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Sulfur and Adenine Metabolisms Are Linked, and Both Modulate Sulfite Resistance in Wine Yeast

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Sulfite treatment is the most common way to prevent grape must spoilage in winemaking because the yeast *Saccharomyces cerevisiae* is particularly resistant to this chemical. In this paper we report that sulfite resistance depends on sulfur and adenine metabolism. The amount of adenine and methionine in a chemically defined growth medium modulates sulfite resistance of wine yeasts. Mutations in the adenine biosynthetic pathway or the presence of adenine in a synthetic minimal culture medium increase sulfite resistance. The presence of methionine has the opposite effect, inducing a higher sensitivity to SO_2. The concentration of methionine, adenine, and sulfite in a synthetic grape must influences the progress of fermentation and at the transcriptional level the expression of genes involved in sulfur (*MET16*), adenine (*ADE4*), and acetaldehyde (*ALD6)* metabolism. Sulfite alters the pattern of expression of all these genes. This fact indicates that the response to this stress is complex and involves several metabolic pathways.

KEYWORDS: Wine yeast; stress resistance; sulfite; fermentation; adenine; methionine

INTRODUCTION

The wine yeast *Saccharomyces cerevisiae* faces a great variety of stress conditions during its role in winemaking (*1*). One of these stress conditions is caused by the addition of sulfite to the grape must (*2*). Sulfite refers to all species of sulfurous acid, including sulfur dioxide (SO_2). This chemical is used in the wine industry to prevent growth of spoiling microorganisms, while *S. cerevisiae* is relatively resistant to sulfite. Such resistance has been studied at the molecular level in laboratory strains. The main protein involved in sulfite resistance is the sulfite pump Ssu1p (*3*, *4*) that mediates sulfite efflux. Deletion of this gene leads to sulfite sensitivity. This membrane protein is regulated at the transcriptional level by the transcription factor *FZF1* (*5*, *6*). Mutations in this gene also lead to sulfite sensitivity.

In wine strains there is a particular version of the *SSU1* gene called *SSU1-R* that was produced by reciprocal translocation between chromosomes VIII and XVI (*7*, *8*). This event leads to a *SSU1* gene with a promoter which contains sequences derived from the *ECM34* gene and results in a higher *SSU1* expression and sulfite resistance. Both alleles of *SSU1* are regulated in different ways (*9*).

An important feature of sulfite is its chemical reactivity, particularly with carbonyl groups (*10*). This fact explains why a high production of acetaldehyde by a yeast strain leads to increased sulfite resistance (*11*, *12*). The opposite is also true, and we have shown that acetaldehyde increases transcription of sulfur metabolism coding genes, such as *MET16* (*13*), establishing another link between both compounds. Regarding sulfur metabolism, it is worth mentioning that methionine addition decreases yeast thermotolerance (*14*), suggesting that sulfur metabolism plays another role in stress resistance.

Transcription of genes encoding proteins involved in metabolism strongly depends on media composition in *S. cerevisiae*. Regarding sulfur metabolism, yeast is able to import sulfate from the medium and reduce it first into sulfite and then into sulfide to finally incorporate it into sulfur amino acids such as methionine and cysteine (*15*). However, whenever a source of organic sulfur is present, this sulfate assimilation pathway is switched off. Methionine is the key regulator of this event (*15*). *MET* genes transcription is repressed in laboratory strains when methionine is present in the medium at a concentration of over 0.05 mM. In a similar way, adenine in the media represses its own biosynthetic pathway (*16*), acting on the first step of the path, the enzyme phosphoribosylpyrophosphate amidotransferase coded by *ADE4*.

This paper studies sulfite resistance of wine yeast and relates it with the metabolic state of the cell. Our analysis demonstrates that a novel link exits between adenine metabolism, sulfur metabolism, and sulfite resistance. Mutations in the adenine biosynthetic pathway or the presence of adenine in the medium lead to an enhanced resistance to sulfite. Gene expression

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Table 1. Strain Resistance to Sulfite

strains	sulfite stress resistance[a]	acetaldehyde stress resistance[b]	oxidative stress resistance[c]	relative nitrogen needs[d]	fermentation speed[d]
L2056	8	>99%	39.4 ± 0.6	medium	moderate
T73	7	>99%	36.8 ± 1.4	low	moderate
EC1118	6	>99%	39.8 ± 0.2	low	fast
RC212	6	>99%	39.11 ± 0.7	high	moderate
R2	6	>99%	38.5 ± 0.5	high	moderate
BM45	6	>99%	40.9 ± 0.9	high	moderate
DV10	4	>99%	38.5 ± 0.2	low	fast
QA23	4	>99%	39.8 ± 0.5	low	fast
M2	4	>99%	40.3 ± 0.5	medium	moderate
CY3079	4	>99%	37.7 ± 0.3	high	moderate
CSM	4	>99%	37.4 ± 0.5	high	moderate
BDX	4	>99%	38.8 ± 0.6	high	moderate
71B	2	96.2 ± 0.7%	36.7 ± 0.5	low	moderate

[a] Strains were replica-plated from YPD plates to YPD+TA plates buffered at pH 3.5 and containing increasing amounts of Na_2SO_3. The highest sulfite (in mM) concentration in which cells grow is shown. [b] Exponentially growing cultures in YPD were treated with 20 g/L acetaldehyde for 1 h in closed tubes. Viability was measured by the citrate–methylene blue method (*20*) in three independent experiments. [c] 2×10^5 cells from a culture grown to saturation in YPD were spread on a YPD plate, and a circle of Whatman paper with 10 μL of H_2O_2 (33%) was placed in the middle of the plate. The diameter of the halo (in mm) of inhibition was measured in three plates. [d] Information from Lallemand Inc. (www.lallemandwine.us/products/yeast_chart.php).

analysis was carried out on *MET16*, *ADE4*, *SSU1*, and *ALD6* genes during synthetic must fermentation in order to study all these interactions at the molecular level. SO_2 acts by modulating the transcription of genes of the sulfur, adenine, and acetaldehyde metabolism.

MATERIALS AND METHODS

Yeast Strains and Media. Yeast strains shown in **Table 1** were provided by Lallemand Inc. (Canada). L2056 haploid derivatives were a gift from Michelle Walker (*17*). In the experiments under laboratory conditions YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose) and SD medium (0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% (w/v) glucose) were used. Media with sulfite were buffered with 75 mM tartaric acid (TA) according to Hoon et al. (*18*). For microfermentation experiments, synthetic must containing 300 mg of N/L (MS300) was prepared according to Riou et al. (*19*) but with the following differences: it contains equimolar amounts of glucose and fructose instead of only glucose, no cysteine was added, and variable amounts of methionine (from 1 to 24 mg/L) and adenine (from 4 to 15 mg/L) were included depending on the condition to be tested (see below).

Growth and Stress Conditions. Sulfite resistance on plate was tested by replica plating cells grown in YPD plates on YPD+TA (tartaric acid) plates buffered at pH 3.5 and containing increasing amounts of Na_2SO_3 (*18*). In the experiments on liquid medium, 10 mg/L of $K_2S_2O_5$ was added to SD+TA (pH 3.3) and growth was followed measuring the OD_{600} in three independent cultures. To study acetaldehyde resistance, 20 g/L acetaldehyde was added to exponentially growing cultures in YPD and incubation continued for 1 h. Viability was measured by the citrate−methylene blue method (*20*) in three independent experiments. Oxidative stress was measured by plating 2×10^5 cells from a culture grown to stationary phase in YPD on a YPD plate and placing a circle of paper with 10 μL of H_2O_2 (33%) in the middle of the plate. The diameter of the halo of inhibition in three independent experiments was measured.

For microfermentation experiments cells from overnight cultures in YPD were inoculated at a final concentration of 2×10^5 cells/mL in the synthetic must mentioned above in three independent cultures. Incubations were carried out at several (22 and 30 °C) temperatures without shaking. Evolution of the fermentations was followed by determination of sugar consumption, as previously described (*21*).

Table 2. Oligonucleotides Used in This Work

name	sequence
MET16a	GCTGGAAACGCCACAGGAG
MET16b	ATTGCGCGAATCGGCTGGC
ADE4a	AAACCACTCCAGTAGCTCCG
ADE4b	CCCTCTCTATAAGGCTTCCC
SSU1a	TGATGGTCATGGGTGTCGGC
SSU1b	AGCCCATAGCGAGCAATGCC
ALD6a	ACGACACTGAATGGGCTACC
ALD6b	CTTCAACATCTTGGCCACCT

Analysis of Gene Expression. RNA was isolated by the hot-phenol procedure described by Kohrer and Domdey (*22*). It was quantified, and its quality and concentration were checked in a 1% agarose gel in TAE buffer.

RNA analysis by Northern blot, hybridization, and quantification were carried out as previously described (*23*). Probes were obtained by PCR from genomic DNA of T73 strain using the oligonucleotides described in **Table 2**.

RESULTS

Mutations in the Adenine Synthesis Pathway Lead to Increased Sulfite Resistance. As the starting point for the study of sulfite resistance, we first characterized several commercial yeast strains (Lallemand Inc.; www.lallemandwine.us/products/yeast_ chart.php) for their resistance to this compound. We performed replica plating of these strains on Petri dishes containing increasingly higher amounts of sulfite in order to estimate the threshold of sulfite resistance (**Table 1**). The most sulfite-sensitive strain was 71B, and the more resistant one was L2056. T73 is the second most resistant strain. There was no correlation between sulfite resistance and other oenological parameters such as nitrogen needs and fermentation speed (see **Table 1**). A link between acetaldehyde and sulfite has been clearly established. It has been shown that production of acetaldehyde leads to an increased sulfite resistance (*11*, *12*). We wanted to know if resistance to acetaldehyde was correlated to resistance to sulfite. We have previously shown that industrial strains *of S. cerevisiae* are very resistant to this chemical (*23*). This fact was true with all strains tested (see **Table 1**), although strain 71B, the least resistant strain to sulfite, showed a slight sensitivity to the presence of 20 g/L acetaldehyde. Therefore, in strain 71B there is a reduced ability to face both sulfite and acetaldehyde stress conditions, and this indicates that the mechanisms that deal with these stress conditions may be related. We also tested resistance to other stress conditions such as oxidative stress to rule out that 71B has a generally lower stress resistance. This strain has a good behavior against oxygen peroxide compared to other strains more resistant to sulfite and acetaldehyde.

Previous data from our group have established a link between acetaldehyde and sulfur metabolism (*13*). For this reason we began studying a set of haploid strains derived from L2056 wine strain (*17*) that show different acetaldehyde and SO_2 production during vinification. When we tested the sulfite resistance of these strains we did not find a correlation between acetaldehyde or sulfite production (*17*) and sulfite resistance (data not shown). However, the most intriguing result was that a large number of red colonies arose on plates containing sulfite. **Figure 1** shows the behavior of several selected red mutants (dark patches) derived from two of those haploid derivatives C9 and 11D (light patches). The mutants grew better that the parental white strains when replicated on sulfite-containing plates. Red colonies are

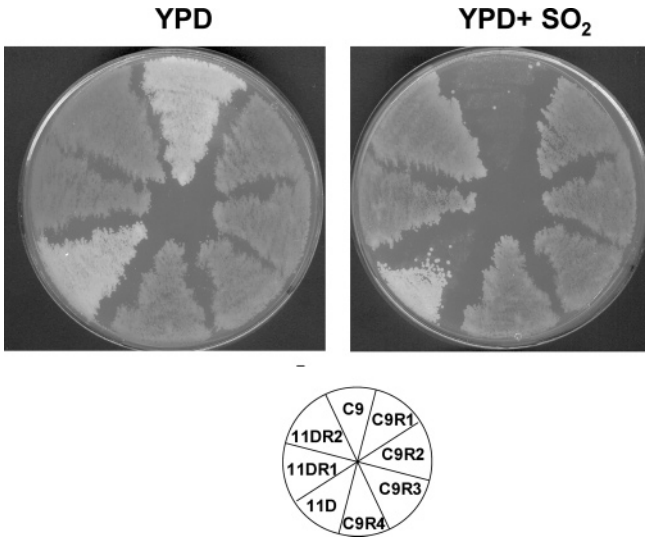

Figure 1. Sulfite resistance of *ade*⁻ mutants. Strains C9 and 11D (derived from L2056 strain) and their red derivatives (C9R1, C9R2, C9R3, C9R4, 11DR1, 11DR2; darker patches) grown in YPD were replica-plated to YPD+TA pH 3.5 containing 9 mM Na_2SO_3.

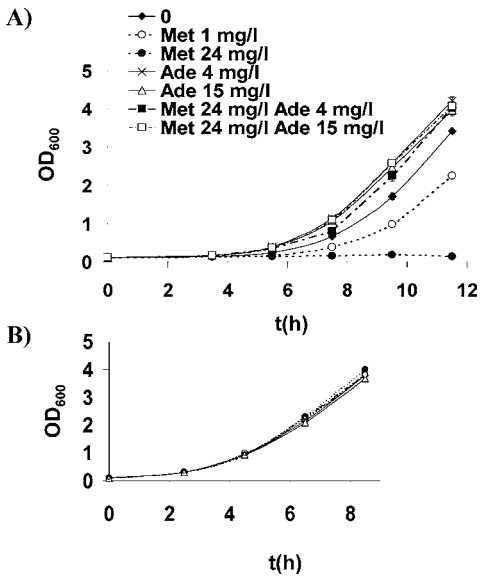

Figure 2. Effect of methionine and adenine in sulfite sensitivity. 71B strain was grown in SD+TA pH 3.3 with (**A**) or without (**B**) 10 mg/L $K_2S_2O_5$ containing different amounts of methionine (1 or 24 mg/L) or adenine (4 or 15 mg/L). Growth was followed by measuring OD_{600}.

typical of mutants in the adenine synthetic pathway such as *ade1* and *ade2* (*24*). The red strains obtained were unable to grow in synthetic medium unless adenine was added. We picked the mutants derived from C9 to further characterize their phenotype. Most of them (C9R1, C9R3, and C9R4 but not C9R2) grew on minimal medium when transformed with the centromeric plasmid pRS412 that contains the *ADE2* gene (data not shown). These results indicate that C9R1, C9R3, and C9R4 are *ade2* mutants. C9R2 could be an *ade1* mutant because this is the other mutation in the adenine biosynthetic pathway that leads to a red color. Laboratory strains that lack the *ADE2* gene were also more resistant to sulfite, but the difference was less dramatic than in the industrial strain (data not shown). All these results suggest that mutations in the adenine biosynthesis pathway make yeast more resistant to the toxic effects of SO_2.

Adenine and Methionine Modulate Sulfite Resistance. To study the influence of the medium composition in sulfite resistance we chose 71B and T73 strains as sulfite-sensitive and -resistant strains, respectively (**Table 1**). These strains have been described by their manufacturers as strains with low nitrogen needs, while L2056 (which has the highest sulfite resistance) shows medium nitrogen requirements. As nitrogen depletion may affect incorporation of sulfur to amino acids (*25*) producing high amounts of H_2S, an unfavorable metabolite from the organoleptic point of view, we chose T73 instead of L2056 as the sulfite-resistant strain to rule out the potential effect of nitrogen metabolism. Besides, T73 is our model strain in terms of gene expression (*13*, *23*).

We tested the growth of 71B on SD medium containing sulfite (**Figure 2A**). In this minimal medium we could analyze the importance of several metabolites in sulfite resistance. Sulfite sensitivity was increased when methionine was added to the media. We tested two amounts of methione, 1 and 24 mg/L. These amounts cover the range of methionine found in natural grape musts (*26*). The higher the amount of methionine, the more acute the toxicity of sulfite. A 24 mg/L amount of methionine caused a dramatic stop in growth compared to the control strain grown on SD. Even the smaller amount of 1 mg/L methionine caused a relevant defect in cell proliferation. Methionine is the signal that mediates repression of the sulfur assimilation pathway (*15*). Our results suggest that repression of sulfur metabolism blocks a pathway for sulfite assimilation (its reduction into organic sulfur) and leads to an increased sulfite sensitivity. In laboratory conditions the nonrepressive concentration is under 0.05 mM (*15*), i.e., 19.9 mg/L. According to our data wine strains are very sensitive to external methionine concentration because a very small amount of methionine (1 mg/L) is sensed.

Due to the results described in the previous section, we also investigated the effect of adenine addition in growth in the presence of sulfite. We used the amounts of adenine usually found in grape must, i.e., 4 and 15 mg/L (*26*). Surprisingly, addition of any amount of adenine lead to an increased sulfite resistance. Therefore, blocking the adenine biosynthesis pathway by mutation (**Figure 1**) or adding exogenous adenine (**Figure 2**) leads to a better tolerance to sulfite. Adenine addition relieves the negative effect of methionine. Even when the highest amount of methionine tested is present (24 mg/L), adenine addition causes the culture to grow better than the control condition, and 15 mg/L is more effective than 4 mg/mL. All cultures reached similar final densities (data not shown), indicating that this effect is transient. Therefore, in this range of concentrations the less methionine and more adenine in the growth media the better it is for 71B to grow in the presence of sulfite. All these differences disappear when sulfite is absent (**Figure 2B**), indicating that addition of methionine and adenine per se does not affect yeast growth in a relevant way in this condition. When T73 (a more sulfite-resistant strain) was tested in these conditions, similar but less dramatic changes in growth were observed (data not shown). This indicates that the effect of adenine and methionine amount in the media is shared by all strains tested.

Methionine Levels Affect Growth, Sulfite Sensitivity, and Gene Expression during Fermentation. To test the effect of adenine and methionine on yeast performance during fermentation, microfermentations on synthetic must with different amounts of these compounds were set up. Synthetic must was prepared according to Riou et al. (*19*), but the concentration of sulfur amino acids was altered. No cysteine was added because this amino acid is undetectable in most grape musts analyzed (*26*). Methionine concentration was adjusted according to the amounts normally found in natural grape juices, from 1 to 24

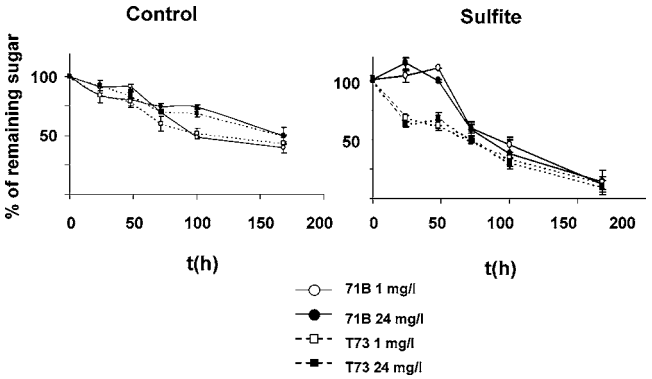

Figure 3. Fermentations with variable amounts of methionine. Fermentation of synthetic must containing 1 or 24 mg/L of methionine and 10 mg/L of adenine was carried out with strains 71B and T73 without (Control) or with 100 mg/L $K_2S_2O_5$ (Sulfite). Fermentations were carried out in triplicate and followed measuring sugar consumption; data shown correspond to average and standard deviation.

mg/L. Adenine, which is ignored in most synthetic musts, was supplemented at the amounts indicated. The incubation was at 22 °C without shaking. In our lab we noticed that fermentations on synthetic must without shaking tend to be slower than fermentations carried out with shaking or with grape juice. This is particularly true in the experiments made with this must; we do not know if it is due to the lack of cysteine or the presence of adenine. However, initial growth is good, and cultures reach up to 5×10^7 cfu/mL and OD_{600} around 10 (data not shown). Because our main interest is to see the effect of sulfite at the beginning of the fermentation, when cells face this exogenous stress, we only analyzed the first hours of fermentation, when most of the sugar consumption is achieved.

First, we tested the effect of methionine, using 1 or 24 mg/L of amino acid in the presence of an average amount of adenine (10 mg/L; *26*) both with or without sulfite (50 mg/L). **Figure 3** shows the fermentation rate of 71B and T73 strains in these conditions measuring sugar consumption. In the absence of sulfite, the maximal amount of methionine slows down sugar consumption of T73 while it helps 71B to start fermentation and causes a lower rate of sugar consumption later on. When SO_2 is present there is a delay in the onset of the fermentation in 71B for both amounts of methionine, but there is better performance during the second half of the fermentation. Under this condition there is no difference between the two amounts of methionine during fermentation. In T73 the advance of fermentation in the presence of sulfite is not affected by methionine either, and there is a better overall rate in sugar consumption compared to the fermentation when sulfite is absent. Therefore, SO_2 is only toxic for some strains at the beginning of fermentation, but it helps yeast grow in most assays. This effect seems to be strain independent. It has been shown that sulfite may help yeast to deal with ethanol stress (*27*), and this may be the reason it helps yeast especially during the second half of fermentation.

Regarding gene expression (**Figure 4**), we studied several genes related with sulfur, adenine, and acetaldehyde metabolism. *MET16* codes for the 3′-phosphoadenyl sulfate reductase, an enzyme involved in the sulfate assimilation pathway. This gene is under a typical sulfur metabolism transcriptional control (*15*), and it is activated by acetaldehyde (*13*). According to the transcriptional regulation described for laboratory strains, *MET16* transcription is repressed in T73 strain by methionine at the beginning of fermentation. However, in the case of 71B, this effect is not found. In fact, fermentation conducted by this strain under maximal methionine concentration shows higher *MET16* levels, indicating that this sulfite-sensitive strain has an altered sulfur metabolism. When sulfite is present, the results change. The typical *MET16* mRNA maximum at 24 h is shifted to 48 h in all conditions and strains, indicating that sulfite is able to promote at least a partial repression of *MET* genes in fermentation experiments, even when growth is not affected, as is the case of T73 strain (see **Figure 3**).

ADE4 codes for the first step in adenine biosynthesis. In the absence of sulfite the pattern of expression of this gene in T73 is similar despite methionine concentration, with a peak of induction around 24 h. However, 71B shows an activation of *ADE4* transcription when more methionine is added. The expression pattern changes in both strains when sulfite is added, indicating at the molecular level that *ADE4* transcription responds to sulfite in the media. When SO_2 is present, *ADE4* transcription in T73 is repressed by methionine.

SSU1 codes for the sulfite efflux pump. Levels of this mRNA are very low in the sulfite-sensitive strain 71B compared to T73. The presence of a specific *SSU1* allele called *SSU1-R* has been linked to sulfite resistance (*7*, *8*). We looked for the presence of this allele in 71B and found it absent. It has the same chromosomal arrangement as laboratory strains (data not shown). Transcription of *SSU1* in this strain is not significantly affected by the amount of methionine. In T73 (which contains the SSU1-R allele; 8) methionine seems to repress the sulfite pump, particularly in the presence of sulfite, although the overall pattern of transcription is not significantly affected when this chemical is present. This indicates a transcriptional control by the sulfur source. Sulfite itself is not a powerful inducer of the pump gene transcription.

As a marker of acetaldehyde metabolism, the main cytosolic aldehyde dehydrogenase gene *ALD6* was monitored. In the absence of sulfite there is no effect in methionine addition, but important differences in the expression levels at the time point of maximum mRNA levels are found among strains. *ALD6* transcription is higher in T73 than in 71B. However, sulfite again dramatically changes the expression pattern in both strains, particularly in the T73 strain that shifts its maximum from 24 to 8 h, indicating a function of aldehyde dehydrogenase in sulfite metabolism.

Adenine Levels Affect Gene Expression during Fermentation. Our next experiments were aimed at studying the role of different amounts of adenine during fermentation with a fixed amount of methionine (**Figures 5** and **6**). **Figure 5** shows sugar consumption in microfermentations with different amounts of adenine (4 and 15 mg/L), typical levels of this purine found in must (*26*), in the presence of 10 mg/L methionine, an average level for this amino acid in different natural musts. There is little difference in terms of growth between the two amounts of adenine with or without sulfite. T73 tends to grow better than 71B in the presence of sulfite, as expected.

Gene expression was also studied in these fermentations (**Figure 6**). The *MET16* expression pattern was similar between both strains in the absence of sulfite. However, in 71B, expression of this gene during the first hours seems to be activated by adenine while it is repressed in T73. Sulfite addition globally changes the expression pattern in a similar way to that described in the case of variations in the amount of methionine (**Figure 4**). Regarding *ADE4* expression, as expected according to its regulation by adenine, there is lower initial expression of this mRNA when the highest amount of adenine is added in both strains. However, in the 71B strain this pattern changes

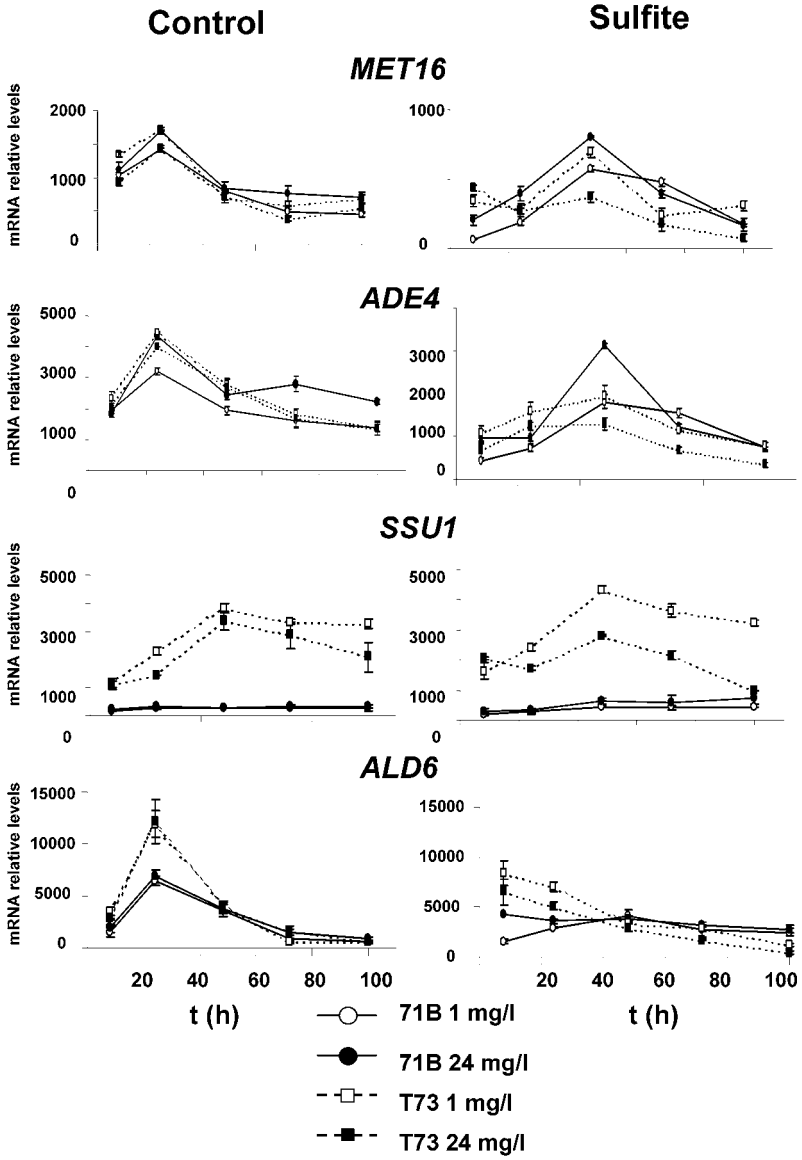

Figure 4. Gene expression in fermentations containing different amounts of methionine. Gene expression of the fermentations described in **Figure 3** is shown. mRNA levels normalized against rRNA are shown for four genes (*MET16*, A*DE4*, *SSU1*, and *ALD6*). Probes were obtained by PCR from primers of **Table 2**. Fermentations were carried out in triplicate; data shown correspond to average and standard deviation

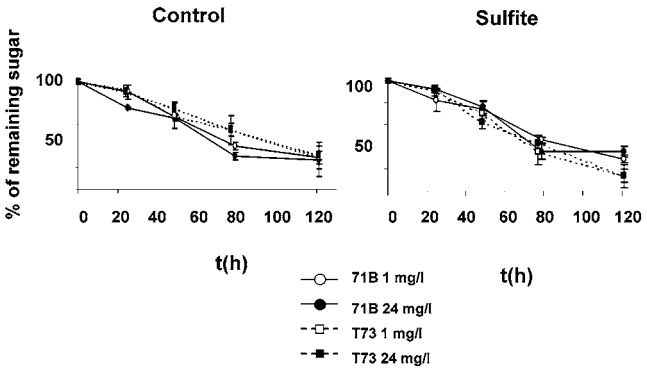

Figure 5. Fermentations with variable amounts of adenine. Fermentation of synthetic must containing 4 or 15 mg/L of adenine and 10 mg/L of methionine was carried out with strains 71B and T73 without (Control) or with 100 mg/L $K_2S_2O_5$ (Sulfite). Fermentations were carried out in triplicate; data shown correspond to average and standard deviation

rapidly and *ADE4* levels are higher and maintained in the fermentation with 15 mg/L adenine in the following points. T73 shows a peak of expression at 24 h, and then *ADE4* amount falls for both amounts of adenine. Sulfite addition does not significantly change T73 strain *ADE4* expression pattern but makes null the difference between 4 and 15 mg/L adenine in 71B strain. *SSU1* expression is not affected by adenine in 71B strain. In T73 strain adenine seems to repress slightly *SSU1* expression in the presence of sulfite during most of the time points studied, although the initial level is similar. *ALD6* levels are not affected by adenine in 71B strain, although sulfite slightly changes its relatively flat pattern. In T73 strain, adenine seems to induce this gene in the absence of sulfite, but sulfite addition changes this pattern into a repression by adenine. That indicates a strong control by adenine of this gene in this particular strain.

Effect of Methionine Concentration in Thermotolerance. Jakubowski and Goldman (*14*) proved that methionine addition increased yeast sensitivity to heat shock in laboratory conditions. To test if this is the case during must fermentation, fermentations by 71B and T73 strains on synthetic must containing different amounts of methionine (1 and 24 mg/L) were carried out at 30 °C, a high temperature in vinification terms (**Figure 7**). The

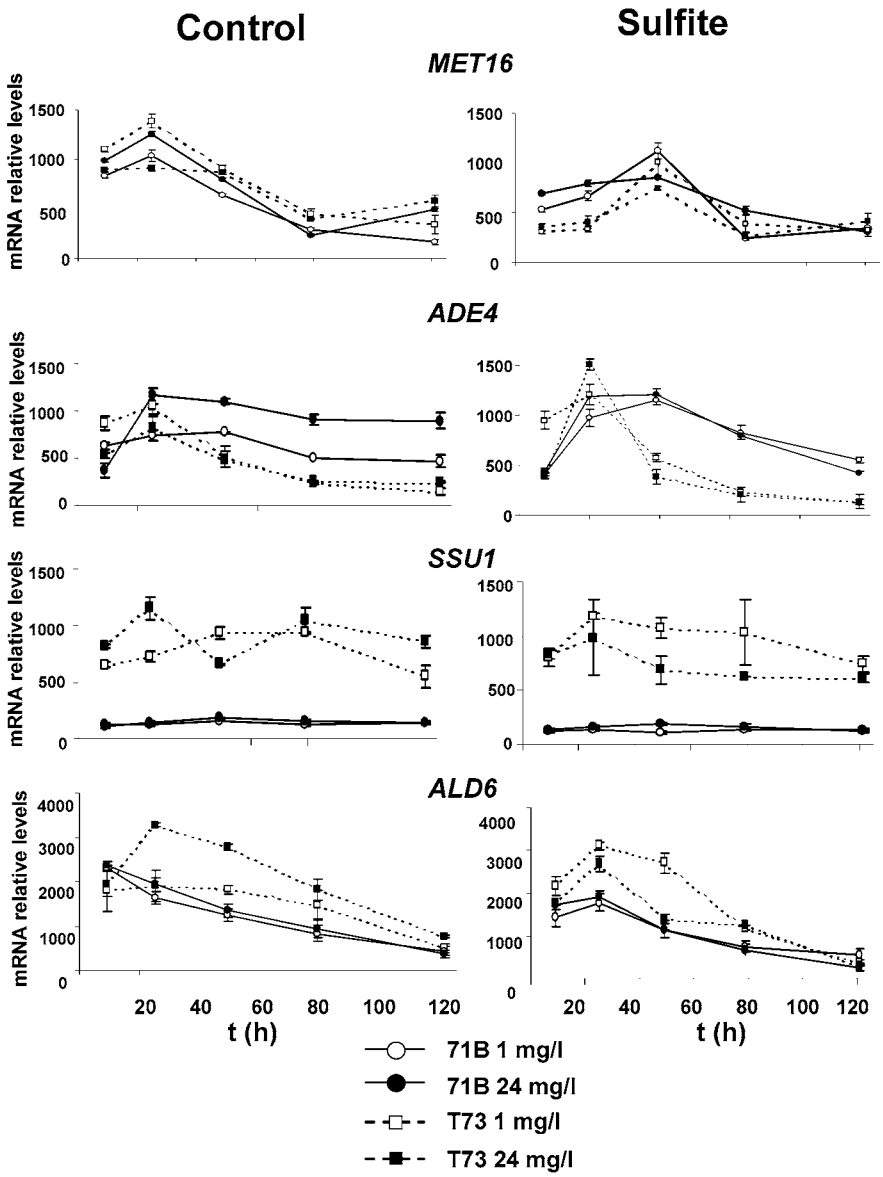

Figure 6. Gene expression in fermentations containing different amounts of adenine. Gene expression of fermentations is described in **Figure 5**. Conditions as in **Figure 4**.

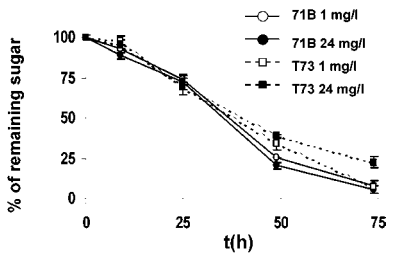

Figure 7. Effect of methionine in thermotolerance. Fermentation with 71B and T73 strains in synthetic must containing 1 and 24 mg/L of methionine was carried out at 30 °C. Fermentations were carried out in triplicate; data shown correspond to average and standard deviation.

advance of fermentation was followed measuring sugar consumption. Strain 71B does not show a significant sensitivity to methionine in those conditions. This could be explained by the abnormal sulfur metabolism shown by this strain (see above). However, T73 showed a decrease in sugar fermentation at the end of the fermentation, when methionine in the media is high, pointing to a role of methionine in stress response and cell viability also in industrial yeasts during winemaking.

DISCUSSION

The aim of this work is to achieve a better understanding of the molecular mechanisms involved in resistance to sulfite in wine yeast. First, we selected two strains as models of high (T73) and low (71B) sulfite resistance. The molecular study of sulfite resistance until now has been focused mainly on the role of the sulfite pump Ssu1p (*3*, *4*) as the main determinant in sulfite resistance. We found that *SSU1* transcription in fermentation conditions is much higher in a sulfite-resistant strain (T73) than in a sulfite-sensitive strain, such as 71B (see **Figures 4** and **5**). Sulfite-resistant strains usually have a specific allele, called *SSU1-R* (*7*, *8*). We found that this is the case in T73 but not in sensitive strain 71B, which contains the allele found in laboratory strains (data not shown), and may explain the difference in the levels of *SSU1* mRNA, much higher in T73 compared to 71B.

In this report we describe the role of other players in sulfite resistance. The biochemical pathways involved in sulfur and adenine metabolism play a role in challenging the negative effects of SO_2. Methionine is the key metabolite in sulfur metabolism. Its presence represses genes involved in sulfate

assimilation, from sulfate to organic sulfur through sulfite and sulfide (*15*). According to our data, methionine increases the toxicity of sulfite in laboratory conditions, even at the lowest levels in which this amino acid is found in grape must (1 mg/L, **Figure 2**). This indicates that enzymes involved in assimilating sulfite into sulfur amino acids are relevant to deal with the excess of sulfite. Besides, sulfite alters the expression of a typical gene in the sulfur amino acid biosynthetic pathway, *MET16*. Expression of this gene is repressed in the presence of sulfite in the resistant strain T73 (**Figure 4**). However, 71B strain, which is very sensitive to sulfite, shows an irregular sulfur metabolism with its *MET16* gene not being repressed but activated by methionine during the early stages of fermentation. This indicates that normal sulfur metabolism may be important for efficient sulfite resistance. In spite of this, the effect of methionine amount on oenological conditions is not as being determinant in sulfite resistance (**Figure 3**). In this condition many other factors may be affecting yeast performance. For instance, it has been proposed that sulfite may act as a protection against osmotic stress (*27*), so its toxicity may be less relevant in a high osmolarity medium such as grape must than in laboratory conditions.

More surprising is the unexpected role of adenine in sulfur metabolism and sulfite resistance. Spontaneous *ade*$^-$ mutants were isolated from haploid derivatives of L2056 wine strain. Those mutants showed an increased sulfite resistance. Our results demonstrate that addition of adenine enhances sulfite resistance and even reverses the negative effect of methionine (**Figure 2**). Therefore, blocking the adenine biosynthesis pathway by mutation (**Figure 1**) or repressing it by adding its final product (**Figure 2**) lead to the same result: better protection against sulfite. These results suggest that an unknown protein or metabolite used in detoxification of sulfite is regulated or produced by the purine synthetic pathway. Adenine nucleotides are required for sulfate assimilation but in a step prior to its reduction to sulfite (*15*). Adenine enters yeast metabolism via two pathways, one of which involves deamination by adenineaminohydrolase (AAH) into hypoxanthine (*28*). Hypoxanthine has been proposed to be a cofactor of mammalian sulfite oxidase (*29*). This enzyme has not been described in *S. cerevisiae*, but other enzymes in this yeast contain the cytochrome b_5 heme-binding domain that constitutes the active domain of sulfite oxidase (*30*) and could be involved in sulfite detoxification using hypoxanthine as cofactor.

There is another link between adenine and sulfur metabolism. Mutations in *ADE1* or *ADE2* genes lead to accumulation of a vacuolar red pigment (*31*). This pigment is the result of conjugation of glutathione (which contains an active sulfhydryl group) with the toxic adenine precursors phosphoribosylaminoimidazole (AIR) and phosphoribosylaminoimidazole carboxylate (CAIR) and its transport to the vacuole mediated by pumps such as Ycf1p. A similar mechanism may be participating in sulfite detoxification if sulfite reacts with AIR or CAIR. However, mutations in the *GSH1* gene involved in glutathione metabolism or in the vacuolar transporter *YCF1* do no alter sulfite sensitivity (data not shown).This indicates that those proteins do not play a role in sulfite detoxification, so maybe an unidentified protein may be linking sulfite to AIR or CAIR or another adenine metabolite could be used in this case. Besides, we measured the red pigment in the C9R1 mutant strain as described in Sharma et al. (*31*) and found no increase in the amount of red pigment when sulfite is present (data not shown). For these two reasons it seems the pathway that leads to formation of the red pigment is not involved in sulfite detoxification.

In addition, transcription of *ADE4* gene, the first step in the synthesis of adenine, is affected by the presence of sulfite and methionine during wine fermentation, establishing a molecular link between these molecules. For instance, in the presence of sulfite, *ADE4* is repressed by methionine (**Figure 4**).

In a previous work we showed that there is a relationship between acetaldehyde stress and sulfur metabolism (*13*). Acetaldehyde formation is a way to neutralize an excess of sulfite (*11*) because of its high affinity to form a complex called hydroxysulfonate. We found that sulfite alters the pattern of expression of the main aldehyde dehydrogenase gene, *ALD6* (**Figure 4**), reinforcing the link at the molecular level between both metabolites.

This work shows a complex picture of the interplay between different metabolic routes, in this case the adenine and sulfur amino acid biosynthetic pathways, and their role in protection against chemical compounds that are normal metabolites of the cell, as sulfite or acetaldehyde, but can be harmful to cells when present in excess. All these experiments were done in synthetic defined media in order to gain a better understanding of the effect of each compound. A broader study using natural grape must with different amounts of sulfur and/or adenine should be done to confirm these results.

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