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The Wine Yeast Strain-Dependent Expression of Genes Implicated in Sulfide Production in Response to Nitrogen Availability

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Sulfur metabolism in *S. cerevisiae* is well established, but the mechanisms underlying the formation of sulfide remain obscure. Here, we investigated by real-time RT–PCR the dependence of expression levels of *MET3*, *MET5/ECM17*, *MET10*, *MET16*, and *MET17* along with *SSU1* on nitrogen availability in two wine yeast strains that produce divergent sulfide profiles. *MET3* was the most highly expressed of the genes studied in strain PYCC4072, and *SSU1* in strain UCD522. The strains behaved differently according to the sampling times, with UCD522 and PYCC4072 showing the highest expression levels at 120 h and 72 h, respectively. In the presence of 267 mg assimilable N/l, the genes were more highly expressed in strain UCD522 than in PYCC4072. *MET5/ECM17* and *MET17* were only weakly expressed in both strains under any condition tested. *MET10* and *SSU1* in both strains, but *MET16* only in PYCC4072, were consistently upregulated when sulfide production was inhibited. This study illustrates that strain genotype could be important in determining enzyme activities and therefore the rate of sulfide liberation. This linkage, for some yeast strains, of sulfide production to expression levels of genes associated with sulfate assimilation and sulfur amino acid biosynthesis could be relevant for defining new strategies for the genetic improvement of wine yeasts.

Keywords: *Saccharomyces cerevisiae*, nitrogen, H_2S, *MET* genes, *SSU1* gene

The volatile sulfur compounds generated during fermentation by yeast *Saccharomyces cerevisiae* are responsible for much of the sensory quality of wine. They include hydrogen sulfide (H_2S) and various ethyl and methyl mercaptans, most of which are, like H_2S, associated with unpleasant odors [23]. Racking and aeration, and/or the addition of trace quantities of copper sulfate, represent effective means of eliminating the most volatile sulfides, but the remainder left behind by these treatments represent a troublesome source of off-odors in wine.

S. cerevisiae uses either elemental sulfur, sulfate, or sulfite as its sulfur source, *via* the sulfate reduction sequence (SRS) pathway (Fig. 1); in brief, this consists of the transfer of an ATP adenosyl phosphoryl moiety to sulfate to yield adenylyl sulfate (APS), which is in turn phosphorylated to phosphoadenylyl sulfate (PAPS). These reactions are catalyzed by both ATP sulfurylase (encoded by *MET3*) and APS kinase (encoded by *MET14*). Activated sulfate is first reduced to sulfite and then is further reduced to sulfide by sulfite reductase, a heterotetrameric protein consisting of two α- and two β-subunits encoded by, respectively, *MET10* and *MET5/ECM17*. The final step in the sulfate assimilation pathway consists of the *MET17* gene product (*O*-acetyl-homoserine-*O*-acetyl serine sulfhydrylase)-facilitated incorporation of sulfide into a carbon source during the biosynthesis of cysteine, methionine, and *S*-adenolsylmethionine (SAM) [24].

Sulfur amino acid biosynthesis is transcriptionally regulated [9], with the expression of the various *MET* genes being dependent on the activator Met4p and on the transcriptional factors Cbf1p, Met28p, Met31p, and Met32p [19]. *MET* genes are repressed by the addition of methionine, SAM, homocysteine, or high concentrations of cysteine [6], and are induced by low concentrations (<200 μM) of methionine [19]. *MET17* overexpression has been shown to reduce H_2S formation in some yeast strains [21]. The rate-limiting factor for H_2S production is OAH, so its flux from aspartate is particularly important [27]. The *MET16* product 3'-phosphoadenylsulfate reductase reduces 3'-phosphoadenylyl sulfate to adenosine-3',5'-bisphosphate and free sulfite, and is regulated by the transcriptional

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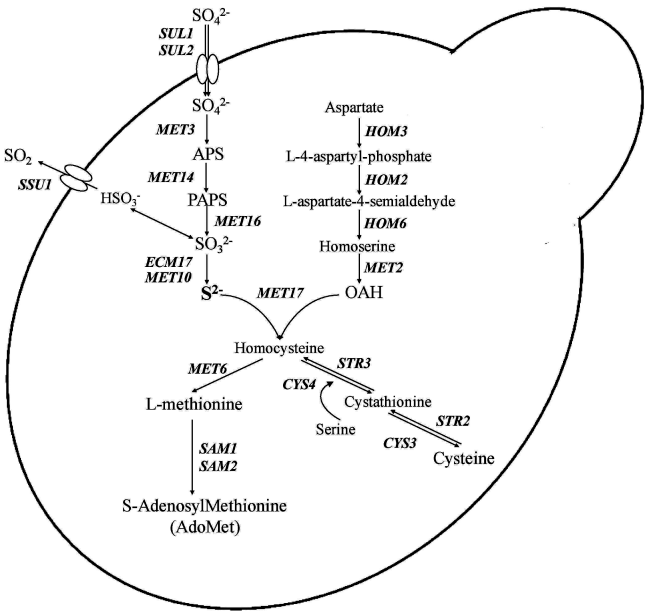

Fig. 1. The sulfate reduction sequence pathway and the biosynthesis of sulfur amino acid, with reference to the particular genes analyzed in this study.

activator Gcn4p. As a result, its expression increases when cells are starved of amino acids [5]. A recent analysis of gene expression in the yeast strain PYCC4072 subjected to a range of nitrogen regimes has demonstrated changes in the expression of many genes involved in several metabolic processes [12]. In particular, several *MET* genes involved in the sulfate to sulfide assimilatory/reduction pathway were downregulated under conditions of nitrogen deficiency [13]. Here, we contrast the expression patterns of the sulfur metabolism genes *MET3*, *MET5/ECM17*, *MET10*, *MET16*, and *MET17*, along with *SSU1*, a plasma membrane sulfite pump required for sulfite efflux (Fig. 1), in two yeast strains that differ markedly from one another for the production of H_2S.

Materials and Methods

Yeast Strains and Culture Conditions

The two yeast strains used were *Saccharomyces cerevisiae* UCD522 (obtained from the Department of Viticulture and Enology, University of California, Davis, U.S.A.) and S*accharomyces cerevisiae* PYCC4072 (from the Portuguese Yeast Culture Collection, New University of Lisbon, Portugal). Both yeasts were maintained at 4°C on yeast peptone dextrose agar (YPD) slants, containing (per liter) glucose 20 g, peptone 10 g, yeast extract 5 g, and agar 20 g. Immediately before use, the cells were transferred to a fresh YPD slant and cultured for 24–48h at 25°C. For fermentations experiments, a chemical defined media [7] instead of natural grape juice was used, to avoid variability found in natural grape juice whose composition is dependent upon the grape variety, year, and climate as well as being correlated with the soil and with the fertilization applied to vineyards. Glucose (200 g/l) was included as the sole carbon and energy source, and two different nitrogen concentrations (supplied as diammonium phosphate, DAP) were added: (i) with 267 mg N/l (GJM_{267}), the nitrogen concentration required by the yeast strain to complete fermentation, and (ii) with 402 mg N/l (GJM_{402}), the condition of excess of nitrogen in which there still is nitrogen available in the media at the end of alcoholic fermentation [11]. Each set of fermentations was performed in triplicate.

Inocula and Fermentation Conditions

Starter cultures were prepared by shaking (150 rpm in an orbital shaker) the yeast cultures overnight at 25°C in 250-ml flasks containing 100 ml of GJM (same composition for all assays). The test cultures were inoculated with 5×10^5 CFU/ml starter culture, and fermentation continued in 500-ml flasks filled to 2/3 of their volume, following established methods [14]. The data reported here represent mean values derived from at least three replicated experiments.

Determination of Growth Parameters During Fermentation

During fermentation, the optical density (660 nm) of appropriately diluted culture aliquots was used to estimate yeast cell population density. At the end of alcoholic fermentation, two 50-ml and/or three 15-ml aliquots were pelleted by centrifugation in pre-weighed tubes (10 min at 2,300 ×*g*), and the pellet was washed twice in deionized water, dried (24 h at 100°C), and stored in a desiccator before weighing. The coefficient of variation between these duplicate or triplicate determinations was uniformly less than 1%.

Analytical Methods

Ammonium content was determined during fermentation according to the methodology previously described [11]. At the end of the fermentation, the quantity of glucose remaining was assessed using the 2,4-dinitrosalicylic acid method [15]. Other enological parameters (total SO_2, volatile acidity, pH, and ethanol) were determined by standard methods [16]. Quantification of H_2S released by the cultures was carried out colorimetrically following the collection of fermentation gases within a modified fermentation lock and sulfide-trapping system, as described elsewhere [8, 14]. The sulfide content was calculated from a calibration curve established from known quantities of sulfide (0–12 μg), following standard methods [1, 18].

RNA Isolation, cDNA Synthesis, and Real-Time RT–PCR Analysis

Gene expression analysis was performed at two time points chosen on the basis of the quantities of extracellular nitrogen and sulfide released by the strains. A 25-ml aliquot was centrifuged (2,205 ×*g*, 5 min), and the pellets were immediately frozen in liquid nitrogen and held at −80°C. RNA was extracted from 5×10^8 cells using the phenol-chloroform method [4]. The synthesis of cDNA was based on a 5 μg sample of RNA, using the SuperScript III RT kit (Invitrogen). To ensure the absence of contamination by genomic DNA, a PCR targeting the intron-containing *ACT1* gene was conducted. PCR amplifications were performed in a 25 μl final volume, containing 2 μl of cDNA, 4 μl of each specific primer (sequences given in Table 1), and 12.5 μl of SYBR Green Supermix – UDG with Rox (Invitrogen). All amplifications were carried out in a DNA Engine Opticon instrument (Bio-Rad) with an initial step at 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Samples were analyzed at least in triplicate.

Table 1. Sequences of the PCR primers used.

Name	Sequence (5' to 3')
PDA1 - F	AATTAGCTGATGCTGCTCC
PDA1 - R	TCCCTAGAGGCAAAACCTTG
MET3 - F	GGTGGTGGCAGGTATTAC
MET3 - R	AACAGAGTCCTTGTCATC
MET5 - F	GAGCCTGAGAGACCATTT
MET5 - R	GAGGCAAATCTGGTGTAT
MET10 - F	AGAGGATTTGGTTACTCC
MET10 - R	AGTTCCTCAAGAGATGGG
MET16 - F	GACGTTCGAGCAGGTTAAA
MET16 - R	TCTTCCTGCTCTCTCATCT
MET17 - F	AGTTGGCATCTGGTGTTA
MET17 - R	TCATGGTTTTTGGCCAGC
SSU1 - F	GAGTCCTAGGAACCATAT
SSU1 - R	TTTCCGTACCTGACTCTG
ACT1 - F	GGATCTTCTACTACATCAGC
ACT1 - R	CACATACCAGAACCGTTATC

For each primer pair, a calibration curve was first prepared from a serial dilution of the cDNA prepared from each strain grown in YPD under standard conditions. Coefficients of efficiency were derived from the expression $E=(10^{-1/slope})-1$ [20] and $\Delta\Delta CT$ was given by $(CT_{target\ gene}-CT_{reference\ gene})\times test-(CT_{target\ gene}-CT_{reference\ gene})\times calibrator$. Expression levels were normalized using the constitutively expressed gene *PDA1* as the reference gene [26].

Statistical Analysis

Statistical analysis was performed using JMP software v.7 (SAS Institute Inc., U.S.A.). The main effects on gene expression of (and interactions between) the initial nitrogen concentration, the gene considered, the sampling time, and the strain were defined by an analysis of variance (ANOVA), as were the main effects on (and interactions between) sulfide formation of the initial nitrogen concentration, sampling time, and strain. Tukey's test was used to compare multiple means, and the Student's t-test for paired comparisons.

RESULTS AND DISCUSSION

Saccharomyces cerevisiae produces sulfide as an intermediate product of both the SRS pathway and sulfur amino acid biosynthesis. Here, we compared the transcript abundance of a number of SRS pathway and sulfur amino acid biosynthesis genes in two strains that contrast in their production of sulfide. A deficiency of vitamins has been associated with sulfide liberation [3, 25]. However, these effects were overlooked in the current study, as the initial levels of these vitamins (125 μg/l of biotin, 500 μg/l of thiamine, and 1,000 μg/l of pantothenic acid) were largely in excess [7].

Growth and Fermentative Behavior of Yeast Strains Under Different Nitrogen Availability

The growth patterns and fermentation rates of the two strains were quite similar to one another (Table 2). Regarding nitrogen consumption, within 48 h of inoculation, it was largely depleted from the GJM_{267} medium, whereas 25% (UCD522) and 35% (PYCC4072) of the initial nitrogen remained in GJM_{402} (Fig. 2). Total SO_2 (Table 2), as well as final pH, volatile acidity, and ethanol produced (Fig. 2), were similar in both strains under both nitrogen conditions. The amount of sulfide released in response to nitrogen availability was, however, strain-dependent (Table 2 and Fig. 2). The sulfide production by PYCC4072 in GJM_{267} peaked at 72 h (83.4 μg/l), which corresponds to early entry into the stationary growth phase, after nitrogen depletion from the media (Fig. 2). Very low sulfide production occurred in GJM_{402}, where approximately 106 mg N/l remained in the medium at this same time point. Strain UCD522 produced much more H_2S than PYCC4072 in both media, with a peak production at 48 h (Fig. 2). In GJM_{402}, even though 14% of the initial nitrogen remained at the end of the experiment, a substantial amount of H_2S (Fig. 2B) was still produced in UCD522. Despite the contrasting behavior of the two strains with respect to the basal level of H_2S formation, the amount of sulfide liberated by both was reduced in response to the higher quantity of nitrogen present in GJM_{402}.

According to the statistical analysis of data obtained, despite the absence of significant differences in fermentative behavior, growth rate, or maximum fermentation rate between the strains (Table 2), results on sulfide production showed differences ($P<0.001$) depending on yeast strain identity, sampling time, and initial nitrogen concentration (Table 3).

Table 2. The fermentation performance of yeast strains UCD522 and PYCC4072 cultured in chemically defined grape juice media at 20°C, as affected by initial nitrogen levels.

Yeast strain	Initial nitrogen (mg/l)	Time to reach dryness (h)	Max. fermentation rate[a] (g/h)	Final nitrogen (mg/l)	Specific growth rate (μ)	Total SO_2 (mg/l)	Total H_2S (μg/l)
UCD522	267	144	0.219±0.005	0.0±0.0	0.235±0.007	16.38±0.51	731.2±34.6
	402	144	0.226±0.003	55.0±2.6	0.219±0.009	13.74±0.29	178.1±43.6
PYCC4072	267	144	0.217±0.002	0.0±0.0	0.226±0.003	17.07±1.48	222.7±43.7
	402	144	0.210±0.004	71.2±6.7	0.221±0.001	14.85±0.68	4.18±1.1

[a]Maximum fermentation rate was determined using time points corresponding to the steepest decline in weight.
Data means are shown associated with their standard deviation.

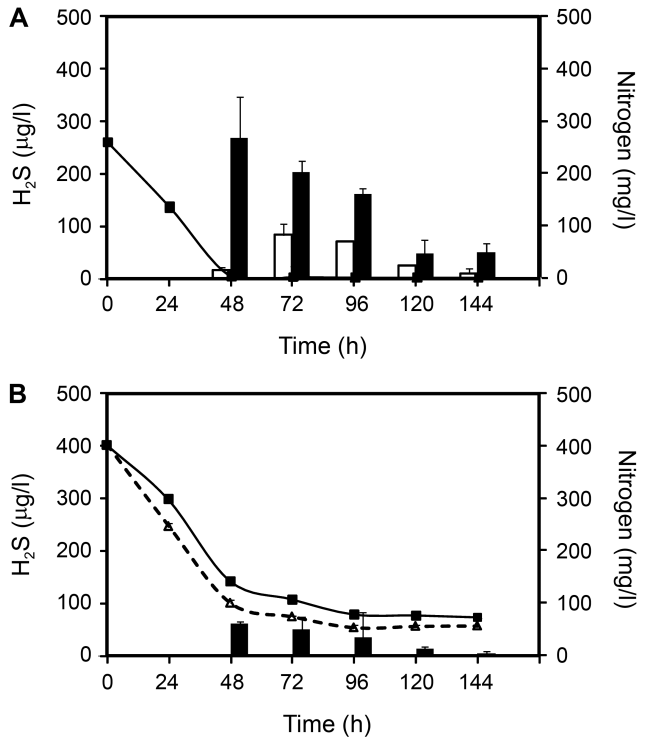

Fig. 2. Hydrogen sulfide production by yeast strains UCD522 (■) and PYCC4072 (□) cultured in synthetic grape juice medium at 20°C, containing an initial nitrogen concentration of either 267 mg (**A**) or 402 mg N/l (**B**).
Time course of nitrogen consumption by UCD522 (dashed line) and PYCC4072 (solid line). Data shown represent mean values calculated from triplicate fermentations.

Both strains produced more sulfide in GJM_{267}, and particularly more at 72 h than at 120 h.

Cross-Talk Between H_2S Liberation and Nitrogen Availability with Gene Expression Levels

To evaluate whether differences between strains in respect to liberation of H_2S and nitrogen availability could be associated with gene expression levels, the expression levels in both UCD522 (high sulfide producer, according to the results described above) and PYCC4072 (medium producer) of *MET3*, *MET5/ECM17*, *MET10*, *MET16*, and *MET17*, along with *SSU1*, were analyzed by real-time RT–PCR (Table 3). The analysis was performed at two different sampling time points (72 and 120 h), because nitrogen was completely consumed at both time points in GJM_{267} and there still was nitrogen available in GJM_{402}, and distinct amounts of sulfide were produced by both strains at each time point.

According to the results obtained in the current study, in the lower sulfide producer (PYCC4072), *MET 10*, *MET16*, *SSU1*, and *MET3* were all more highly expressed when there was nitrogen available in the extracellular media (GJM_{402}, Table 3). These findings corroborate with the analysis of previous results obtained in genome-wide analysis in PYCC4072 [12, 13]. In fact, *in silico* analysis of the output of a prior gene expression analysis of strain *Saccharomyces cerevisiae* PYCC4072 (http://www.ncbi.nlm.nih.gov/geo/; Accession No. GSE5842) showed that *MET3*, *MET14*, *MET16*, *MET5/ECM17*, and *MET10* were all higher expressed at the early stages of the control (GJM_{267}) and N-limiting (GJM_{66}) fermentations (Fig. 3A), with increased values in GJM_{267}. Additionally, *MET2* and *MET17*, encoding L-homoserine-*O*-acetyltransferase and *O*-acetyl homoserine-*O*-acetyl serine sulfhydrylase, respectively, which are both involved in the first steps of sulfide incorporation during the biosynthesis of methionine and cysteine, behaved in a similar fashion (data not shown). The expression of these genes may have been induced by the lack of sulfur amino acids in the media, which could act to derepress various genes in the SRS pathway. On the other hand, genes involved in sulfur amino acid biosynthesis (Fig. 3B) also show higher expression levels early in the process, but in this case, being increased in nitrogen-limited fermentation compared with control fermentation. The differing expression pattern of genes involved in

Table 3. Mean gene expression levels and sulfide production in yeast strains UCD522 and PYCC4072.

INC (mg/l)	TS	Strains	N concentration (mg/l)	Gene	Expression level	H_2S (µg/l)
267	72	PYCC4072	1.23±0.17	*MET3*	11.0±1.16	83.4±20.5
				MET5/ECM17	0.26±0.05	
				MET10	3.49±0.32	
				MET16	3.28±0.56	
				MET17	0.36±0.05	
				SSU1	4.25±1.04	
		UCD522	1.42±0.52	*MET3*	4.03±1.08	202.1±23.3
				MET5/ECM17	0.63±0.04	
				MET10	1.04±0.07	
				MET16	3.58±0.11	
				MET17	0.25±0.18	
				SSU1	11.92±3.32	

Table 3. Continued.

INC (mg/l)	TS	Strains	N concentration (mg/l)	Gene	Expression level	H_2S (μg/l)
	120	PYCC4072	0.0	*MET3*	4.84±1.62	24.5±0.8
				MET5/ECM17	0.15±0.04	
				MET10	1.53±0.62	
				MET16	5.18±0.01	
				MET17	0.41±0.17	
				SSU1	1.44±0.497	
		UCD522	0.0	*MET3*	10.02±2.29	48.60±26.5
				MET5/ECM17	1.31±0.23	
				MET10	1.27±0.33	
				MET16	19.49±0.29	
				MET17	0.45±0.12	
				SSU1	13.78±1.02	
402	72	PYCC4072	105.77±3.35	*MET3*	29.29±4.25	0.96±0.8
				MET5/ECM17	0.39±0.24	
				MET10	9.95±2.79	
				MET16	13.31±2.21	
				MET17	0.74±0.19	
				SSU1	7.08±1.60	
		UCD522	72.55±2.95	*MET3*	12.51±1.22	50.50±24.4
				MET5/ECM17	1.70±0.23	
				MET10	3.83±0.62	
				MET16	7.27±3.07	
				MET17	0.34±0.00	
				SSU1	14.86±5.71	
	120	PYCC4072	74.95±7.50	*MET3*	18.68±4.97	0.43±0.3
				MET5/ECM17	0.16±0.01	
				MET10	7.11±1.38	
				MET16	9.05±0.93	
				MET17	0.85±0.26	
				SSU1	6.08±1.55	
		UCD522	54.29±3.36	*MET3*	7.35±2.50	12.30±6.2
				MET5/ECM17	1.58±0.18	
				MET10	3.66±2.93	
				MET16	11.24±6.58	
				MET17	0.66±0.33	
				SSU1	22.17±7.03	
			Effects	INC	$P<0.001$	$P<0.001$
				TS	0.6843	$P<0.001$
				S	0.1337	$P<0.001$
				G	$P<0.001$	
				INC*TS	0.0073	$P<0.001$
				INC*S	$P<0.001$	0.0235
				INC*G	$P<0.001$	
				TS*S	$P<0.001$	0.0011
				TS*G	$P<0.001$	
				S*G	$P<0.001$	
				INC*TS*S	0.3156	0.0965
				INC*TS*G	$P<0.001$	
				INC*S*G	$P<0.001$	
				TS*S*G	0.4752	
				INC*TS*S*G	$P<0.001$	

Data means are shown associated with their standard deviation. INC, initial nitrogen concentration; TS, time of sampling; S, strain; G, gene.

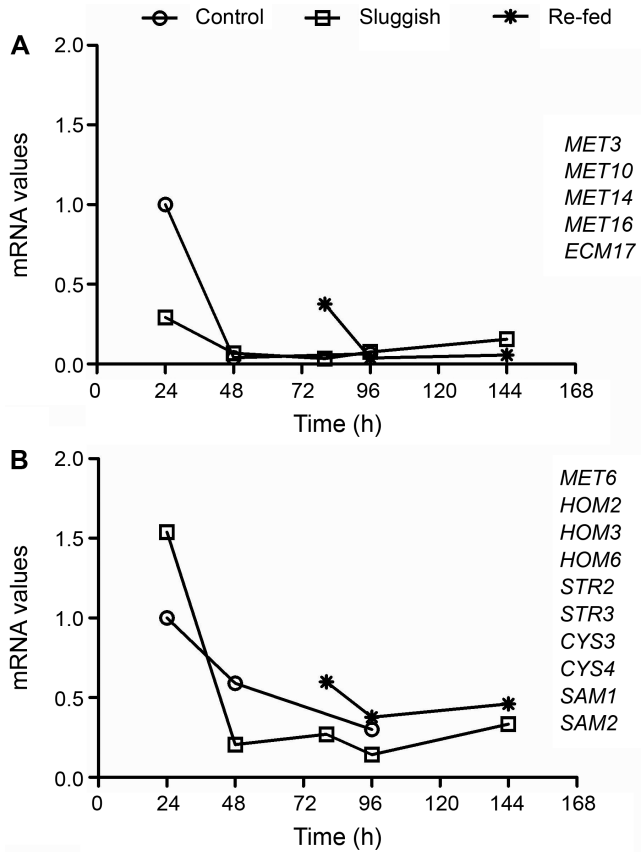

Fig. 3. Transcript profiles of *S. cerevisiae* PYCC4072 genes involved in sulfur assimilation (**A**) and sulfur amino acids biosynthesis (**B**) in culture solutions containing either 267 mg N/l (control conditions, ○), 66 mg N/l (□), or 66 mg N/l followed by supplementation of 200 mg N/l (✻) (data taken from the analysis of results found by Mendes-Ferreira *et al.* [12]).
The values are based on the ratio between the median expression level at the indicated stage and that measured after 24 h of fermentation in non-limiting conditions.

sulfur assimilation and sulfur amino acid biosynthesis between control and limiting conditions is consistent with the fall in H_2S production observed when fermentation is limited by nitrogen availability [14]. The addition of nitrogen under these conditions stimulated the expression of all the *MET* genes, even though there was a delay in the onset of regrowth [12]. Thus, here, nitrogen availability had more of an impact on gene expression than did the growth stage.

The expression levels differed between the two strains and varied ($P<0.001$) among genes. The results also showed that the initial nitrogen concentration affected gene expression levels ($P<0.001$), being higher in GJM_{402}, irrespective of sampling time or strain identity. The interaction ($P<0.001$) between sampling time and strain identity could be due to the fact that the *MET* genes expression levels peaked in UCD522 at 120 h, whereas in PYCC4072 these peaked at 72 h. In GJM_{267}, higher mRNA levels were generally found in UCD522 than in PYCC4072.

The expression levels of *MET5/ECM17* and *MET17* were weak in both strains, and were independent of both the initial nitrogen concentration and the sampling time; this was consistent with their downregulation when yeast stops growing, as has been suggested elsewhere [17]. Our results do, however, differ from those obtained with strain VIN13, in which all the genes involved in the reduction of sulfate and the incorporation of sulfide were highly expressed following the addition of DAP to a natural grape juice [10].

For *MET3*, the expression level was highest in GJM_{402}, particularly at the early stationary phase (72 h). There was also a significant interaction ($P<0.001$) between gene expression level and strain identity, with *MET3* being the most highly expressed gene in PYCC4072 and *SSU1* in UCD522. The expression of *SSU1*, which was the most highly expressed of the genes analyzed here, in UCD522, rather than being responsive to nitrogen status, was influenced more heavily by the timing of the stationary growth phase, since it was more expressed at 120 h. Similarly, *MET16* was the only gene more highly expressed in the lower nitrogen media (GJM_{267}) at this time point. Overall, *MET10* was the gene that responded most readily to nitrogen status in UCD522.

Within the range of nitrogen made available to the yeast in the present study, the quantity of sulfide produced was inversely correlated to the initial nitrogen concentration. Both strains, but in particular strain UCD522, produced significant amounts of this undesirable compound in GJM_{267}, whereas in GJM_{402}, strain PYCC4072 (but not UCD522) liberated only an undetectable trace of H_2S. The regulation of yeast's sulfur and nitrogen metabolism is thus highly genotype-dependent, and may have reflected in these two strains differences in the activity level of both the upstream enzymes involved in SRS, and the downstream ones involved in sulfur amino acid biosynthesis [22]. Considering the equivalent amounts of SO_2 released by the two tested strains, the differences in their rate of sulfide formation must reflect both the activity of sulfite reductase as well as a higher efficiency of OAH sulfhydrylase to incorporate H_2S [21]. Since the expression levels of the various genes were higher in GJM_{402}, whereas the levels of H_2S released were higher in GJM_{267}, it was difficult to propose a mechanism for cross-talk between gene expression and H_2S liberation. However, when gene expression levels were correlated with the amounts of sulfide produced, the indication was that *MET10* and *SSU1* in both strains, but just *MET16* in PYCC4072, were upregulated when sulfide production was repressed. One explanation for this correlation is related to the energy demand of the upstream SRS pathway; when nitrogen is plentiful, the yeast cell is

better able to obtain sufficient energy for the efficient assimilation of sulfate. This sulfate is then reduced to sulfide, in which form it can be readily incorporated into the carbon skeleton accessible in the intracellular pool. Inter-strain variability for the ability to incorporate sulfide has been explained as reflecting variation in the expression level of *MET17* [21]. However, in this study, neither strain expressed this gene very strongly; it was marginally more expressed in PYCC4072 than in UCD522, but the difference was insufficient to account for the more effective incorporation by PYCC4072 of sulfide into OAH, and its reduced tendency to release sulfide in high nitrogen conditions. With respect to *SSU1*, an additional explanation could be given: since this gene is expressed more strongly in UCD522, which is more resistant to sulfite than PYCC4072 (results not shown), corroborating previous observation in T73 (sulfite-resistant strain) and 71B (sulfite-sensitive strain) [2], there may be a direct relationship between *SSU1* expression and resistance to sulfite. In our strains, *SSU1* expression was higher in the high- than in the medium-sulfide producing strain, consistent with there being a connection between sulfite resistance and H_2S production.

Our experiments have given some insights on how yeast strains respond to nitrogen availability at the molecular level and how this response is associated with sulfide production. Nevertheless, further research is clearly needed to elucidate how strain dependent the production of sulfide is.

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