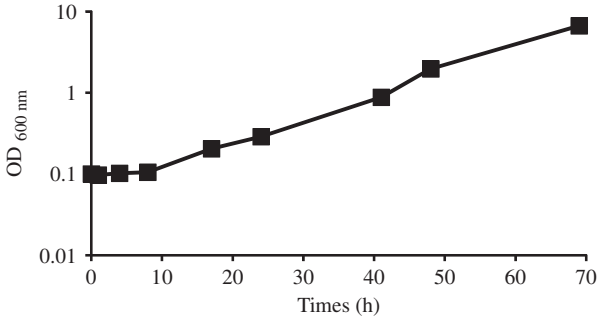

Fig. 1. Evolution of population (measured as $OD_{600\,nm}$) of QA23 wine yeast strain during the firsts hours of fermentation in SM.

Table 1. Number of modified proteins on 2-D gels during first 24-h of SM fermentation

		Number of spots Time after inoculation		
		1 h	4 h	24 h
	Reference gel	13 °C	13 °C	13 °C
Detected	336	357	252	380
Matched with reference gel	–	204 (61)	228 (68)	197 (52)
Statistically significant*	–	69 (33)	66 (29)	77 (39)
At least twofold induced	–	1 (0.4)	13 (5.9)	2 (0.1)
At least twofold repressed	–	18 (8.8)	1 (0.4)	11 (5.6)

Percentage values are given in parentheses. Confidence level was set at 95%.
*Number of matched spots that show significant differences with 95% of confidence in Student's *t*-test.

Table 2. Proteins whose concentration increased (positive numbers) or decreased (negative numbers) at least twofold after 1 h of yeast inoculation

Gene name	Protein name	Metabolic function	Cellular location	Fold change
CPR3	Mitochondrial peptidyl-prolyl *cis–trans* isomerase	Protein folding	Cytoplasm, mitochondrion	−2.3
DLD1	D-Lactate dehydrogenase	Carbohydrate metabolism	Cytoplasm, mitochondrion, membrane, mitochondrial envelope	−3.5
ENO1	Enolase I, phosphopyruvate hydratase	Glycolysis	Cytoplasm, mitochondrion, vacuole	−2.2
ENO2	Enolase II, phosphopyruvate hydratase	Glycolyis	Cytoplasm, mitochondrion, vacuole	−6.3
FBA1	Fructose-1,6-bisphosphate aldolase	Glycolyis	Cytoplasm, mitochondrion	−2.7
GDH1	Glutamate dehydrogenase	Amino acid biosynthesis	Cytoplasm, nucleus	−2.9
GPD1	Glycerol-3-phosphate dehydrogenase	Response to osmotic stress	Cytoplasm, peroxisome	ND
HSP12	Heat shock protein 12	Stress response	Cytoplasm, membrane, nucleolus, plasma membrane	−2.3
HSP60	Heat shock protein 60	Protein refolding after heat shock	Cytoplasm, mitochondrion	−3.6
LEU2	β-Isopropylmalate dehydrogenase	Amino acid biosynthesis	Other	−4.0
LYS9	Saccharopine dehydrogenase	Amino acid biosynthesis	Cytoplasm	ND
MET6	Cobalamin-independent methionine synthase	Amino acid biosynthesis	Cytoplasm	−4.8
PDC1	Pyruvate decarboxylase	Glycolyis, glucose fermentation	Cytoplasm, nucleus	−3.7
PDI1	Protein disulfide isomerase	Protein folding	Cytoplasm, endoplasmic reticulum	−2.4
PGK1	3-Phosphoglycerate kinase	Glycolyis	Cytoplasm, mitochondrion	−2.6
TDH3	Glyceraldehyde-3-phosphate dehydrogenase	Glycolyis	Cytoplasm, mitochondrion and cell wall	3.8
TPS1	Trehalose-6-phosphate synthase	Stress response	Cytoplasm	−5.6
TSA1	Thioredoxin peroxidase	Oxidative stress	Cytoplasm	−2.1

ND, not detected at 1 h.

decided to perform an in-depth analysis of these proteins as their concentrations were modified at least twofold (Table 1).

Changes in protein profile during the lag phase

The concentration of some yeast proteins statistically increased or decreased twofold during the lag phase (1- and 4-h samples) as shown in Tables 2 and 3. The concentration of only one protein increased 1 h after inoculation while that of 18 proteins decreased (8.8% of matched proteins) in the same period. Four hours after the inoculation, 13 proteins (5.9%) increased at least twofold and just one protein was detected to decrease significantly in comparison with the rehydrated yeast. Most of the proteins that were modified were involved in glycolysis and glucose fermentation. However, the changes were dependent on the time of fermentation. Thus, most of these proteins were present at lower concentrations after 1 h (i.e. Fba1p, Pgk1p, Eno1p, Eno2p, and the key fermentative enzyme Pdc1p) and at higher

Table 3. Proteins whose concentration increased (positive numbers) or decreased (negative numbers) at least twofold after 4 h of yeast inoculation

Gene name	Protein name	Metabolic function	Cellular location	Fold change
ADH1	Alcohol dehydrogenase	Glycolyis, glucose fermentation	Cytoplasm	3.4
ATP1	α-Subunit of mitochondrial ATP synthase	Mitochondrial transport and homeostasis	Cytoplasm, mitochondrion, membrane, mitochondrial envelope	2.2
ATP2	β-Subunit of mitochondrial ATP synthase	Mitochondrial transport and homeostasis	Cytoplasm, mitochondrion, membrane, mitochondrial envelope	2.2
CYS3	Cystathionine γ-lyase	Amino acid biosynthesis	Cytoplasm	2
ENO1	Enolase I, phosphopyruvate hydratase	Glycolyis	Cytoplasm, mitochondrion, vacuole	2.1
FBA1	Fructose 1,6-bisphosphate aldolase	Glycolyis	Cytoplasm, mitochondrion	2.8
GPM1	Phosphoglycerate mutase	Glycolyis	Cytoplasm, mitochondrion	2.5
MDH1	Mitochondrial malate dehydrogenase	Tricarboxylic acid (TCA) cycle	Cytoplasm, mitochondrion	2
PGK1	3-Phosphoglycerate kinase	Glycolyis	Cytoplasm, mitochondrion	2.6
POR1	Mitochondrial porin	Mitochondrial homeostasis	Cytoplasm, mitochondrion, membrane, mitochondrial envelope, golgi apparatus, endomembrane system	5.2
RPS0B	40S Ribosomal protein	Protein synthesis	Cytoplasm, ribosome	5.6
TDH1	Glyceraldehyde-3-phosphate dehydrogenase	Glycolyis	Cytoplasm, mitochondrion, cell wall	> 10
YHB1	Nitric oxide reductase	Oxidative and nitrosative stress	Cytoplasm, mitochondrion	−2.6

concentrations 4 h after inoculation than they were in the rehydrated cells. Tdh3p and Adh1p increased at 1 and 4 h, respectively. All these proteins, with the exception of Fba1p, represent the enzymes of the lower part of glycolysis and glucose fermentation.

Other enzymes related to the carbohydrate metabolism were the mitochondrial malate dehydrogenase (Mdh1p), whose concentration increased after 4 h, and the D-lactate dehydrogenase (Dld1p), which decreased after 1 h. Mdh1p catalyses interconversion of malate and oxaloacetate in the tricarboxylic acid (TCA) cycle and Dld1p, which oxidizes lactate to pyruvate, is repressed by glucose and is derepressed in ethanol or lactate (Lodi *et al.*, 1999).

Four hours after the inoculation, a ribosomal protein (Rps0bp), three mitochondrial proteins (Atp1p, Atp2p and Por1p) and a protein involved in the synthesis of cysteine (Cys3p) were also induced.

Among the proteins whose concentrations reduced, we detected an important set related to osmotic (Gpd1p), oxidative (Sod1p, Tsa1p and Yhb1p) and general (Tps1p, Hsp12p and Hsp60p) stresses. The remaining proteins with reduced concentrations are involved in protein folding (Hsp60p, Pdi1p and Cpr3p) and in amino acid biosynthesis (Gdh1p, Leu2p, Lys9p and Met6p). In many cases, the degradation mechanism could be involved in the decrease of these proteins. We detected fragments of different sizes belonging to Sod1p in the 1-h sample (data not shown). This degradation was confirmed by a lower concentration of this protein 24 h after inoculation.

Changes in protein profile during the early-exponential phase

Cell population almost duplicated 24 h after inoculation in the SM. Therefore, the cells were at the beginning of the exponential or proliferating phase and the changes in protein concentration should reflect the adaptation of the cellular machinery to biomass production. However, the number of proteins whose amount changed in comparison with those of the rehydrated cells was not very significant. Only two and 11 proteins were found at higher and lower concentrations, respectively. The two increased proteins (Fba1p, Eno1p) (Table 4) are key enzymes of the glycolytic pathway. We also detected a glycolytic protein (Tdh3p) with reduced concentration. Other proteins with decreased concentration were involved in stress response (Hsp12p, Sod1p and Ald3p), amino acid biosynthesis (Gdh1p, Ald3p and Lpd1) and carbohydrate metabolism (Dld1p, Gor1p and Lpd1p). Some proteins can be catalogued into more than one functional category. This is the case with Ald3p, which is induced by stress, repressed by glucose and involved in alanine synthesis (Navarro-Aviño *et al.*, 1999; White *et al.*, 2003), or Lpd1p, whose mutation stops the activity of the glycine decarboxylase, 2-oxoglutarate dehydrogenase and

Table 4. Proteins whose concentration was modified at least twofold during the early exponential phase

Gene name	Protein name	Metabolic function	Cellular location	Fold change
Proteins found in higher concentration				
ENO1	Enolase I, phosphopyruvate hydratase	Glycolyis	Cytoplasm, mitochondrion, vacuole	2.5
FBA1	Fructose-1,6-bisphosphate aldolase	Glycolysis	Cytoplasm, mitochondrion	4.8
Proteins found in lower concentration				
ALD3	Aldehyde dehydrogenase	Amino acid biosynthesis	Cytoplasm	ND
DLD1	D-Lactate dehydrogenase	Carbohydrate metabolism	Cytoplasm, mitochondrion, membrane, mitochondrial envelope	4.5
GDH1	Glutamate dehydrogenase	Amino acid biosynthesis	Cytoplasm, nucleus	ND
HSP12	Heat shock protein 12	Stress response	Cytoplasm, membrane, nucleolus, plasma membrane	6.7
LPD1	Dihydrolipoamide dehydrogenase	Amino acid biosynthesis	Cytoplasm, mitochondrion	3.2
MRP8	Mitochondrial ribosomal protein	Translation	Cytoplasm, mitochondrion, ribosome	2.7
PIB2	Proteinase B inhibitor	Proteolysis regulation	Cytoplasm, mitochondrion	3
SOD1	Superoxide dismutase	Oxidative stress	Cytoplasm, mitochondrion, mitochondrial envelope	2.1
TDH3	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	Cytoplasm, mitochondrion, cell wall	2.4
GOR1	Putative hydroxyisocaproate dehydrogenase	Oxidoreductase activity	Cytoplasm, mitochondrion, nucleus	2.4

ND, Not detected at 24 h.

pyruvate dehydrogenase complexes (Sinclair & Dawes, 1995; Dickinson *et al.*, 1997; Zaman *et al.*, 1999). Gor1p is a glyoxylate reductase that reversibly reduces glyoxylate to glycolate (Rintala *et al.*, 2007). An important trend of most of these proteins with decreased concentration was that seven out of 11 proteins were mainly located in the mitochondria.

Discussion

The use of ADWY is a widespread practice in wine technology. These ADWY are, after a rehydration process in water at 37 °C, inoculated in large fermentation tanks with grape must. This inoculation exposes the yeast cells to strong osmotic, acidic and thermal stresses (especially in refrigerated musts), and the adaptation to the new medium is crucial for successful fermentation. Our aim in this study was to monitor the proteome changes of an *S. cerevisiae* commercial wine yeast during the first hours of fermentation at a low temperature. We were interested in both the cellular response to inoculation in a fermentable growth medium and the cold adaptation of the yeast cells. Different DNA microarray analyses have dealt with the evolution of the transcriptome of a wine yeast under these conditions (Rossignol *et al.*, 2006; Novo *et al.*, 2007). The genome-wide transcriptional changes during low-temperature wine fermentations have also been analyzed recently (Beltran *et al.*, 2006). The main relevant differences comparing global transcriptome and proteome analysis is that proteomic changes are much more limited than transcriptomic ones. Thus, 1874 genes changed their expression more than threefold after inoculation in a synthetic grape must (Rossignol *et al.*, 2006) and 535 ORFs were differentially expressed as a consequence of the temperature of fermentation (Beltran *et al.*, 2006), in comparison with fewer than 20 proteins with modified concentrations detected at each time point in this study.

Protein changes during the first hour reflected a degradation or modifications in proteins present in the rehydrated yeasts. Our results indicated that the initial stages after rehydration may involve critical changes in yeast metabolism, and one of these may be the active degradation of useless protein and mRNAs (Novo *et al.*, 2007). On the contrary, most proteins increased their concentrations 4 h after inoculation. These protein changes may reflect transcriptional reprogramming after yeast inoculation in a new fermentable medium.

Proteins involved in stress response

An increase in proteins related to a stress response may be expected after yeast inoculation. The high sugar concentration, low pH and low temperature may subject the cells to various stressful situations. However, our results showed a decrease in proteins related to stress during the time points studied (especially 1 h after inoculation). In this case, the decrease in proteins related to stress matched with the downregulation of most of the genes involved in stress response after yeast inoculation (Rossignol *et al.*, 2006). We should keep in mind that the ADWY have been already subjected to highly stressful conditions during their industrial production (Perez-Torrado *et al.*, 2005). Inoculation in a fresh fermentation medium seems to alleviate the stress exerted during biomass propagation and dehydration.

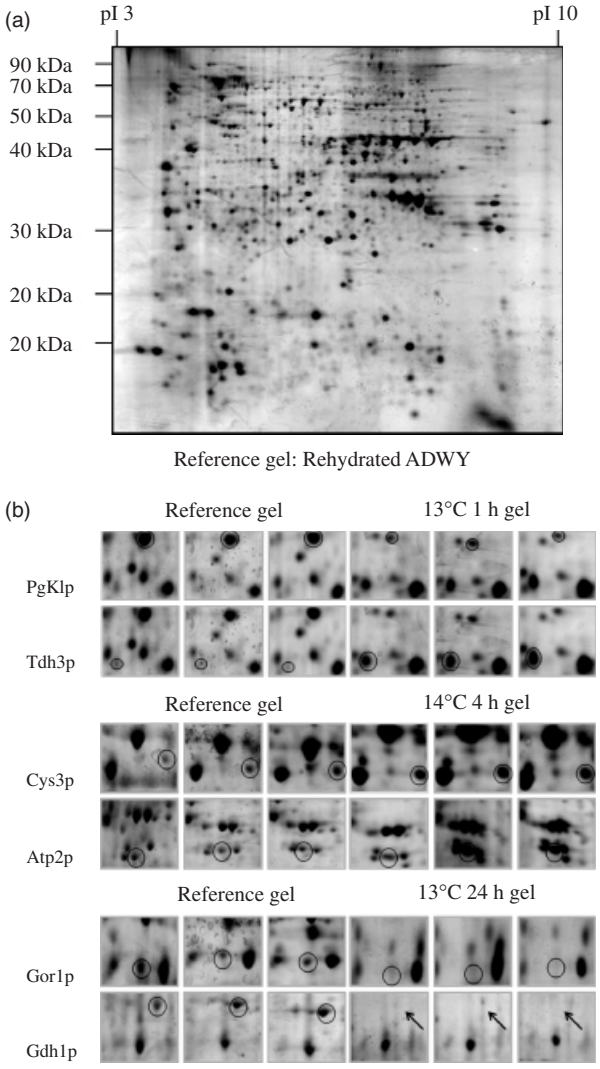

Fig. 2. (a) Reference gel corresponding to ADWY after rehydrated on water 30 min at 37 °C. (b) Evolution of some of the most representative proteins at the different time-points studied.

Schade *et al.* (2004) defined two groups of genes that are transcriptionally activated by cold: the early cold response (ECR) genes (time < 2 h and independent of the transcriptional activator Msn2p/Msn4p) and the late cold response (LCR) genes (time > 12 h and dependent on the transcriptional activator Msn2p/Msn4p). ECR genes were made up of genes implicated in RNA metabolism and lipid metabolism whereas LCR genes included metabolic and stress genes. In contrast to expectations, we did not detect significant differences in the proteins encoded by ECR genes and, as mentioned above, stress proteins, included in the LCR genes, decreased their cellular concentration

Tps1p is a typical stress protein, that is encoded by the Msn2p/Msn4p regulated gene *TPS1* and involved in trehalose synthesis. This disaccharide plays an important role as a cell protector and it is found at high levels in ADWY because the industrial conditions of dry cell production cause it to be synthesized. We previously reported a decline in intracellular trehalose in the same wine strain 1 h after inoculation (Novo *et al.*, 2003). In this instance, the decrease in this metabolite was matched by a decrease in the enzyme responsible for its synthesis.

It was surprising that Gpd1p, a key enzyme in the osmotic stress response, decreased 1 h after inoculation without any further modification being detected. Two issues must be considered. The first one is the high levels of Gpd1p expected in ADWY due to drying conditions (Perez-Torrado *et al.*, 2005). These can mask the response to the high osmotic pressure in the growth media (Rossignol *et al.*, 2006). The other issue could be again the influence of the rehydration media. Low osmotic pressure supported by the yeast during rehydration process could lead the cell to repress the glycerol catabolic pathway.

The only protein with a higher concentration that could be involved in oxidative stress response is the cystathione-γ-lyase Cys3p. This enzyme is responsible for the novo synthesis of cysteine in yeasts. Matiach & Schröder-Köhne (2001) reported that the *cys3* mutant grew perfectly on rich medium at 25 °C but was unable to grow on the same medium at 37 °C. They provided enough evidence to indicate that this heat-sensitive phenotype was due to oxidative stress rather than simple starvation of cysteine as an amino acid for protein biosynthesis. Cysteine is a constituent of the tripeptide glutathione, the most abundant antioxidative compound in yeast. The *cys3* mutant is depleted significantly in this antioxidant. However, the level of oxidative stress was not increased by a high temperature alone. A rapid downshift in the growth temperature of *S. cerevisiae* from 30 to 10 °C resulted in an increase in transcript levels of the antioxidant genes *SOD1*, *CTT1* and *GSH1* (Zhang *et al.*, 2003). Although this hypothesis should be confirmed by trials carried out under the same condition but at a higher temperature, the increase of Cys3p may fit with this antioxidant response as a consequence of the low growth temperature.

Protein of glycolysis and carbohydrate metabolism

We did not expect most of the glycolytic proteins to decrease after inoculation in a glucose-rich SM. However, Rossignol *et al.* (2006) had already reported a downregulation of most of these genes 1 h after inoculation. These authors explained this repression as a general phenomenon during the lag phase in response to new available nutrients. Conversely, most of these proteins increased their concentration 4 and 24 h after inoculation. Most of these enzymes participate in the lower part of the glycolysis, the part of the pathway that

leads to ATP generation. Peter-Smits *et al.* (2000) enhanced the glycolytic flux and the fermentative capacity of *S. cerevisiae* through the simultaneous overexpression of these enzymes. The huge amount of sugars in the SM might produce a high ATP demand for sugar phosphorylation (Rizzi *et al.*, 1997). The increase of enzymes from the lower part of glycolyis may balance the drain of ATP. In this strategy, to pull the flux through the ATP production stage, a key protein is the glyceraldehyde-3-phosphate dehydrogenase Tdh3p. This enzyme diverts the glycolytic flux to pyruvate production vs. the dihydroxyacetone and glycerol production. Tdh3p is the first glycolytic protein increasing (after 1 h) and decreasing (after 24 h) its concentration. Moreover, this increase is matched by a decrease in the glycerol-3-phosphate dehydrogenase Gpd1p, a key enzyme in glycerol synthesis. Therefore, the cellular increase in Tdh3p being concomitant with a decrease in Gpd1p may be considered to be a mechanism that activates the glycolytic flux.

Proteins of nitrogen metabolism

All the proteins related to nitrogen metabolism were reduced at the time-points studied, with the exception of Cys3p. In agreement, Rossignol *et al.* (2006) also detected a repression of the genes involved in nitrogen utilization after inoculation in fermentation medium. SM is rich in ammonium and amino acids at the beginning of the process. It is known that a rich-nitrogen medium triggers a rapid catabolite repression of the main genes involved in amino acid biosynthesis (Beltran *et al.*, 2004) and this could lead to a reduction in the proteins encoded by these genes.

As mentioned above, the cellular demand of Cys3p may be more closely related to a response to oxidative stress resistance than to protein synthesis. The decrease in Met6p may also be related to the cell's need to synthesize glutathione because both Cys3p and Met6p use homocysteine as a substrate for synthesizing cysteine (glutathione) and methionine, respectively.

Mitochondrial proteins

ADWY shifts from a respiratory metabolism during its industrial propagation to a fermentative metabolism during wine fermentation. The inoculation of *S. cerevisiae* cells in a medium with a high sugar concentration triggers the Crabtree effect, which means that respirative metabolism is blocked in favour of the fermentative metabolism. The downregulation of many genes involved in oxidative metabolism, respiration, TCA cycle, glyoxylate cycle and neoglucogenesis is a typical regulation of the Crabtree effect. However, several studies have proved that the Crabtree effect is not immediate and that yeast cells have a period of respirofermentative metabolism (Postma *et al.*, 1989; Van Urk *et al.*, 1990). In our proteome analysis, we detected several proteins that produce energy through oxidative phosphorylation (Atp1p, Atp2p), mitochondrial homeostasis (Por1p) and TCA cycle (Mdh1p) at higher concentrations after 4 h. On the other hand, most of the mitochondrial proteins that showed significant differences 24 h after inoculation were present at lower concentrations, suggesting that the change to fermentative metabolism has been completed. A clear indicator of the shift from respiratory to fermentative metabolism is the decrease in the protein Lpd1p. This protein is a component of the pyruvate dehydrogenase complex, the key enzyme in respiratory metabolism.

In conclusion, a set of proteins involved in the adaptive response of a wine *S. cerevisiae* strain to a new fermentative medium has been identified. These proteins mainly included enzymes of the nitrogen and carbon metabolism and proteins related to the cellular stress response. The ADWY inoculation in an SM produced a metabolic change in the cell. However, the shift from respiration to fermentation was not immediate in the inoculation because some mitochondrial proteins involved in oxidative metabolism were induced in the 1-h sample. Inoculation in this fresh medium also reduced the cellular concentration of stress proteins produced during industrial production of the ADWY. The only exception was Cys3p, which might be involved in glutathione synthesis as a response to oxidative stress. Further studies should confirm the need for glutathione synthesis during the lag phase of the alcoholic fermentation. In contrast to our expectations, we did not detect growth-related proteins during the early exponential phase. A decrease in some enzymes related to amino acid biosynthesis was detected in the three samples analysed. Further transcriptional analysis should confirm the catabolite repression of the genes encoding these proteins and also determine their relation to the protein decrease. A better understanding of the yeast stress response to rehydration and inoculation will lead to improved ADWY handling efficiency in winemaking and presumably better control of fermentation startup.

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