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ORIGINAL PAPER

Identification of genes related to nitrogen uptake in wine strains of *Saccharomyces cerevisiae*

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Abstract The yeast *Saccharomyces cerevisiae* is the main microorganism responsible for wine fermentation and its development influences the quality of wine. A problem affecting these types of fermentations, generating important losses in this industry, are the slow or stuck fermentations which may result from low nitrogen availability in the must. Therefore, several studies have been directed towards identifying genes involved in nitrogen metabolism using high throughput strategies which include subjecting the yeast to changes in the type or concentration of the available nitrogen source. However, this type of approach can also generate responses in the yeast that do not necessarily alter the expression of genes related to nitrogen metabolism. In this work, by using intraspecific hybridisation of wild wine yeast strains we obtained genetically and oenologically similar strains with differences in the consumption of nitrogen sources. Using the same must, the global expression patterns of these yeasts were compared by microarrays, the analysis of which identified 276 genes that varied in their expression between the strains analysed. The functional analysis of the genes with a known function indicates that some participate in nitrogen metabolism, alcoholic fermentation, ion transport and transcriptional regulation. Furthermore, differences were observed in the expression of genes which have been partially associated to nitrogen, as in the case of *ZRT1* and *ATO2*. Interestingly, many of the genes identified have no known function or have not been previously associated to this phenotype.

Keywords Fermentation · Nitrogen uptake · Transcription analyses · Wine · Yeast

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Introduction

The yeast *Saccharomyces cerevisiae* is responsible for alcoholic fermentation and for many of the organoleptic characteristics that the must acquires during its transformation into wine (Ribereau-Gayon et al. 2000). During this process, the yeast is subjected to a medium containing excess carbon sources but a limited amount of assimilable nitrogen (Boulton et al. 1996). Variations in the availability of nitrogen in the must have been indicated as one of the causes of slow or stuck wine fermentations (Bely et al. 1990; Jiranek et al. 1995; Pretorius 2000), leading to important economic losses for this industry at a global scale.

The wine yeast strains have differing nitrogen requirements to complete the fermentation (Jiranek et al. 1995) and these different phenotypes would result from the high genetic polymorphism that exists between them as documented by various molecular techniques (Fernández-Espinar et al. 2003; Fernández-Espinar et al. 2001) and by genome sequencing of various strains (Liti et al. 2009).

This would result from mutations that have occurred during their evolution as well as from chromosomal reorganisations that generate polyploidy or aneuploidy and which affect the copy number and/or expression of genes involved in this phenotype (Bakalinsky and Snow 1990; Dunn et al. 2005).

Several research works (Jimenez-Marti and del Olmo 2008; Marks et al. 2003; Mendes-Ferreira et al. 2007; Rossignol et al. 2003) have focused on the study of the wine yeast transcriptome as a means of answering the variations occurring in complex traits such as the fermentative characteristics (Marullo et al. 2004). Global expression studies have identified changes in gene expression produced during alcoholic fermentation indicating that around 2,000 genes alter their expression (Rossignol et al. 2003). Many of these genes are related to stress, given that the yeast is exposed to extreme conditions such as high osmotic pressure, high ethanol and sugar concentrations and also changes in the culture conditions. Within this context Jimenez-Marti and del Olmo (2008), identified the changes in the expression of genes when the yeast was exposed to nitrogen starvation and then supplied with ammonium or amino acids. The addition of ammonium promotes the expression of genes involved in the biosynthesis of amino acids was reported (Marks et al. 2003). The addition of amino acids produced an overexpression of genes involved in the transport of amino acids and ribosomal biogenesis (Pizarro et al. 2008). On the other hand, Mendes-Ferreira et al. (2007) analysed the changes in gene expression of a yeast following exposure to limiting nitrogen conditions and to 72 h of supplementing the must with nitrogen, showing that many cellular processes such as carbohydrate metabolism, oxidative stress and protein synthesis are affected during fermentation and that some of these variations respond to nutritional stress caused by the lack of assimilable nitrogen.

These studies expose the yeast to different culture conditions that may trigger stress responses that would hinder the detection of genes directly related to the phenotype under study. To avoid this issue, in this work we cultured wine yeast with similar oenological phenotypes and genotypes but differing capacities to consume nitrogen sources under the same culture conditions. These strains were obtained from an hybrid strain by sporulation and are therefore genetically related. This strategy was performed to identify genes involved in nitrogen metabolism by comparing their expression profiles. Hence, genes already related with this phenotype as well as other genes not previously associated to this process and which could be involved in nitrogen metabolism in wine strains were identified.

Materials and methods

Strains, media and growth conditions

The yeasts used in this study were the wine strains L3217 and L3218 both collected in Chile and strains L3145 and L3147 obtained from the native strains by sporulation, and which correspond to the parental strains in this study. Furthermore, we used the commercial strain EC1118 (Lalvin®) as control for the evaluation of fermentation parameters.

For fermentation studies, 1×10^6 cells obtained from a 16 h culture with shaking in synthetic must were inoculated in 10 ml of the same must in 15 ml tubes. They were maintained at 28°C for 20 days without shaking. The synthetic must is composed of tartaric acid 5 g/l, malic acid 5 g/l, calcium chloride (dihydrate) 0.3 g/l, magnesium sulfate 1.3 g/l, fructose 100 g/l, sucrose 5 g/l, glucose 100 g/l, potassium hydroxide 2.5 g/l and vitamin solution 2 ml/l. The must was autoclaved for 21 min at 15 psi and the vitamin solution added. The vitamin solution contains the following: thiamine 1.152 g/l, biotin 4.8×10^{-3} g/l, nicotinic acid 2.3 g/l, pyridoxine hydrochloride 0.23 g/l, calcium pantoneate 1.152 g/l and sulfuric acid 0.25 mol/l. This must was supplemented with 518 mg/l of total nitrogen obtained from 1,600 mg/l of $(NH4)_2HPO_4$, which equates to 339 mg/l of assimilable nitrogen and a mixture of 9 amino acids: L-arginine 300 mg/l, L-serine 100 mg/l, L-threonine 100 mg/l, L-glutamic acid 100 mg/l, L-aspartic acid 100 mg/l, L-lysine 100 mg/l, L-asparagine 50 mg/l, L-leucine 75 mg/l and L-glutamine 50 mg/l, which correspond to 179 mg/l of assimilable nitrogen (Salinas et al. 2010).

Sporulation and production of a hybrid and its offspring

Sporulation was induced on acetate medium (1% potassium acetate, 2% agar) at 23°C. Crossbreeding was achieved by spore conjugation as described (Winge and Laustsen 1938). Ascus dissection, spore isolation and crossbreeding were performed with a Nikon model Eclipse 50*i* micromanipulator.

The offspring were obtained from the hybrid by sporulation and dissection of the asci by micromanipulation and both confirmed by interdelta fingerprinting (Legras and Karst 2003). Briefly, total genomic DNA was isolated and diluted to 50 ng/µl and 2 µl was used for PCR amplification on a PTC-100 Peltier Thermal Cycler (MJ Research, USA) with the primers d1 and d2 (Table 1). PCR products were visualized by electrophoresis in agarose (2%) and gels were recorded with a Photo Enhancer System (Kodak sciences, USA).

Table 1 Oligonucleotides used in this study

Genes	Primers sequences (5′–3′)
TVP23	TCT GGT GGT CGA TGT ATG TGA
	ACA GAA GGA AGA GCG AAC CA
ARR3	TCA GAT TGC TGG AGG AGA CA
	CAG TGG TAT GCC GAG AAA AAC
ZRT1	AAT GGA CCC TGC TTA TGG TG
	GTG GGT ATG GTC ATG GGA AA
VPS8	GAC ATG GTT GCA TCA GTT GG
	CAG TCG AGG GCT TGG TTA GA
RDL1	CGC ATT TGC CTT AGA TCC TT
	TAT CAC CCC CAT GAG AAA CC
NHA1	TTG AGC ACA GCC GTC AAT AC
	TTC GCT TTC AAT CTC GCT TT
ARR1	GCA ACT TAG AGC ATC CCA AAA
	ATG TGG GCG GTT TTT CTT C
YMR195W	CGA GAG ACA CCG GGA AGT
	TTG GCA TAA AGA TCG GTC AA
ATO2	AAA ACA ACG ATC AGA GCC GC
	GCA AAA CCG GAA AGT CCT AGT
CMP2	AAG CTG GTA AGT CGG AAG CA
	CAC GCC TGA ATT GTG ATC TTT
ACT1	TTG GCC GGT AGA GAT TTG AC
	CCC AAA ACA GAA GGT GGA A
d1	CAA AAT TCA CCT ATA TTC TCA
d2	GTG GAT TTT TAT TCC AAC A

Kinetic analysis

The growth curves were carried out in 50 ml of synthetic must at 28°C without shaking. Growth was measured at an absorbency of 600 nm and the biomass produced was estimated according to Salinas et al. (2010). The specific growth velocity (μ) was determined from the slope of the Biomass versus Time curve during exponential growth using the Excel software (Microsoft, USA). Generation time was calculated from the quotient between ln2 and μ.

Oenological analysis

The residual sugar, glycerol and ethanol production were determined following the methods of Somogyi (1952) and Varela et al. (2004). To determine the concentration of ammonium phosphate in the fermented must, a rapid enzyme assay from Amonio Megazyme® was used. The sample analysis was performed through a multiwell plate reader (Biotek, USA) using the Gen5 software (Biotek, USA). The nitrogen uptake from ammonium phosphate was defined as the difference between the initial and final amount following fermentation. On the other hand, the concentration of amino acids in the fermented must was determined by HPLC according to Janssen et al. (1986). The nitrogen uptake from amino acids was calculated by multiplying the amount of each amino acid consumed by the percentage of nitrogen content in each amino acid. All the oenological analyses were determined at end of the fermentation.

RNA isolation and microarray analysis

RNA extraction was performed from cultures grown in synthetic must without shaking at OD of 0.50 (600 nm) using the RNeasy Mini Kit® (Qiagen, USA). Whole genome yeast Y6.4K7 cDNA microarrays consisting of double-spotted glass slides (6,240 ORFs) were purchased at the University Health Network Microarray Centre, Toronto, Canada. Microarray experiments were conducted as a dye-swap replicate resulting in a quadruplicate data set. A labelled cDNA was done following Salinas et al. (2010). Clean-up of labelling reactions was done with the MiniElute PCR Purification Kit (Qiagen, USA). Labelled cDNAs were combined with 30 µl hybridisation solution consisting of 50% deionised formamide, 10× SSC, 0.2% SDS and 1 µg of sheared fish sperm DNA. The hybridisation mixture was denatured at 95°C for 3 min and deposited on the microarray surface. Microarray hybridisation, washes, scan, image analysis and normalisation of the data were performed according to Salinas et al. (2010). Briefly, slides were dried by centrifugation at 1,000 g for 10 min and immediately scanned in a Scan-Array Lite fluorescence scanner (Perking Elmer, USA). Images were saved in tiff-format and analysed with the GenePix Pro 6.0 software (Molecular Devices, USA). Data normalisation was performed with the DMAD tool at Asterias website (Vaquerizas et al. 2004). The difference in gene activity between strains compared was obtained from the $\log_2$ ratio of gene expression. The functional analysis was carried out using the Gene Set Analysis Toolkit V2 (Duncan et al. 2010; Zhang et al. 2005).

Validation of microarray results by QPCR

The reverse transcription (RT) reaction was based on Zuzuarregui et al. (2006). The cDNA was used as template in the real time PCR reaction (QPCR). The QPCR reaction was carried out in a final volume of 20 µl. The reaction mixture contained 10 µl of 2× Brilliant II SYBR Green QPCR Master mix (Stratagene, USA), 0.1 mg/ml of BSA (New England BioLabs, USA) and 2 µM of each primer (Table 1). The QPCR reaction was carried out in a LightCycler 1.5 equipment (Roche, Germany) under the following conditions: 95°C for 10 min, 45 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, a melting analysis at 95°C for 1 s, 65°C for 15 s and 95°C for 1 s with a 0.1°C/s

increase in temperature, finally a cooling stage at 40°C for 30 s. Results were analysed using the LightCycler 4.0 software (Roche, Germany) and quantification of relative gene expression was done using the mathematic method (Livak and Schmittgen 2001) and normalised with *ACT1*.

Statistical analysis

The oenological trait data were subjected to analysis of variance (ANOVA) and the mean values of the experiments were statistically analysed using the LSD test. Differences were considered significant when the probability was <0.05.

Results

This work was concerned with identifying genes involved in nitrogen metabolism in wine yeasts by comparing the transcriptional profile of strains, which although they are genetically related, differ in the nitrogen consumption phenotype. Therefore, the genes which showed different levels of expression between both strains could be involved in this phenotype. To obtain genetically related strains the hybrid L3044 obtained from crossing the monosporic cultures L3145 and L3147, was used. From this hybrid, 115 offspring were obtained and their enological profile was evaluated. From these, strains which mainly differed in the consumption of nitrogen available in the must were selected and their transcriptional profiles compared by microarray.

Selection of strains with high and low nitrogen consumption

To obtain genetically related strains but which differ in their consumption of nitrogen, the native strains L3217 and L3218 were sporulated and their spores were isolated by micromanipulation. From 9 monosporic cultures analysed, strains L3145 and L3147 showed adequate parameters for wine fermentation (Table 2). These strains were crossed generating the hybrid L3044, which was confirmed by interdelta fingerprinting whereby the presence of bands from both parental strains confirms its hybrid condition. The hybrid L3044 was sporulated obtaining 115 offspring which showed some of the hybrid band profile, also confirmed by interdelta fingerprinting (data not shown). The consumption of nitrogen from ammonium and amino acids between the parental strains, the hybrid and commercial strain EC1118 did not show significant differences (Table 2), however, the 115 offspring showed variations in their nitrogen uptake from ammonium with values between 14.2 ± 5.9 and 206.5 ± 12.5 mg/l and nitrogen uptake from amino acids from 12.8 ± 9.2 to 106.4 ± 10.4 mg/l (Fig. 1). From 115 offspring analysed, strains AC115, AC114 and AC19 were selected. These strains completed the fermentation (residual sugar lower than 5 g/l) and showed statistically significant differences in the consumption of nitrogen but not in the other parameters evaluated; glycerol, ethanol, duplication time and growth rate, suggesting that the differences in nitrogen consumption from ammonium and amino acids, would not result from differences in growth or fermentation capacity.

Genes involved in the consumption of ammonium

To identify genes related to the consumption of nitrogen sources, the gene expression profiles of strains AC19 and AC114, selected for presenting significant differences in the consumption of nitrogen from ammonium, were compared (Table 2). The gene expression analysis indicates that these strains differ in the expression of 121 genes related to ion transport, alcohol metabolism, hexose catabolism and transcription regulation (Online Resource 1). From these we selected the genes *ICY1*, *ZRT1*, *CMP2*, *NHA1*, *RDL1* and

Table 2 Oenological and kinetics properties of yeast strains in synthetic must

Strain	Residual sugar (g/l)[a]	Ethanol production (% v/v)[a]	Nitrogen uptake from ammonium (mg/l)[a]	Nitrogen uptake from amino acids (mg/l)[a]	Glycerol (g/l)	Growth rate (h^{-1})	Generational time (h)
L3145	$2.62 \pm 0.18^{2,3}$	9.24 ± 0.49^{1}	70.90 ± 13.55^{1}	67.58 ± 15.49^{2}	$4.86 \pm 0.23^{1,2}$	ND[b]	ND[b]
L3147	2.21 ± 0.77^{2}	10.17 ± 0.15^{3}	$86.16 \pm 10.54^{1,2}$	58.98 ± 7.62^{2}	$5.00 \pm 0.28^{1,2}$	ND[b]	ND[b]
L3044	$2.62 \pm 0.35^{2,3}$	10.28 ± 0.49^{3}	62.40 ± 16.22^{1}	55.90 ± 2.81^{2}	5.20 ± 0.14^{2}	ND[b]	ND[b]
AC19	3.04 ± 0.12^{3}	$9.33 \pm 0.26^{1,2}$	110.98 ± 6.05^{2}	32.62 ± 11.17^{1}	$4.95 \pm 0.07^{1,2}$	0.12 ± 0.09^{1}	2.46 ± 0.18^{1}
AC114	2.13 ± 0.77^{2}	$9.38 \pm 0.77^{1,2}$	77.27 ± 5.44^{1}	35.68 ± 13.56^{1}	$5.15 \pm 0.35^{1,2}$	0.13 ± 0.01^{1}	2.37 ± 0.02^{1}
AC115	1.69 ± 0.72^{1}	$9.96 \pm 0.16^{2,3}$	73.99 ± 2.11^{1}	66.67 ± 5.15^{2}	4.70 ± 0.14^{1}	0.12 ± 0.03^{1}	2.44 ± 0.07^{1}
EC1118	$2.52 \pm 0.50^{2,3}$	$9.31 \pm 0.26^{1,2}$	68.76 ± 24.40^{1}	58.26 ± 8.03^{2}	$4.95 \pm 0.07^{1,2}$	0.17 ± 0.09^{2}	1.79 ± 0.01^{2}

[a] Numbers with different numbers within a same column differ at $P \leq 0.05$ level

[b] *ND* no determined

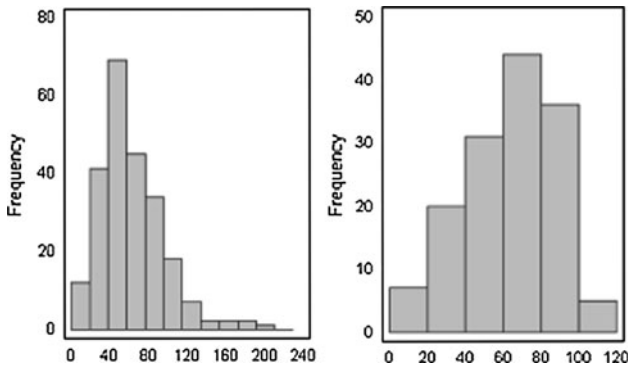

Fig. 1 Distribution of nitrogen uptake in hybrid L3044 offspring (n = 115). Nitrogen uptake in mg/l from ammonium (*left*) or amino acids (*right*)

VPS8 which have not previously been related to nitrogen metabolism, along with gene *ATO2* which encodes for a putative ammonium transporter. The differences in expression detected by microarrays were confirmed by QPCR (Table 3).

Genes involved in the consumption of amino acids

To identify genes involved in the consumption of amino acids, the gene expression profiles of strains AC115 and AC19 were analysed since they showed significant differences in the consumption of nitrogen from both sources (Table 2). In this case, the expression analysis reveals that 155 genes show differences in expression between these strains (Online Resource 1). The functional analyses of these genes suggest that they are mainly related to inorganic substance response, regulation of nitrogenated compound metabolism and RNA metabolism. Using a similar methodology to that used for genes involved in nitrogen uptake from ammonium, genes *ARR1*, *ARR3*, *RDL1* and *TVP23* previously not related to amino acid consumption, were selected. The expression profile detected by microarrays was confirmed by QPCR (Table 3).

Discussion

Several genes related to nitrogen metabolism have been identified in *S. cerevisiae* by changes in their expression according to the nitrogen source, the amount of available nitrogen or the nutritional stage of the yeast during alcoholic fermentation (Mendes-Ferreira et al. 2007; Rossignol et al. 2003). However, some genes identified by this approach may not have any relationship to nitrogen metabolism but may be associated to other physiological responses generated by the stress to which the yeast is exposed as a result of the changes in the culture conditions used. In the present study, to avoid putting the yeast through fluctuating culture conditions, we used an experimental approach that would allow us to associate phenotypic differences in the consumption of nitrogenated sources in synthetic must with the existing genetic variability in genetically related wine yeast strains.

The comparison of the global gene expression between strains AC19 and AC114 that differ in the consumption of ammonium permitted the detection of 121 genes affected in their expression. Some of these genes have previously been associated to nitrogen metabolism, for example gene *URE2*

Table 3 Genes involved in nitrogen uptake

Gen name	Open reading frame	Description	Strain with more mRNA	Fold change by Microarray[a,c,d]	Fold change by QPCR[b,c,d]
ATO2	YNR002C	Putative protein involved in the ammonium transport	AC19	1.46	2.64
ICY1	YMR195W	Unknown function	AC19	1.50	3.03
ZRT1	YGL255W	Protein involved in the Zn transport	AC19	3.72	4.72
CMP2	YML057W	Isoform of calcineurin A	AC114	1.43	1.66
NHA1	YLR138W	Na^+/K^+ pump	AC114	2.03	1.25
RDL1	YOR285W	Unknown funtion	AC114	2.52	1.87
VPS8	YAL002W	Vacuolar localization	AC114	3.25	2.60
ARR3	YPR201W	Arsenite transporter	AC115	5.73	3.08
ARR1	YPR199C	Transcriptional factor	AC115	1.57	3.24
RDL1	YOR285W	Unknown funtion	AC115	1.59	3.04
TVP23	YDR084C	Integral membrane protein of Golgi	AC115	9.51	2.02

[a] Average of four replicates

[b] Average of two replicates

[c] The number is the $\log_2$ ratio of gene expression between the strains evaluated

[d] The data used in microarrays and QPCR comes from independent experiments, and could explain the differences in expression observed

that encodes for a regulator involved in catabolic repression by nitrogen (Blinder et al. 1996), *MET31*, that encodes for a regulatory factor of methionine biosynthesis (Blaiseau et al. 1997) and *ATO2* an exporter of ammonium (Palkova et al. 2002). On the other hand, the global gene expression comparison between strains AC19 and AC115, that also differ in the consumption of amino acids, showed that they differ in the expression of some genes, such as *URA2*, *HXT12*, *STR3* and *ILV5* that are regulated by the transcriptional factor Gcn4p, an essential regulator of catabolic repression by nitrogen (Hinnebusch and Natarajan 2002).

Within this context, genes *ATO2* and *ZRT1* were overexpressed in strain AC19 which shows a greater preference for ammonium as nitrogen source. Of the two genes, *ATO2* had already been associated with the transport of ammonium cations (Palkova et al. 2002), but gene *ZRT1* has been associated to zinc transport (Tamura and Yoshimura 2008; Zhao and Eide 1996). Nevertheless, we cannot rule out that *ZRT1* is involved in the transport of ammonium to the interior of the cell and thus explain its increase in this strain. Furthermore, it has been described that *ZRT1* is regulated by Gln3p, an activator of catabolic regulation by nitrogen (Cox et al. 1999) however, its expression would not completely depend on this regulator (Gitan et al. 1998). Similarly, it was observed that strain AC114 shows an upregulation of the gene *NHA1*, a Na^+/H^+ proton pump involved in K^+ homeostasis (Kinclová et al. 2001) and elimination of toxic cations. This gene also plays an important role in the regulation of the cell cycle (Simón et al. 2001) and intracellular pH (Sychrova et al. 1999) or in the immediate cell response to osmotic shock (Kinclová et al. 2001; Proft and Struhl 2004). This proton pump has been involved in osmotic stress regulation produced by the transport of ions, or stress produced by the entry of ammonium to the cell. Furthermore, it has been described that during the stress produced by nitrogen starvation, there is an induction of vacuolar recycling by proteolysis (Rossignol et al. 2003) which could explain the increase in the expression of proteins associated to vacuolar transport such as *VPS8* (Chen and Stevens 1996; Horazdovsky et al. 1996).

On the other hand, many genes of unknown function also alter their expression under the conditions assayed, for example genes *ARR3*, *ARR1*, *RDL1* and *ICY1*. Genes *ARR3* and *ARR1* are overexpressed in strain AC115 and would be related to ion transport, such as arsenic and its activation, respectively (Bobrowicz et al. 1997). Gene *RDL1* showed a high expression in strains AC114 and AC115, which were characterized by showing a low consumption of total nitrogen and nitrogen from ammonium, respectively. Although this gene encodes for a protein of unknown function induced by the lack of zinc (Wu et al. 2008), it has a cystiol domain which would participate in its regulation by nitrosylation. In the case of *ICY1*, its upregulation has been described in amino acid starvation (Dunn and Jensen 2003; Kleinschmidt et al. 2005; Suzuki et al. 2003).

Although there are previous reports where some of the genes identified have been related to the consumption of nitrogen, 35% of the genes determined in this study have no known function and the results obtained support their participation in the metabolism of nitrogen, as well as contributing information for future studies. Finally, the genes associated to nitrogen metabolism identified in this work, may be useful biomarkers for stuck or slow fermentations.

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