The role of *GAP1* gene in the nitrogen metabolism of *Saccharomyces cerevisiae* during wine fermentation

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Running headline: Role of *GAP1* in the wine yeast

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ABSTRACT

Aim: The aim of this study was to analyze the relevance of the general amino acid permease gene (*GAP1*) of the wine yeast *Saccharomyces cerevisiae* on nitrogen metabolism and fermentation performance.

Methods and Results: We constructed a *gap1* mutant in a wine strain. We compared fermentation rate, biomass production and nitrogen consumption between the *gap1* mutant and its parental strain during fermentations with different nitrogen concentrations. The fermentation capacity of the *gap1* mutant strain was impaired in the nitrogen-limited and nitrogen-excessive conditions. The nitrogen consumption rate between the wild strain and the mutant was different for some amino acids, especially those affected by the Nitrogen Catabolite Repression (NCR). The deletion of *GAP1* gene also modified the gene expression of other permeases.

Conclusions: The Gap1 permease seems to be important during wine fermentations with low and high nitrogen content, not only because of its amino acid transporter role but also because of its function as an amino acid sensor.

Significance and Impact of Study: A possible biotechnological advantage of a *gap1* mutant is its scarce consumption of arginine, whose metabolism has been related to the production of the carcinogenic ethyl carbamate.

Keywords: gene deletion, functional analysis, yeast, ammonium, amino acids, permeases, wine.

INTRODUCTION

Nitrogen availability is one of the main limitations of alcoholic fermentation. Both the quantity and quality of nitrogen sources in grape must affect the growth and metabolism of yeasts, the rate of fermentation and the completion of fermentation (Monteiro and Bisson 1991). A minimal concentration of more than 140 mg/L is often cited as necessary to complete wine fermentation (Bell and Henschke 2005). The wine industry has partially avoided these problems by adding nutritional supplements, usually inorganic forms of nitrogen such as ammonium salts, to grape must prior to fermentation.

Qualitatively, a wide variety of nitrogen-containing compounds are present in grape juice. These depend on the variety of grape and the time of harvest. The two main sources of yeast assimilable nitrogen compounds are amino acids and ammonium ions. Ammonium ions make up a large percentage of total assimilable nitrogen (up to 40%) while proline and arginine are the most common organic nitrogenous compounds in grape juice (30-65% of total amino acid content). *S. cerevisiae* can use different nitrogen sources for growth but not all nitrogen sources support its growth equally well. *S. cerevisiae* selects nitrogen sources that enable the best growth through a mechanism called Nitrogen Catabolite Repression (NCR) (Magasanik 1992). Good nitrogen sources such as glutamine, asparagine and ammonium decrease the use of other nitrogen sources by reducing the enzyme concentration and gene expression required for poorer nitrogen sources.

Other important targets of the NCR mechanism are the permeases involved in nitrogen uptake. Amino acids are transported into the cell by general and specific transport systems. The general high-capacity permeases like *GAP1* and *AGP1* or the specific proline permease *PUT4* are nitrogen-regulated and become down-regulated at the transcriptional as well as the posttranslational level, in response to high-quality nitrogen sources like ammonium and glutamine (Forsberg and Ljungdahl 2001). However, specific permeases like the histidine

permease (*HIP1*), the lysine permease (*LYP1*) and the basic amino acid permease *CAN1* are expressed constitutively (Ter Schure et al. 2000).

In previous works we have studied this NCR in *S. cerevisiae* during wine fermentations (Beltran et al. 2004) as well as the effect of the nitrogen supplements on the pattern of nitrogen uptake (Beltran et al. 2005). In wine fermentations the cells evolved from a nitrogen-repressed situation at the beginning of the process to a nitrogen-derepressed situation as the nitrogen was consumed. These nitrogen-repressed/derepressed conditions determined the different patterns of ammonium and amino acid consumption because they determined the permeases activated in each situation. However, grape must rich in nitrogen or supplemented with ammonium maintained a repressed condition throughout the process. This NCR condition inhibited the uptake of arginine and alanine, and stimulates the consumption of branched-chain and aromatic amino acids. Changes in the nitrogen uptake patterns influence the production of aroma and spoilage compounds (especially hydrogen sulfide) and the amount of urea, the major precursor to the carcinogen ethyl carbamate (Marks et al. 2003; Beltran et al. 2005; Bohlscheid et al. 2006).

The Gap1p which transports all naturally occurring amino acids is repressed during growth on good nitrogen sources (Ter Schure et al. 2000). This repression is carried out by transcriptional and post-transcriptional mechanisms which involve a cessation in the *GAP1* gene transcription and a degradation of Gap1p in the vacuole respectively (Magasanik and Kaiser 2002). Beltran et al. (2004) monitored its transcription activity during fermentation with different nitrogen concentrations and concluded that *GAP1* transcription is a good indicator of the availability of nitrogen during wine fermentations. These results led us to investigate the role of this permease in the nitrogen metabolism during wine fermentation. In this study we try to answer questions such as: Is the only role of Gap1p to transport amino acids in nitrogen-poor conditions? Is this permease unnecessary during growth in nitrogen-

rich conditions? Does Gap1p have other functions during wine fermentation? To go into these questions in depth we constructed a *gap1* mutant in a wine strain. Fermentation rate, biomass production and nitrogen consumptions were compared between the *gap1* mutant and its parental strain during fermentations with different nitrogen concentrations. We also monitored the transcriptional activity of other permeases involved in the transport of ammonium and specific amino acids in both strains.

MATERIAL AND METHODS

Strains, Fermentations and Sampling

The commercial *S. cerevisiae* wine strain QA23 (Lallemand S.A., Toulouse, France) and its derivative *gap1* mutant strain were used in this study. Fermentations were carried out in a synthetic grape must (pH 3.3) described by Riou et al. (1997) but with some modifications. Carbon source (g l^{-1}): glucose 100, fructose 100, malic acid 6, citric acid 6. Assimilable nitrogen source: 300 mg N l^{-1} (detailed below). Mineral salts (mg l^{-1}): KH_2PO_4 750, K_2SO_4 500, $MgSO_4$ 250, $CaCl_2$ 155, NaCl 200, $MnSO_4$ 4, $ZnSO_4$ 4, $CuSO_4$ 1, KI 1, $CoCl_2$ 0.4, H_3BO_3 1, $(NH4)_6Mo_7O_{24}$ 1. Vitamins (mg l^{-1}): myo-inositol 20, calcium panthothenate 1.5, nicotinic acid 2, chlorohydrate thiamine 0.25, chlorohydrate pyridoxine 0.25, biotine 0.003.

The Yeast Assimilable Nitrogen (YAN) content in the control synthetic grape must was 300 mg N l^{-1}: 120 mg N l^{-1} as ammonium (NH_4Cl) and 180 mg N l^{-1} in amino acid form. The High Nitrogen Content (HNC) and Low Nitrogen Content (LNC) conditions contained four-fold (1200 mg l^{-1}) and 1/5-fold (60 mg l^{-1}) the YAN of the control must, respectively. The proportions of the different amino acids and ammonium were maintained in all the fermentations.

Fermentations took place at 25 ºC without agitation in laboratory-scale fermenters: 2 l bottles filled with 1.8 l of medium and fitted with closures that enabled the carbon dioxide to

escape and the samples to be removed. Fermentations were performed in semi-anaerobic conditions, as limited aeration was necessary in order to harvest samples for the subsequent analysis. The population inoculated in every flask was 2 x 10^6 cell ml^{-1} from an overnight culture in YPD.

Sugar consumption was monitored by measuring the decrease in density of the fermentation media. Density was measured throughout the fermentation process by weighing 5 ml of the media. In the latter stages of the fermentation, sugar consumption was assayed using enzymatic kits (Roche Applied Science, Germany). Fermentation was considered to be complete when residual sugars were below 2 g l^{-1}. Cell growth was determined by plating in YPD medium.

Five ml samples were harvested at different points during the fermentation process so that mRNA could be analyzed. The fermentation bottles were magnetically stirred to re-suspend settled biomass, transferred to centrifuge tubes and centrifuged at 2300 g for 5 min at room temperature to prevent temperature shock. Cell pellets were transferred to 1.5 ml Eppendorf tubes and frozen immediately in liquid nitrogen. They were then kept at -80 ºC until they were analyzed. The supernatant of these samples was stored at -20 ºC for nitrogen content analysis.

Nitrogen content analysis

YAN was analyzed using the formol index method (Aerny 1996), and the ammonium content was quantified using an enzymatic method (Roche Applied Science, Germany). The individual amino and imino acids were analyzed by OPA and FMOC derivatizations, respectively, using the Agilent 1100 Series HPLC equipped with a low pressure gradient quaternary pump, a thermostatted autosampler, a DAD ultraviolet detector and a fluorescence detector (Agilent Technologies, Germany). The sample (2 μL) was injected into a 4.6 x 250 mm x 5 μm Hypersil ODS column (Agilent Technologies, Germany). The gradient solvent

system was: solvent A (16 mol l^{-1} sodium acetate and 0.022% triethylamine, adjusted to pH 7.2 with 1-2% acetic acid, and 0.6% tetrahydrofuran) from 100% at time 0 to 0% at time 18 min, and solvent B (20% of 66 mol l^{-1} sodium acetate, adjusted to pH 7.2 with 1-2% acetic acid, 40% acetonitrile and 40% methanol) from 0% at time 0 to 100% at time 18 min. The analysis temperature was 40 ºC, and the flow was 1.5 ml min^{-1}. Several dilutions of each sample were analyzed and averaged using the analysis software. The concentration of each amino acid was calculated using external and internal standards, and expressed as mg l^{-1}. The software used was Agilent ChemStation Plus (Agilent Technologies, Germany).

Construction of *gap1* mutant

GAP1 was deleted using the short flanking homology (SFH) method based on the kanMX4 deletion cassette and the Cre-*lox* recombination system, which allows marker recycling (Guldener et al. 1996). The primers GAP1-S1 and GAP1-C2 were used for amplification of the *loxP-kanMX4-loxP* cassette from plasmid pUG6 (Guldener et al. 1996) (Table 1). The primers have 60-nucleotide extensions corresponding to regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). The PCR fragment was used to transform the industrial QA23 strain using the lithium acetate procedure (Schiestl and Gietz, 1989). The transformants that were resistant to G418 geneticin were analyzed by PCR with total DNA using primers upstream and downstream from the deleted region (GAP1-A1 and GAP1-A2) combined with primers of the *KanMX* gene (Table 1). The use of GAP1-A1and GAP1-A2 yielded two bands of 3121 and 2203 bp corresponding to the wild-type allele and the *gap1*::*KanMX* allele. The similar band intensity for each allele (deleted or not) suggested that this strain was diploid for *GAP1*. The KanMX marker was excised using the Cre-*lox* system. The heterozygous disruption strain was transformed with the plasmid Yep351-Cre-Cyh (Güldener et al. 1996) which carries the positive marker CYH^R,

conferring resistance to cycloheximide, and the *CRE* gene under the control of the inducible *GAL1* promoter. Expression of the Cre recombinase was induced by shifting cells from YPD to YPG (galactose) medium. The remaining wild-type *GAP1* copy was deleted using the same process described above but using a new primer (2GAP1-S1) that hybridized in a new region of the *gap1* gene no longer present in the gap1 deleted copy. The aim of this new strategy was to avoid repeated insertion of the *loxP-kanMX4-loxP* cassette in the already deleted allele. The deletion of the wild-type copies was also verified by PCR. The absolute lack of *GAP1* transcription was further verified by real-time quantitative PCR (QPCR).

RNA extraction and cDNA synthesis

Total RNA was isolated from yeast samples as described by Sierkstra et al. (1992) and re-suspended in 50 µL of DEPC-treated water. Total RNA suspensions were purified using the High Pure Isolation kit (Roche Applied Science, Germany) following the protocol provided by the manufacturer. RNA concentrations were determined using a GenQuant spectrophotometer (Pharmacia, Canada) and the quality of RNA was verified electrophoretically on 0.8 % agarose gels. Solutions and equipment were treated so that they were RNase free, as outlined in Sambrook et al. (1989).

Total RNA was reverse-transcribed with Superscript^{TM} II RNase H^{-} Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystem, USA). 0.5 µg of Oligo $(dT)_{12-18}$ Primer (Invitrogen, USA) was used with 0.8 µg of total RNA as template in a reaction volume of 20 µl. Following the protocol provided by the manufacturer, after denaturation at 70 ºC for 10 min, cDNA was synthesized at 42 ºC for 50 min. Finally, the reaction was inactivated at 70 ºC for 15 min.

Real-time quantitative PCR

The PCR primers used in this study are listed in table 1. The primers of the housekeeping gene *ACT1*, *GAP1* and *MEP2* were those previously described by Beltran et al. (2004). The remaining primers were all designed with the available GenBank sequence data and the Primer Express software (Applied Biosystems, USA) in accordance with the Applied Biosystems guidelines for designing PCR primers for quantitative PCR. All amplicons were shorter than 100 bp, which ensured maximal PCR efficiency and, therefore, the most precise quantification.

For each gene, a standard curve was made with yeast genomic DNA. DNA extraction was performed as described by Querol et al. (1992), digested by RNase and isolated by two-fold phenol-chloroform extractions and ethanol precipitation. Concentration was determined using a GeneQuant spectrophotometer (Pharmacia, Canada). Serial 10-fold dilutions of DNA were conducted to yield DNA concentrations from 400 to 4 x 10^{-2} ng μl^{-1}. These dilution series were amplified (in triplicate) by SYBR PCR for each gene to obtain standard curves (see above). The standard curve displays the Ct value *vs.* $\log_{10}$ of the starting quantity of each standard. The starting quantity of the unknown samples was calculated against the standard curve by interpolation. Gene expression levels are shown as the concentration of the studied gene normalized with the concentration of the housekeeping *ACT1* gene.

The Real-Time Quantitative PCR reaction was performed using SYBR® Green I PCR (Applied Biosystems, USA). The 25 µl SYBR PCR reactions contained 300 nmol l^{-1} of each PCR primer, together with 1 µl cDNA (or 5 µl of each DNA serial dilution for standard tubes) and one time SYBR master mix (Applied Biosystems, USA).

All PCR reactions were mixed in 96-well optical plates (Applied Biosystems, USA) and cycled in a PE Applied Biosystems 5700 thermal cycler under the following conditions: 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles at 95 ºC for 15 sec and at 60 ºC for 60 sec. All

samples were analyzed in triplicate and the expression values were averaged by the analysis software (Applied Biosystems, USA).

Statistical data processing

Each fermentation condition was performed in triplicate. The data were statistically treated using SPSS 13 software package. By analyzing variance (ANOVA, Tukey test), we determined the differences between the wild strain and the mutant strain (the statistical level of significance was set at $P \leq 0.05$).

RESULTS

Fermentation kinetics

Fermentations with a normal (300 mg l^{-1}; CNC), a low (60 mg l^{-1}; LNC) and a high nitrogen content (1200 mg l^{-1}; HNC) were conducted with the *gap1* mutant strain and its wild strain, the commercial *S. cerevisiae* QA23. Fermentation evolution was monitored by measuring media density and yeast population growth throughout these fermentations (Fig 1; Table 2). Although all the fermentations showed a similar fermentation rate, regardless of the nitrogen content or the strain used, low nitrogen content and the *GAP1* deletion affected the end of the fermentation process. Wild strain fermentations were completed after 18, 18 and 25 days for the HNC, CNC and LNC respectively. The mutant strain also ended after 18 days in the CNC fermentation as did the wild strain. However, the fermentation capacity of the *gap1* mutant strain was impaired by nitrogen limitation or nitrogen excess. HNC and LNC fermentations with the *gap1* strain were completed after 30 and 32 days respectively (Fig 1). Curiously, the main differences in fermentation capacity between the wild strain and the mutant strain occurred during the last days of fermentation. At day 18, when both CNC fermentations had finished, residual sugar concentrations were 6.7 and 11.5 g l^{-1} in HNC and

LNC fermentations with the *gap1* strain and two more weeks were required to lower this amount below 2 g l^{-1} (considered as final fermentation).

Nitrogen concentration did not influence population size. The maximum population reached by the wild strain was approximately 3.5 x 10^7 cfu ml^{-1} in the three fermentations with different nitrogen content. However, this maximum population only peaked at 2.5 x 10^7 cfu ml^{-1} in the mutant strain (Table 2).

Nitrogen consumption

With both strains, nitrogen was only completely exhausted in the LNC condition. The CNC and HNC fermentations with the wild strain only consumed 45% and 15% of the total YAN and 45% and 7% of the initial ammonium, respectively (Table 3). Nitrogen consumption by the *gap1* mutant was very similar to the wild strain in the LNC and CNC fermentations. However, the mutant strain showed significant differences in nitrogen consumption during the HNC fermentation. The mutant strain consumed 90 mg l^{-1} of YAN more than the wild strain, which was mainly accounted for by an increase in amino acid consumption.

Extracellular amino acids were also determined at different points during all the fermentations. Table 4 shows the consumption of both strains at the end of the CNC and HNC fermentations. We have not included the data of the LNC fermentation because both strains consumed all the amino acids (only a few mg of residual alanine, asparagine and glycine were left in the culture of the mutant strain). In the CNC fermentation, seven amino acids were consumed more by the wild strain than by the mutant strain: Asp, Glu, Ser, Ala, Arg, Val, Ile and Gln which account for the significant difference observed in the total amino acid consumption (Table 3). In the HNC fermentation, the excess nitrogen yielded greater differences in consumption between both strains. Quantitatively, most of the amino acids were consumed more by the mutant strain. Remarkably, the mutant strain consumed two-fold

more glutamine than the wild strain. Also Arg and Ala were much more consumed by the mutant strain in the HNC.

Gene expression analysis

To test the effect of *GAP1* deletion on other nitrogen permeases, we monitored the transcriptional activity of *MEP2*, *GNP1*, *TAT1* and *TAT2* throughout fermentation. These permeases are involved in the transport of ammonium, glutamine and tryptophan, respectively. These three nitrogen compounds represented approximately 80% of the total YAN consumed. *GAP1* was also monitored and, as expected, was undetectable in the mutant strain. All the transcriptional changes were expressed relative to time zero of the wild strain (control condition). Time zero was the expression of yeast before inoculation (after overnight growth in YPD). As previously reported (Beltran et al., 2004), *GAP1* and *MEP2* were clearly subjected to NCR, with much lower activity at higher nitrogen content (Fig 2). Comparing gene expression between both strains, with the exception of the first hours in the LNC fermentation, the wild strain showed a higher *MEP2* expression than the mutant strain in all the samples analyzed. These differences in *MEP2* gene expression did not correlate with a differential rate of ammonium uptake. The *GNP1*, *TAT1* and *TAT2* gene expressions were much lower than those of *GAP1* and *MEP2* (Fig 3). *GNP1* showed higher expression in the wild strain than in the mutant with the highest expression values at the beginning of the fermentation. Most of the fermentation samples showed a repression for *TAT1* after inoculation (IF sample) while the highest values for *TAT2* were in the media with an excess of nitrogen (CNC and HNC fermentations). As for *GNP1*, the wild strain generally presented higher expression values than the mutant strain for the same time-point in the fermentations.

DISCUSSION

The mechanism known as “nitrogen catabolite repression (NCR)” enables to the *S. cerevisiae* cells to select the best nitrogen sources in a rich-medium. The nitrogen content in grape must depends on the grape variety, degree of maturation, fertilization, viticulture practices, etc. These differences in nitrogen content influence fermentation rate, yeast growth and the organoleptic quality of the wine. Therefore, nitrogen metabolism plays a pivotal role in the biotechnological issues of wine fermentation.

The metabolism of nitrogen depends heavily on its uptake through different nitrogen transporters. These permeases can be expressed constitutively or transcriptionally regulated. A clear example of permease repressed by NCR is *GAP1*. In previous studies, we have showed that the expression of this gene dropped sharply when the cells were inoculated in the grape must and nitrogen was still available, and increased continuously when good nitrogen sources, especially ammonium, were consumed (Beltran et al. 2004; Beltran et al. 2005). However, its transcriptional activity was almost negligible throughout fermentation when the grape must had high nitrogen content. Therefore, this gene seemed to be operative only in poor-nitrogen fermentation media. Gene disruption is one of the most powerful methods for studying and verifying the function of a gene product. However, due to the higher-ploidy nature of industrial strains, most of these functional studies have been carried out in haploid laboratory-derived strains which are not suitable for industrial production purposes. In this study, we have studied the consequences of *GAP1* deletion on yeast physiology and technological performance in commercial wine yeast under enologically relevant conditions.

We also pursued other aims with this study. Because industrial strains are usually prototrophic, dominant selectable markers such as antibiotic resistance are mainly used. The introduction of antibiotic resistant genes into industrial wine yeasts would be absolutely unacceptable for marketing (Cebollero and Gonzalez 2004). The construction of auxotrophic wine strains based on nutritional markers like *URA3*, *LEU2* or *LYS2* have been proposed as

alternative markers to antibiotic resistance (Puig et al., 1998). However most of these nutritional markers necessarily disturb intermediary metabolism, possibly interfering with the phenomena to be studied. Regenberg and Hansen (2000) proposed *GAP1* as a novel selection and counter-selection marker for multiple gene disruptions in *S. cerevisiae*. This marker did not lead to special nutritional requirements of the industrial strain when deleted. The mutant *gap1* strain is unable to grow in L-citrulline at low concentration (1-2 mM) as its sole nitrogen source (its uptake is exclusively mediated by Gap1p) but can grow in the presence of D-histidine, since this compound is toxic to the yeast cell upon transport by Gap1p (Regenberg and Hansen 2000). The repression of this gene during most of the stages of wine fermentation could make this gene a suitable marker in wine strains, where it is desirable to maintain prototrophic strains.

No significant differences were detected between the mutant and the wild strain in the fermentation performance of the control fermentation where nitrogen content was neither limiting nor excessive. As expected, the *gap1* mutant strain showed differences in fermentation length with respect to the wild strain in the nitrogen-limited condition (LNC fermentations). The rapid depletion of the good nitrogen sources yielded a nitrogen-derepressed situation very early on during the fermentation process. In this condition, the lack of *GAP1* must impair the transport of some amino acids and, therefore, decrease the fermentation rate. However, the fermentation performance of the mutant strain was also affected by the excess of nitrogen. This result was unexpected because a continuous repressed situation was kept throughout the HNC fermentation with a very low activity of the *GAP1* gene. A possible explanation for the fermentation problems of the mutant can be found in the dual role assigned to this permease, which not only functions as an amino acid transporter but also plays a role as an amino acid sensor (Donaton et al. 2003). There are two major signaling pathways in yeasts, the Ras2/cAMP pathway and the fermentable growth medium (FGM)

pathway, which signal sugar and nitrogen source availability and converge through the regulation of protein kinase A (Thevelein and deWinde, 1999). Donaton et al. (2003) showed that Gap1p acts as an amino acid sensor for rapid activation of the FGM signaling pathway which controls the PKA targets. PKA pathway plays a major role in the control of metabolism, stress resistance and proliferation, in particular in connection with the available nutrient conditions. Therefore, the deletion of *GAP1* may affect the correct activation of the PKA pathway, yielding an impaired metabolism regard to the wild strain. In this study, we did not detect differences in the lag and exponential phases between the wild type and the mutant. The differences were only detected in the late phases of fermentation. In these phases, a proper fitness of the stress mechanism is necessary and the deletion of *GAP1* may affect the correct induction of this response.

Nevertheless, the transport role of the permease should not be discarded, because differences in the consumption of amino acids were detected between the wild type and the mutant. The first remarkable result was that no amino acid was exclusively transported by Gap1p because the mutant strain also consumed all the amino acids in the LNC fermentation. However, the consumption rate between the wild strain and the mutant was different for some amino acids. In the LNC fermentation, aspartate, glutamate, alanine and arginine were more quickly consumed by the wild strain than by the mutant during the first days of fermentation (data not shown). These amino acids were almost two-fold more consumed by the wild strain than the mutant at the end of the CNC fermentation. In our previous works (Beltran et al. 2004; Beltran et al. 2005) we determined that these amino acids are affected by the NCR because they are hardly consumed when there is an excess of good nitrogen sources. Although these amino acids are transported by specific permeases, the main transporter must be the general amino acid permease. However, in the HNC fermentation, where the NCR was stronger, alanine and arginine, which accounted for approximately 20% of the total amino

acids in the must, were hardly consumed in the wild strain while the mutant consumed few milligrams.

Highly surprising was the high consumption of nitrogen by the mutant in the nitrogen-excess medium (HNC). Practically all the amino acids were consumed more by the mutant than by the wild type. However, the main difference was in the consumption of glutamine. The mutant consumed more than two-fold the glutamine consumed by the wild strain. Glutamine is a key amino acid in the central nitrogen metabolism and its intracellular concentration induced gene repression (NCR). Moreover, the higher consumption of nitrogen was not translated into a higher population size in the mutant fermentation. All these results point to a deregulation problem of the *gap1* mutant. The deletion of *GAP1* permease might not only affect PKA pathway activation but also other nitrogen signaling pathways. To confirm this, we also tested the effect of *GAP1* deletion on the other main specific permeases. Higher glutamine consumption could not be related to the higher activity of its specific permease. *GNP1* expression was higher in the wild strain than in the mutant in all the fermentation phases. *GNP1* expression was three times higher during the first days of HNC fermentation, when most of the nitrogen was consumed. Didion et al. (1996) demonstrated that Bap2 permease is induced at the transcriptional level by the presence of certain amino acids in the medium. In this case, both *GNP1* and *TAT2* might also be induced by the external concentration of amino acids because higher activity of these permeases was detected in the HNC fermentation. We found that the more nitrogen available, the more repressed the *MEP2* gene was. This higher repression was also consistent with a lower proportion of ammonium consumed in the high nitrogen fermentations.

In conclusion, we have analyzed the effect of *GAP1* deletion on fermentation performance and nitrogen uptake. The lack of this permease in the commercial wine strain QA23 did not affect the fermentation rate in grape must with optimal nitrogen content. However, both

limited and excess nitrogen in the media yielded a delay at the end of fermentation. This occurrence seemed to be more connected with the sensing function attributed to Gap1p than to its role as transporter. This deletion also impaired biomass production and the uptake of certain amino acids which must be mainly transported by this permease. A possible biotechnological advantage of a *gap1* mutant is the scarce consumption of arginine. The metabolism of this amino acid has been related with the production of the carcinogenic ethyl carbamate (Ough et al. 1988; Pretorius and Hoj 2005). Arginine breakdown produces citrulline, urea or carbamyl phosphate which reacts with ethanol forming ethyl carbamate. Previously, with the same objective, the *CAR1* arginase gene was deleted in a sake yeast and urea was not accumulated in the rice wine, but growth was markedly impaired (Kitamoto et al. 1991). Despite the differences in the fermentation performance of the mutant, we think that it is an interesting selectable marker for industrial yeasts. In our experience, auxotrophic markers such as *LEU2* in this strain experience more serious fermentation problems.

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Table 1. Primers used in this study

Gene	Name	Oligonucleotide sequence (5′–3′ end)
GAP1 disruption cassette*	**GAP1- S1**	TCAGCCGATCGCTTATCTGCTCACTAGAATCGTAATCAGTGATATTTTT ATTAATAATTACGTACGCTGCAGGTCGACA
	2GAP1-S1	ATACCATTTCCTTTTGATAAGGGGTCGTTGGTGCCGTGCCGCTATCAGG CAGCCTCACTACGTACGCTGCAGGTCGACA
	GAP1-C2	CAGATTAGTTTTCATCTCGCTGTCTACTAAAAGATTTATTTTTACATTT CCTAGAAAAACACTAGTGGATCTGATATC
GAP1 PCR verification	**GAP1-A1**	CATACTCACTCTGAAGTTGT
	GAP1-A2	AGCGGATTTAATCGGAATCA
KanMX4 PCR verification	**K2-R**	GGGACAATTCAACGCGTCTG
	K3-F	CCTCGACATCATCTGCCAG
ACT primers real time PCR	**ACT-F**	TGGATTCCGGTGATGGTGTT
	ACT-R	CGGCCAAATCGATTCTCAA
GAP1 primers real time PCR	**GAP1-F**	CTGTGGATGCTGCTGCTTCA
	GAP1-R	CAACACTTGGCAAACCCTTGA
MEP2 primers real time PCR	**MEP2-F**	GGTATCATCGCTGGCCTAGTG
	MEP2-R	ACAACGGCTGACCAGATTGG
GNP1 primers real time PCR	**GNP1-F**	TCGTGTGGTTCCTCATTTCATAA
	GNP1-R	CCGTTAGCAACGGAAAGAACA
TAT1 primers real time PCR	**TAT1-F**	GGTGGTGCAGGTGACAGAAGA
	TAT1-R	GGCAAATGGACCAGGATTGT
TAT2 primers real time PCR	**TAT2-F**	CTGGCCACGTGCATTGTCT
	TAT2-R	GCCTTCATCGCCAGTCTAAATC

*Underlining indicates homology to the *loxP-kanMX4-loxP* cassette from plasmid pUG6. The remainder sequences of the primers are homologous to the flanking region of the *GAP1* ORF.

Table 2. Fermentation kinetics and growth of the wild and mutant strains (*Δgap1*) in fermentations with different nitrogen concentrations

	CNC		**LNC**		**HNC**	
	Δgap1	Wild type	*Δgap1*	Wild type	*Δgap1*	Wild type
Final Fermentation (days)	18	18	32	25	30	18
Maximum fermentation rate (density/day)	-8.31	-7.98	-8.21	-7.32	-7.50	-7.41
Maximum population (10^7 cfu ml^{-1})	2.12	3.36	2.44	3.78	1.70	3.26
Time to reach maximum population (hours)	48	48	48	48	24	48

Table 3. Consumption of Yeast Assimilable Nitrogen (YAN; mg N ml^{-1}), expressed as ammonia (YAN-NH_4) and amino acids (YAN-AA) nitrogen for both strains in all conditions. These values are the mean ± SD of the differences between the initial nitrogen in the must and the residual nitrogen at the end of the fermentation.

	LNC		**CNC**		**HNC**	
	Δgap1	Wild type	*Δgap1*	Wild type	*Δgap1*	Wild type
YAN-NH4	26.2 ± 1.09	26.0 ± 1.5	44.4 ± 3.58	48.3± 2.74	45.0 ± 6.22	38.0± 7.81
YAN-AA	34.3 ± 0.10	38.4 ± 0.21 *	77.4 ± 0.16	86.3± 0.22 *	219.7± 0.36	137.5± 0.19 *
YAN-total	60.6 ± 0.57	64.4 ± 1.06	121.7 ± 5.21	134.6 ± 2.84	265.7 ± 11.08	175.5 ± 25.26 *

* = significant differences set at $P \leq 0.05$

Table 4. Total consumption of individual amino acid at the end of the fermentations expressed in mg N ml^{-1}. First column values represent initial amino acid concentration in synthetic grape must.

	CNC				HNC			
		consumption				consumption		
amino acids	aa medium	wild strain	*Δgap1*	Ratio	aa medium	wild strain	*Δgap1*	Ratio
Asp	**4.81**	1.17 ± 0.10	0.74 ± 0.07 *	1.58	**18.57**	0.00 ± 0.00	0.92 ± 0.16	-
Glu	**12.10**	3.24 ± 0.19	1.85 ± 0.14 *	1.75	**60.43**	15.28 ± 0.18	14.30 ± 0.22 *	1.07
Ser	**10.64**	5.11 ± 0.30	4.25 ± 0.12 *	1.20	**44.30**	3.51 ± 0.27	3.96 ± 0.11 *	0.89
His	**2.52**	1.59 ± 0.00	1.36 ± 0.00	1.16	**11.10**	2.49 ± 0.62	1.52 ± 0.13 *	1.64
Gly	**6.24**	0.00 ± 0.00	0.00 ± 0.00	-	**14.14**	0.00 ± 0.00	0.62 ± 0.13	-
Thr	**7.88**	3.57 ± 0.27	3.58 ± 0.15	1.00	**22.52**	0.85 ± 0.11	2.47 ± 0.56 *	0.34
Ala	**27.53**	4.50 ± 0.49	2.32 ± 0.40 *	1.94	**102.51**	0.34 ± 0.32	2.10 ± 0.21 *	0.16
Arg	**30.79**	5.44 ± 0.53	3.11 ± 0.40 *	1.75	**120.90**	0.65 ± 0.17	3.47 ± 0.16 *	0.19
Tyr	**0.87**	0.37 ± 0.01	0.30 ± 0.02	1.24	**5.72**	0.07 ± 0.04	0.37 ± 0.04 *	0.20
Val	**4.26**	3.03 ± 0.03	2.57 ± 0.09 *	1.18	**20.72**	1.02 ± 0.26	2.00 ± 0.18 *	0.51
Met	**2.67**	2.13 ± 0.02	1.82 ± 0.04	1.17	**13.33**	2.56 ± 0.16	2.69 ± 0.09	0.95
Trp	**11.38**	10.01 ± 0.04	10.93 ± 0.04	0.92	**40.35**	32.79 ± 0.42	39.68 ± 0.23 *	0.83
Phe	**2.10**	2.59 ± 0.19	2.15 ± 0.17	1.20	**12.17**	2.63 ± 0.22	2.87 ± 0.16	0.92
Ile	**3.64**	3.03 ± 0.13	2.95 ± 0.12	1.03	**15.71**	2.57 ± 0.13	3.15 ± 0.09 *	0.82
Leu	**3.58**	4.86 ± 0.12	4.60 ± 0.10	1.06	**19.86**	6.34 ± 0.03	6.58 ± 0.09	0.96
Lys	**0.88**	0.84 ± 0.08	0.88 ± 0.03	0.96	**6.24**	5.06 ± 0.00	6.24 ± 0.00	0.81
Gln	**55.32**	35.52 ±0.27	34.46 ± 0.26 *	1.03	**178.55**	60.00 ± 1.21	126.73 ± 1.07 *	0.50

* = significant differences set at $P \leq 0.05$

Figure 1: Fermentation kinetics measured as optical density reduction (black symbols for the wild strain and open symbols for the mutant strain in all conditions).

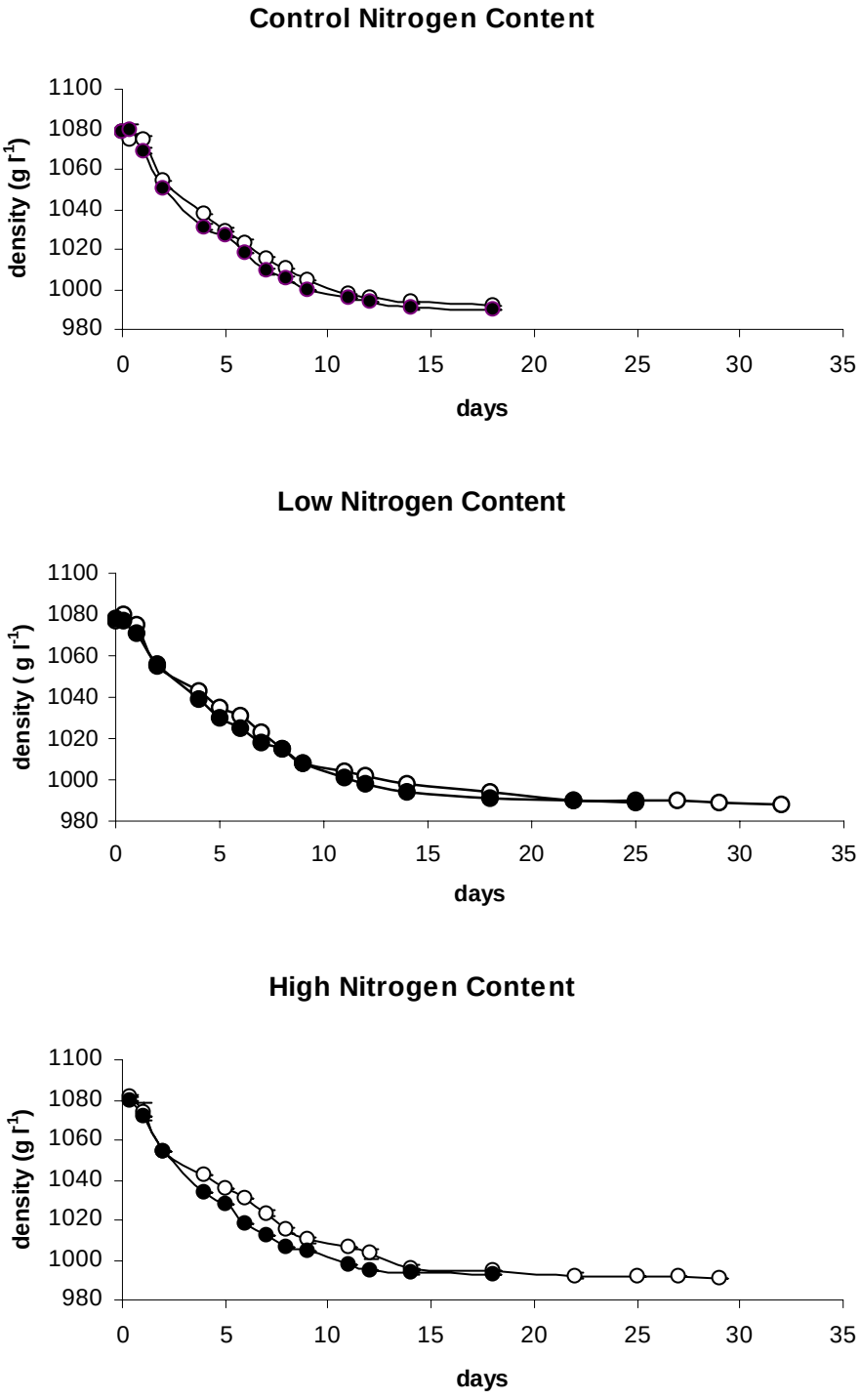

Figure 2: Relative gene expression of the permeases *GAP1* and *MEP2* at time zero (T0) (before inoculation) and the initial (IF), middle (MF) and final (FF) fermentation. Changes in gene expression are expressed relative to the expression of the wild strain at time zero (set as value 1). YAN consumption along the wild strain fermentation (black symbols) and the mutant strain fermentation (open symbols).

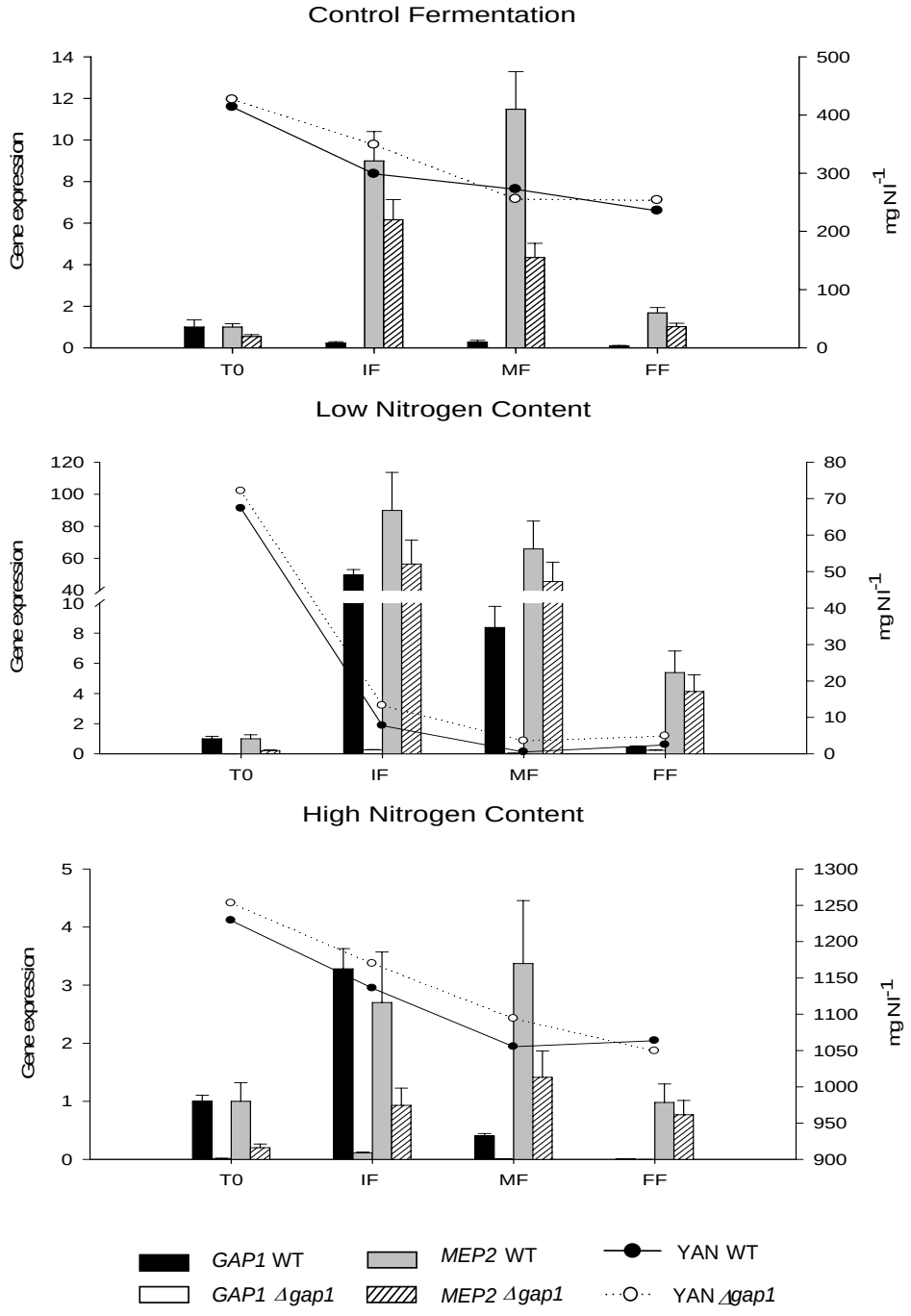

Figure 3: Relative gene expression of the permeases *GNP1*, *TAT1* and *TAT2* at time zero (T0) (before inoculation) and the initial (IF), middle (MF) and final (FF) fermentation. Changes in gene expression are expressed relative to the expression of the wild strain at time zero (set as value 1). YAN consumption along the wild strain fermentation (black symbols) and the mutant strain fermentation (open symbols).