

A sulphite-inducible form of the sulphite efflux gene *SSU1* in a *Saccharomyces cerevisiae* wine yeast

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Sulphite is widely used as a preservative in foods and beverages for its antimicrobial and antioxidant activities, particularly in winemaking where SO₂ is frequently added. Thus, sulphite resistance mechanisms have been extensively studied in the fermenting yeast *Saccharomyces cerevisiae*. Sulphite detoxification, involving a plasma membrane protein encoded by the *SSU1* gene, is the most efficient resistance mechanism in *S. cerevisiae*. In this study, we characterized the unusual expression pattern of *SSU1* in the wine strain 71B. We provide, for the first time, evidence of *SSU1* induction by sulphite. The study of *SSU1* expression during fermentation and in different growth conditions showed that sulphite is the main regulator of *SSU1* expression, explaining its specific pattern. Combining analyses of gene expression and growth behaviour in response to sulphite, we found that 71B displayed unique behavioural patterns in response to sulphite pre-adaptation that may be explained by changes in *SSU1* expression. Examination of the genomic organization of the *SSU1* locus and sequencing of the region revealed three different alleles in 71B, two of which corresponded to translocated VIII–XVI forms. The lack of differences between promoter regions suggests that this inducible *SSU1* expression pattern is due to modification of regulatory/signalling pathways.

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INTRODUCTION

Sulphite is widely used as a preservative in foods, beverages and pharmaceutical products for its antimicrobial and antioxidant activities; SO₂ is frequently used in wine-making. Accordingly, sulphite resistance is one of the first technological traits to be tested for the selection of oenological yeasts. Sulphite resistance mechanisms have been studied extensively in *Saccharomyces cerevisiae*.

SO₂ detoxification, involving the plasma membrane protein Ssu1p, is one of the most efficient resistance mechanisms in *S. cerevisiae* (Avram & Bakalinsky, 1997; Park & Bakalinsky, 2000). Wine yeasts also cope with SO₂ by means of other systems, such as acetaldehyde production and the upregulation of sulphite reduction systems or whole sulphur metabolism (Casalone *et al.*, 1992). The sulphite pump required for efficient sulphite efflux is encoded by the gene *SSU1*. Generally, mutations in *SSU1* cause sensitivity,

whereas overexpression confers heightened resistance to sulphite toxicity (Avram & Bakalinsky, 1997; Goto-Yamamoto *et al.*, 1998; Park & Bakalinsky, 2000).

Another gene involved in sulphite resistance in yeast is *FZF1* (Casalone *et al.*, 1992, 1994), a positive regulator of *SSU1* transcription (Avram & Bakalinsky, 1996; Avram *et al.*, 1999), which interacts with a DNA region immediately upstream from *SSU1* (Avram *et al.*, 1999). Sulphite resistance has been attributed, in particular, to a dominant *FZF1* allele (*FZF1-4*) and to the overexpression of wild-type *FZF1* (Avram & Bakalinsky, 1996; Avram *et al.*, 1999; Casalone *et al.*, 1994).

SSU1 lies on chromosome XVI in the genome of the *S. cerevisiae* laboratory strain S288c. Some highly sulphite-resistant wine strains have an *SSU1* allele (*SSU1-R*) on chromosome VIII (Goto-Yamamoto *et al.*, 1998). The upstream sequence is no longer present on this allele due to a reciprocal translocation between chromosomes VIII and XVI (Perez-Ortin *et al.*, 2002). The *SSU1-R* promoter contains up to six repeats of a 76 bp enhancer sequence from the promoter of *ECM34*, a highly expressed protein of unknown function. *SSU1-R* is expressed at much higher levels than *SSU1* (Goto-Yamamoto *et al.*, 1998). It has also

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are GQ995455–GQ995463.

Two supplementary tables of microarray data, and three supplementary figures showing PCR results, alignments and chromatograms are available with the online version of this paper.

been shown that at least two repeats of the 76 bp enhancer sequence are required for *SSU1-R* expression (Perez-Ortin *et al.*, 2002; Yuasa *et al.*, 2005) and that *SSU1-R* is not directly regulated by *Fzf1p* (Yuasa *et al.*, 2005).

The *SSU1-R* allele is only present in wine yeasts; thus the genomic organization of the *SSU1* locus in oenological strains has been studied extensively as a marker of the adaptive evolution of *S. cerevisiae* (Perez-Ortin *et al.*, 2002). Moreover, a high level of polymorphism has been observed in the *SSU1* gene among vineyard-isolated strains (Aa *et al.*, 2006), suggesting that this transport system is important in the evolution of SO₂ resistance mechanisms.

It has been shown that the expression of *SSU1* varied considerably among vineyard isolates (Townsend *et al.*, 2003) and that high levels of *SSU1* mRNA were correlated with increased sulphite resistance (Goto-Yamamoto *et al.*, 1998; Hauser *et al.*, 2001; Perez-Ortin *et al.*, 2002). Several studies have demonstrated or suggested different mechanisms of activation for *SSU1* (Park & Bakalinsky, 2000; Perez-Ortin *et al.*, 2002; Yuasa *et al.*, 2004, 2005), involving either its conventional activator *FZF1* or an alternative system based on the new promoter resulting from chromosome translocation. The mechanisms described in these studies were constitutive, with no suggestion of inducible transcriptional activation of *SSU1*. Surprisingly, *SSU1* expression has not previously been found to be regulated by sulphite, in either laboratory or wine strains (Park & Hwang, 2008; Yuasa *et al.*, 2005). However, two signal-dependent mechanisms have been proposed for the regulation of *SSU1* and *SSU1-R*, in microaerobic conditions and in response to nitrosative stress (Sarver & DeRisi, 2005; Yuasa *et al.*, 2005). Other studies focusing on wine yeasts have observed transcriptional control of the gene by a sulphur source (Aranda *et al.*, 2006): methionine seems to repress *SSU1*, especially in the presence of sulphite. Moreover, since sulphite is an intermediate of the superpathway of sulfur amino acid biosynthesis, the expression of genes involved in this pathway (such as some MET genes) has been shown to be related with sulphite detoxification and acetaldehyde production (Aranda & del Olmo, 2004; Aranda *et al.*, 2006).

In this study, we analysed the unusual expression pattern of *SSU1* in the wine strain 71B and in other oenological yeasts using real-time quantitative PCR. We correlated these findings to their behavioural response to sulphite and with the genomic organization of the *SSU1* locus. We found, for the first time to our knowledge, that this gene is induced by sulphite and over time, during the fermentation process.

METHODS

Yeast strains and media. In this study, we used commercial yeast strains of *S. cerevisiae*, available as a dried powder for the oenological market: EC1118, 71B and VRB (Lallemand), Premium Zinfandel (Vason), VIN13 (Anchor), Vitilevure GY (VitiGY; Martin Vialette) and L1515 (Sicorex Beaujolais, also known as Zymaflore RB2, Laffort Oenologie).

All yeasts were routinely grown on YPD medium (10 g yeast extract l⁻¹; 20 g proteose peptone l⁻¹; 20 g glucose l⁻¹, pH 6.0–6.2) or YPD agar medium (10 g yeast extract l⁻¹; 20 g proteose peptone l⁻¹; 20 g glucose l⁻¹; 15 g agar l⁻¹). For evaluation of sulphite resistance determinations in aerobic conditions, the growth medium consisted of yeast nitrogen base with amino acids (YNB, Difco, 6.7 g l⁻¹) supplemented with glucose (5 g l⁻¹), at an initial pH of 3.2.

Fermentation experiments and evaluation of sulphite resistance determinations in anaerobic conditions were carried out by using synthetic must MS300 mimicking a standard natural must (containing 200 g glucose l⁻¹ and 300 mg assimilable nitrogen l⁻¹ provided by a mixture of ammonia and amino acids; pH 3.2), as described previously (Rossignol *et al.*, 2003), or in natural white must [Viognier, previously characterized as containing 180 g sugars l⁻¹ and 160 mg assimilable nitrogen l⁻¹ (Nardi, 2007)]. Sodium metabisulphite (Sigma) aqueous solution (14.92 g l⁻¹, corresponding to 10 g SO₂ l⁻¹) was prepared then added in suitable amounts to synthetic must or media.

Fermentation and growth conditions. For fermentation experiments, active dried yeasts were rehydrated in 50 g glucose l⁻¹ at 37 °C for 30 min and used to inoculate synthetic must MS300 at 1 × 10⁶ cells ml⁻¹; SO₂ was added before inoculation at the appropriate concentration. Fermentations were performed under isothermal conditions at 24 °C, in fermenters or Erlenmeyer flasks fitted with stoppers to maintain anaerobiosis. Fermentation was not subjected to strict anaerobic conditions but the fermenter was designed to allow anaerobic fermentation, taking advantage of the CO₂ produced by yeasts. Two independent fermenters were used for each condition analysed. CO₂ production was monitored by automatically weighing the fermenters and calculating weight loss. Sugar consumption and ethanol concentration were calculated from CO₂ production. The number of cells was determined by an electronic particle counter (Beckman Coulter) and by counts on plates of serial dilutions of collected samples (mean of triplicates).

To determine sulphite tolerance, 15 ml synthetic must MS300 was inoculated with yeasts from a 30 h YPD liquid pre-culture at a final OD₆₀₀ of 0.05 in microaerobic conditions (flasks fitted with stoppers to maintain anaerobiosis). For each strain, two different pre-cultures were set up: YPD (pH 4.5) and YPD + 100 mg l⁻¹ total SO₂ (pH 4.5). Five concentrations of total SO₂ were tested (in MS300 must, pH 3.2): 0, 25, 50, 75 and 100 mg l⁻¹. Growth was measured by the increase in turbidity in any medium (A₆₀₀) with a UV1601 (Shimadzu) spectrophotometer. A linear relationship between cell number and A₆₀₀ ranging from 0.1 to 0.9 has been determined previously.

RNA extraction and reverse transcription. Total RNA was extracted using Trizol Reagent (Invitrogen Life Technologies) from samples containing approximately 10⁹ cells, resuspended in 400 µl Trizol and broken up by vortexing for 4 min with 300 µl of glass beads. The total volume was adjusted to 4 ml with Trizol solution; RNA extraction was performed following the protocol provided by the manufacturer. A maximal amount of 100 µg of total RNA was then purified from contaminants using the RNeasy kit (Qiagen) following the cleanup protocol in the manufacturer's instructions. RNA concentration was determined by spectrophotometric analysis; RNA quality was tested by electrophoresis on 1.5% agarose gels under denaturing conditions (2% formaldehyde, v/v, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). RNA (1 µg) was treated with DNase I (Fermentas) according to the manufacturer's instructions. cDNA was synthesized from 0.5 µg total RNA using SuperscriptII reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions using both polyT(16) primers (MWG-biotech; 0.025 µg µl⁻¹) and random hexamers (Promega; 0.025 µg µl⁻¹).

Gene expression analysis by real-time quantitative PCR. The expression levels of *SSU1*, *FZF1*, *MET10* and *MET17* were determined using real-time PCR. All real-time PCRs were performed in an iQ thermal cycler (Bio-Rad). A ready-to-use master-mix (iQ SYBR Green) containing *Taq* polymerase, reaction buffer, dNTPs and SYBR Green was used according to the manufacturer's instructions (Bio-Rad). Optimized reactions were performed in 0.5 ml MicroAmp optical plates (Bio-Rad), each 25 μ l reaction mixture contained 200 nM each primer, 1 \times iQ SYBR Green supermix and 5 μ l cDNA (various dilutions). PCR primers (Table 1) were designed using Primer Select software (DNASTAR) and synthesized by MWG-Biotech (HPSF-purified); primers were designed to amplify a 150 bp fragment with an annealing temperature of 60 °C. For each gene, a standard curve was determined with yeast genomic DNA; yeast DNA was extracted and purified using MasterPure yeast DNA purification kit (EPICENTRE) according to the manufacturer's instructions.

Actin (*ACT1*) and translational elongation factor EF-1 α (*TEF1*) were used as housekeeping reference genes, taking into account primer efficiencies calculated with the iQ5 software (Bio-Rad) (Vandesompele *et al.*, 2002).

All the real-time PCR experiments were carried out using two biological repetitions (independent fermentations). Real-time PCR was repeated twice for each sample (technical repetitions). Results show the overall standard deviation.

PCR assays, gel electrophoresis and DNA sequencing. All PCRs were performed in a PTC200 thermal cycler (MJ Research). A hot-start *Taq* polymerase (Go*Taq*), its buffer and dNTPs (Promega) were

used for all amplification reactions following the manufacturer's protocol with a primer concentration of 200 nM (each). PCR primers (Table 1) were designed using Primer Select software (DNASTAR) and synthesized by MWG-Biotech (HPSF-purified). Amplified samples were run on 1.5% agarose gels containing 0.1 μ g ethidium bromide ml⁻¹, bands were visualized by UV trans-illumination and digital images were acquired with the EDAS290 capturing system (Kodak).

DNA sequencing was performed by BMR-genomics (<http://www.bmr-genomics.it/>). PCR products to be sequenced were purified using the ExoSap cleanup system (United States Biochemical) by adding 1 μ l ExoSap enzyme to 5 μ l PCR products and incubating at room temperature for 30 min. Extraction of DNA bands from agarose gels was performed, when necessary, using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. The sequences obtained were deposited in GenBank under accession numbers GQ995455–GQ995463; sequences were aligned with the known sequence of the laboratory strain S288c (Goffeau *et al.*, 1996) using the CLUSTAL W tool (<http://www.ebi.ac.uk/clustalw/>).

RESULTS

With the aim of characterizing two commercial wine yeasts (EC1118 and 71B), their transcriptomes under wine alcoholic fermentation were compared. These fermentations were carried out in a natural grape must containing 50 mg sulphites l⁻¹. Gene expression was analysed at the

Table 1. Primers used in this study

| Primer | Sequence (5'–3') | Position (5' end/ ATG) | Annealing temp (°C) | PCR product size (bp) |
|------------------------------------|----------------------------------|---------------------------|------------------------|--------------------------|
| Real-time PCR amplification | | | | |
| ACT1-Q-fw | AATGCAAACCGCTGCTCAATCTTCTTCA | 678 | 60 | 145 |
| ACT1-Q-rv | AATACCGGCAGATTCCAAACCCAAAACAG | 822 | | |
| FBA1-Q-fw | CTCCATTGCTGCTGCTTTCGGTAACTGT | 650 | 60 | 153 |
| FBA1-Q-rv | GAACCACCGTGAAGACCAAGAACAATG | 802 | | |
| TEF1-Q-fw | ACGCCATTGAACAACCATCTAGACCAACTG | 692 | 60.3 | 153 |
| TEF1-Q-rv | CAGCTGGGGCGAAAGTAACAACCATACC | 844 | | |
| SSU1-Q-fw | TTTGCCTTTTGGTCAATTCTATGCCCTTTTA | 1187 | 60.2 | 151 |
| SSU1-Q-rv | TCCACGCTTTCAATGCTGTTATACGGAGAA | 1337 | | |
| FZF1-Q-fw | AGAACTCAATGGCAAGGAGGGAGGTTTTG | 351 | 60.4 | 138 |
| FZF1-Q-rv | TGACATGATTTGTATGGGCATTGTAGCACTTG | 488 | | |
| MET10-Q-fw | GTACACCCGTAACCTGCCATTTTCATCTGTGC | 68 | 60.3 | 148 |
| MET10-Q-rv | AATGGCTTCCCACGTGATTCGTTACCA | 215 | | |
| MET17-Q-fw | GCCAAGAGAACCCTGGTGACAATGCTC | 38 | 59.8 | 141 |
| MET17-Q-rv | GAAACCGGAATAGACGTAACCTGGAACCTCT | 178 | | |
| PCR amplification | | | | |
| SSU1pr16_fw | AGTGGTAAGCGTGGTGTCCGGTAAGA | –910* | 55.2 | 956 |
| SSU1pr16_rev | AAGGGGTCAAACCTGCCTCGTAAGA | +46* | | |
| SSU1pr8_fw | CAGAGATGGGGGTCACAGT | –949† | 51.6 | 911–1063 |
| SSU1pr8_rev | TTGCAGGATATGGGAAGC | +114† | | |
| FZF1-seq_fw | TCTCGACAATAAATACGCTAAA | –90 | 49.6 | 1057 |
| FZF1-seq_rev | TTGCCTTTTCTTTGTTTCATAGTTC | +967 | | |
| SSU1-seq_fw | AGTGTAAGAGAAGACAAG | –29*† | 50.1 | 1439 |
| SSU1-seq_rev | GCTTGCCAATTATGTAC | 1409*† | | |

*Relative to ATG in *S. cerevisiae* s288c *SSU1* sequence (GenBank accession no. NC_001148).

†Relative to ATG in *S. cerevisiae* Y-9 *SSU1-R* sequence (GenBank accession no. AB002531).

half-way point of complete fermentation (Nardi, 2007). Microarray data (see also Supplementary Tables S1 and S2, available with the online version of this paper) showed that 277 genes were differently expressed in the two strains: 131 were upregulated in 71B and 146 were downregulated. Some general pathways were upregulated in 71B (i.e. amino acid metabolism, including the methionine biosynthetic pathway) and some were downregulated (i.e. energy metabolism and ATP biosynthesis). Among these expression data, one of the most clear and interesting results was the upregulation of the *SSU1* gene in 71B. The overexpression of *SSU1* was confirmed by real-time quantitative PCR, which showed this gene to be expressed at levels 10 times higher in 71B than in EC1118 (data not shown). Given that 71B has been previously described as a sulphite-sensitive strain, our findings that the sulphite detoxification system is upregulated were unexpected (Aranda *et al.*, 2006; Yuasa *et al.*, 2004). Thus, we used real-time quantitative PCR to further investigate the expression pattern of genes involved in sulphite metabolism during alcoholic fermentation in strain 71B and in the reference wine strain EC1118 (Novo *et al.*, 2009; Rossignol *et al.*, 2003). Active dried yeasts were used to inoculate a natural must. Fermentation dynamics of both strains are shown in Fig. 1. Samples were taken at different stages of the fermentation process: stages 1 and 2 correspond to the active growth phase, stage 3 to the early stationary phase (when half of the sugar had been fermented) and stage 4 to the late stationary phase, when only a small amount of sugar (10 g l^{-1}) was present. The fermentation of 71B was slightly slower than that of EC1118, with the complete process lasting 165 h in 71B and 146 h in EC1118. To ensure yeast cells were collected under the same metabolic conditions, samples were taken at a specified cell population density (stage 1) or after the release of specified

amounts of CO_2 (stages 2, 3 and 4), rather than at specific time points.

For each of the four stages, cells were collected, RNA was extracted and expression was analysed by real-time quantitative PCR. Five putative housekeeping genes (*ACT1*, *PDA1*, *FBA1*, *TEF1* and *APE2*) were amplified for all samples. Raw threshold cycle data were processed using the geNorm online tool (<http://medgen.ugent.be/~jvdesomp/genorm/>) (Vandesompele *et al.*, 2002). This allowed identification of the most stable genes in the dataset (data not shown) and the optimal number of housekeeping genes for normalization, taking into account current constraints for real-time PCR data (Bustin *et al.*, 2005), also recently described for yeasts (Teste *et al.*, 2009). Actin (*ACT1*) and translational elongation factor EF-1 α (*TEF1*) were chosen as reference genes; all data were normalized to both *ACT1* and *TEF1* simultaneously, using the iQ5 software provided by Bio-Rad (Vandesompele *et al.*, 2002). The expression level at stage 1 was used as a reference for normalized gene expression data, as this stage corresponds to the start of the growth phase.

Analysis of the expression pattern for *SSU1* during alcoholic fermentation showed clear differences between the two strains (Fig. 2). We observed only slight changes between the different time points in the EC1118 strain (with a significant 2.6-fold difference observed between stages 1 and 4). The *SSU1* expression pattern differed considerably in 71B cells, consistent with induction of the gene during fermentation: *SSU1* showed a 13.9-fold increase in expression levels at stage 3 (compared with stage 1) and a 21-fold increase at stage 4. We also checked the expression level of *FZF1*, the main transcriptional activator (Sarver & DeRisi, 2005), for the same samples (Fig. 2). *FZF1* expression was increased during stationary

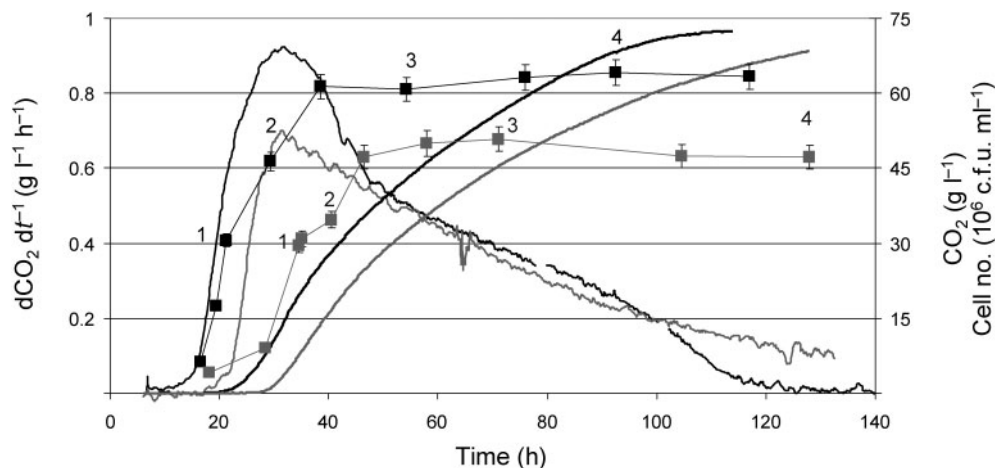


Fig. 1. Fermentation dynamics of strains EC1118 (black) and 71B (grey) in natural grape must. CO_2 production was determined by automatic detection of weight loss. Thick lines show overall CO_2 released, narrow lines show fermentation rate. Mean cell populations (squares) were determined with an electronic particle counter (data from two fermenters). The four sample time points for RNA extraction are indicated by numbers.

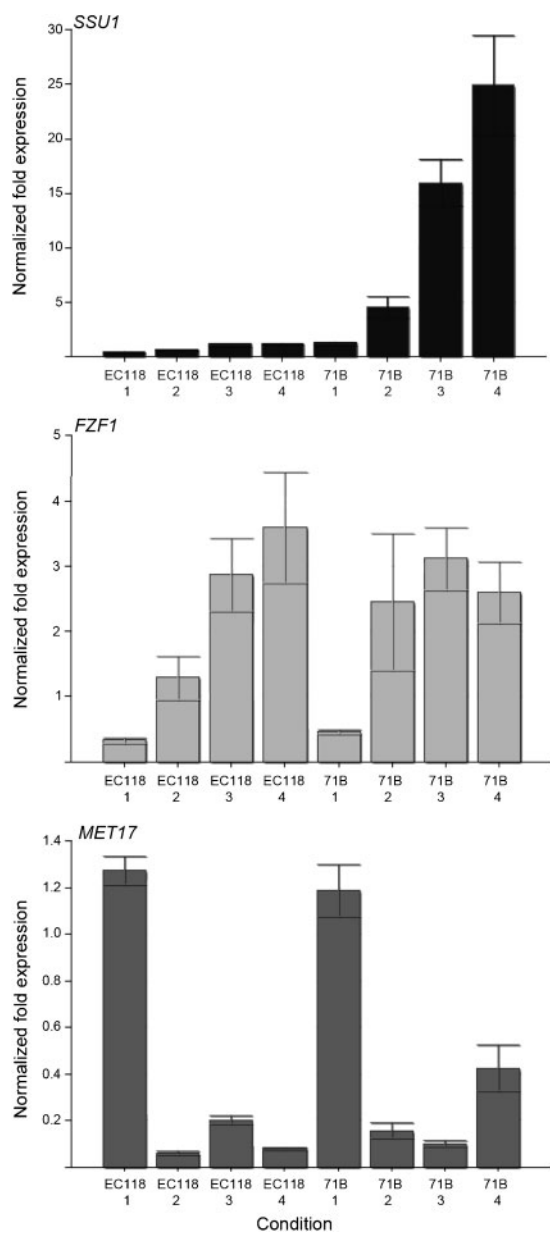


Fig. 2. Expression levels of *SSU1*, *FZF1* and *MET17* in 71B and EC1118 at different stages of alcoholic fermentation. Samples are identified by strain name and fermentation stage (see Fig. 1). Error bars, SD, are from two technical repetitions for two independent biological samples. Relative normalized fold expression was calculated by using the $\Delta\Delta C_t$ method, using *ACT1* and *TEF1* as reference genes (see Methods).

phase for both strains, with the maximal expression level reached at stage 4 by EC1118 and at stage 3 by 71B. *FZF1* was therefore strongly induced during fermentation, but regulation of its expression level was not correlated with *SSU1* expression levels. Data obtained for EC1118 seem to be consistent with previous studies, with a 16-fold increase in *FZF1* expression leading to only a 4-fold increase in *SSU1* expression level (Sarver & DeRisi, 2005). However,

the *FZF1* expression pattern in 71B resembles that of EC1118, and thus cannot explain the strong and specific induction of *SSU1* in this strain.

Moreover, since microarray data indicated that genes involved in sulphur amino acid metabolism were upregulated in 71B and are possibly linked with sulphite detoxification, we monitored the expression levels of two members of the pathway (*MET10* and *MET17*). Only small differences were observed between the two strains (*MET17* is reported in Fig. 2) and no permanent overexpression was detected in 71B. The two *MET* genes exhibited strong expression changes in stationary phase, which may explain the detection of overexpression at a single time point measurement by microarrays. The induction of *SSU1* during fermentation was therefore not associated with the general alteration of sulphur metabolism in 71B.

***SSU1* expression level in 71B at different SO₂ concentrations**

Since *SSU1* showed inducible behaviour in 71B, we decided to investigate whether the main substrate of the pump could affect its induction. *SSU1* gene expression levels during fermentation were determined in synthetic must with or without addition of SO₂. *SSU1* expression levels were also measured in several wine yeasts to determine whether *SSU1* expression is regulated in the same manner in other strains. The following strains were chosen: EC1118 (previously used as reference), three widely used commercial strains (VRB, Premium Zinfandel and VIN13) and two wine strains previously described as being genetically related to 71B (VitiGY and L1515; Legras *et al.*, 2007). Active dried yeasts were used to inoculate synthetic must with or without 50 mg total SO₂ l⁻¹ (pH 3.2). Yeast cells were collected at the beginning of the stationary phase (corresponding to stage 3 in the former experiment) and *SSU1* expression was analysed.

As expected, the basal *SSU1* expression level (in non-sulphited conditions) was strain-dependent (Divol *et al.*, 2006), with 71B showing the lowest expression levels and VitiGY the highest (Fig. 3). These variations in *SSU1* expression were consistent with previous observations of sulphite resistance and *SSU1* expression in wine yeasts (Yuasa *et al.*, 2004). We found that 71B was the only strain tested in which *SSU1* expression was significantly affected by sulphite concentration. The strain L1515 displayed a small but non-significant increase (*t*-test, $P > 0.05$). 71B exhibited the highest *SSU1* expression levels, with a 142-fold increase in *SSU1* expression levels in response to SO₂. These results are consistent with sulphite-driven induction of *SSU1* in 71B. This regulation upon addition of SO₂ was not observed in the other wine yeasts, even those that were genetically related, suggesting that this feature is specific to this strain.

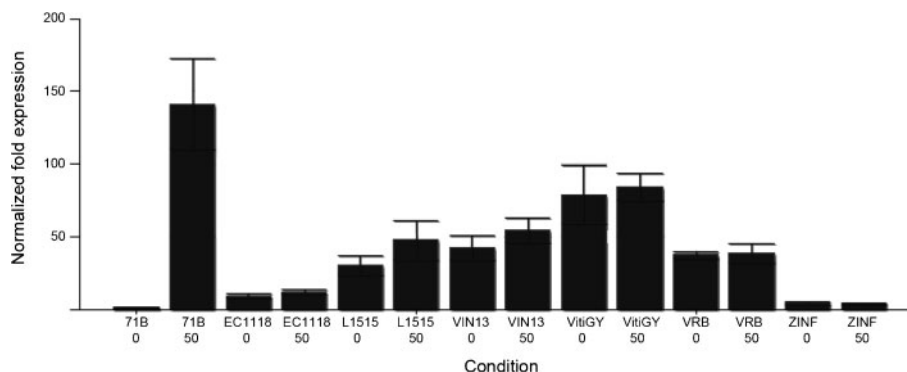


Fig. 3. Expression levels of *SSU1* in seven oenological strains fermenting must supplemented with different quantities of SO₂. Samples are identified by strain ID (2–5 letters) plus sulphite concentration (0 or 50 mg l⁻¹). The lowest dataset was chosen as reference (expression level=1). Error bars, SD, are from two technical repetitions for two independent biological samples. Relative normalized fold expression was calculated by using the $\Delta\Delta C_t$ method, using *ACT1* and *TEF1* as reference genes (see Methods).

Sulphite resistance and *SSU1* expression in aerobic conditions

The results described above suggest that sulphite was involved in *SSU1* induction. Since these results were obtained under anaerobic conditions and *SSU1* has previously been shown to be regulated by anaerobiosis (Yuasa *et al.*, 2005), we examined the potential role of oxygen in *SSU1* induction and the interplay with sulphites. We monitored the growth of 71B and EC1118 and measured *SSU1* expression levels during aerobic growth in minimal medium (YNB, pH 3.2) with or without addition of SO₂ (50 mg l⁻¹). Yeast strains were pre-cultured in YNB medium without sulphites.

For each culture, *SSU1* gene expression was analysed at different time points (24, 30 and 42 h) as described previously. Fig. 4(a) shows that the growth of 71B cells was affected by sulphite, with cells cultured with 50 mg SO₂ l⁻¹ showing a slower growth rate than cells grown in the absence of SO₂. In contrast, EC1118 was not affected by sulphites. *SSU1* gene expression in 71B only slightly increased (by a factor of two) at mid-exponential phase (30 h) in non-sulphited conditions. A much stronger induction of this gene (a 9.9-fold increase) was observed in the presence of sulphite and mainly occurred in stationary phase (42 h) (Fig. 4b). As expected, EC1118 did not show significant differences between sulphited and non-sulphited conditions (data not shown). These data show that *SSU1* is induced by sulphite in 71B, even when oxygen is available. However, the overall level of induction was lower than in anaerobic conditions, suggesting that different factors (anaerobiosis as well as sugar content or fermentation/physiological status and growth rate) contributed at least partly to *SSU1* induction. Slight induction of *SSU1* was observed in the absence of sulphite, associated with entry into stationary phase. This is consistent with previous observations by Gasch *et al.* (2000).

Effect of sulphite pre-adaptation on the growth properties of strains

Given the inducible nature of the *SSU1* gene in 71B and assuming that the *SSU1* gene is strongly involved in sulphite resistance, pre-culture conditions regarding the presence of sulphites may affect the growth properties of this strain. We therefore tested the growth and investigated the effect of pre-adaptation to sulphite in different yeast strains. We inoculated synthetic must containing 0, 25, 50 and 75 mg SO₂ l⁻¹ with strains 71B, EC1118, VitiGY and L1515 in microaerobic conditions. For each strain, two different pre-cultures were set up: YPD medium without sulphite and YPD containing 100 mg total SO₂ l⁻¹ (pH 4.5). All experiments were performed twice (Fig. 5).

For the growth of non-pre-adapted cultures (filled symbols, Fig. 5), we found that EC1118 cells were only slightly affected by the presence of sulphite and showed a good level of resistance to SO₂; the growth of these cells was not affected at 25 mg l⁻¹ and was delayed (up to 28 h exponential phase) at 50 and 75 mg l⁻¹ (Fig. 5a). VitiGY cells were slightly less resistant to sulphite than EC1118, with a delay in exponential phase observed at 25 and 50 mg SO₂ l⁻¹ (up to 24 h). Moreover, growth was both delayed and considerably slower at 75 mg l⁻¹ (Fig. 5b). Similar results were obtained for L1515 (Fig. 5c), although this strain was again more sensitive (exponential phase at 50 mg SO₂ l⁻¹ was reached at 48 h). Strain 71B showed a particularly low resistance to sulphite: growth was considerably affected by the lowest dose (25 mg l⁻¹), giving rise to the exponential phase at 48 h at 50 mg l⁻¹, and no growth was observed at 75 mg l⁻¹ (Fig. 5d). An effect of pre-adaptation to sulphite was barely detectable in EC1118: at high sulphite doses (50 and 75 mg l⁻¹), pre-adapted cells (open symbols) reached stationary phase a little earlier than non-pre-adapted cells (Fig. 5a). VitiGY

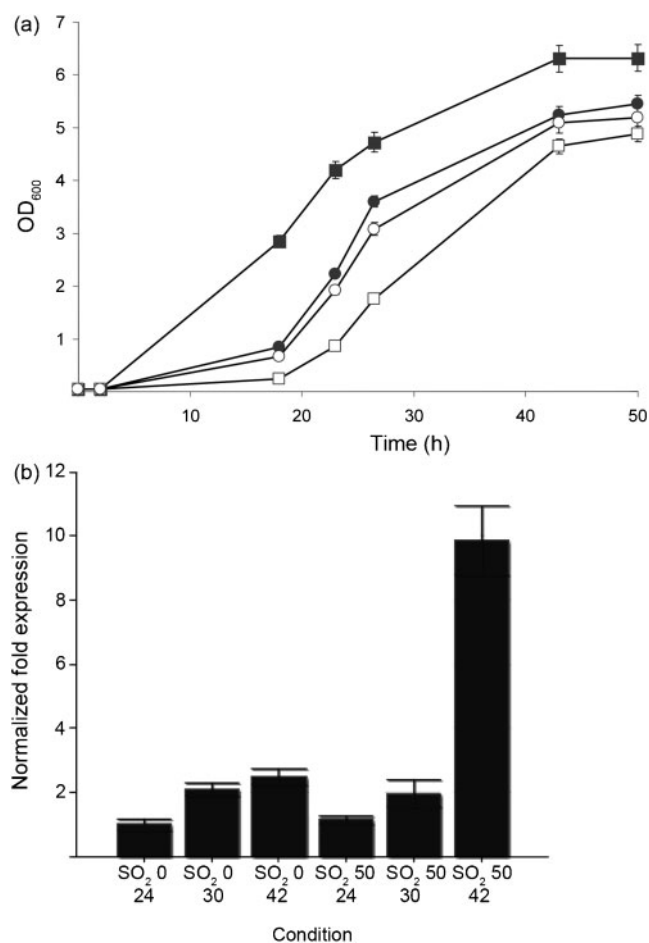


Fig. 4. Sulphite resistance and *SSU1* expression in aerobic conditions. (a) Aerobic growth of 71B (squares) and EC1118 (circles) strains in YNB medium (pH 3.2) supplemented with 0 (filled symbols) and 50 (open symbols) mg SO₂ l⁻¹. Mean \pm SD cell populations were determined from data from two independent cultures. (b) Expression levels of *SSU1* in 71B during the cultures described in (a). Samples are identified by SO₂ concentration and sampling time. Error bars, SD, are from two technical repetitions for two independent biological samples. Relative normalized fold expression was calculated by using the $\Delta\Delta C_t$ method, using *ACT1* and *TEF1* as reference genes (see Methods).

and L1515 cells also showed no visible effect at 25 mg l⁻¹; however, pre-adapted cells grew faster at 50 and 75 mg l⁻¹, despite no change being observed in the exponential phase (Fig. 5b and c). Growth of 71B cells showed significant improvement after pre-adaptation at 25 and 50 mg SO₂ l⁻¹, displaying a reduced exponential phase and subsequent faster growth. Pre-adaptation to sulphite, however, was not sufficient for the survival of 71B cells at 75 mg SO₂ l⁻¹ (Fig. 5d). These data show that pre-adaptation to sulphite improves the growth properties of 71B cells grown in the presence of SO₂ and reduces exponential phase in a manner which was not found in the other wine yeasts. This specific response of 71B to sulphite pre-adaptation is

consistent with *SSU1* induction by sulphite. Indeed, other physiological responses may contribute to improving yeast growth and to reducing exponential phase, such as the production of a sulphite-binding agent (like acetaldehyde) or enhanced sulphite metabolization. In addition, other events such as selection of SO₂-resistant mutants of 71B cannot be ruled out. These seem unlikely, since a similar exponential phase reduction was systematically obtained with this strain only. The other detoxification mechanisms may contribute to the slight improvement of growth rate in 71B and in other strains (L1515 or VitiGY) after pre-adaptation to sulphite. However, their contribution to exponential phase reduction is likely to be weak, since we did not observe a change in the duration of exponential phase with other strains.

Genomic organization and sequence polymorphism of *SSU1* alleles

To investigate the genomic organization of *SSU1* alleles, we amplified their promoter regions in 71B and the other strains previously characterized for *SSU1* expression (Fig. 4) using a set of primers (Table 1) that target the previously described forms of *SSU1* (Fig. 6a). Fig. 6(b) summarizes the amplification results of the *SSU1* and *SSU1-R* promoter regions (see also Supplementary Fig. S1).

In some strains, *SSU1* and *SSU1-R* promoter regions were amplified only in the wild-type or only in the translocated form (such as Zinfandel or L1515, respectively), as observed previously for other wine yeasts (Perez-Ortin *et al.*, 2002; Yuasa *et al.*, 2004). Other strains showed the presence of one copy of *SSU1* on chromosome XVI and one copy on chromosome VIII after translocation (*SSU1-R*), as also reported previously (Novo *et al.*, 2009; Perez-Ortin *et al.*, 2002). In particular, the size of the *SSU1-R* promoter (Fig. 6b), was compatible with two repetitions of the 76 bp *ECM34* enhancer sequence (EC1118) or with four repetitions (VitiGY and VRB). Interestingly, 71B showed both of these amplification fragments. We carried out sequencing of the amplicons and alignment with reference strains (Goto-Yamamoto *et al.*, 1998). The longest *SSU1-R* promoter fragment obtained from 71B actually contained four complete repetitions of the 76 bp enhancer, whereas the shortest contained two repetitions corresponding to the single amplicon obtained from EC1118 [full alignments (Supplementary Fig. S2) are available with the online version of this paper]. Since 71B also harbours the *SSU1* allele on chromosome XVI, this suggested that 71B contains three *SSU1* alleles.

The *SSU1* ORFs for 71B and EC1118 were sequenced. In previous studies, several polymorphic sites were detected within this coding sequence among yeasts from different environments (Aa *et al.*, 2006; Liti *et al.*, 2009; Novo *et al.*, 2009). As expected, EC1118 and 71B sequences differed from the reference strain S288c. Careful analysis of the chromatograms (Supplementary Fig. S3) showed clear double peaks at the six polymorphic positions of the

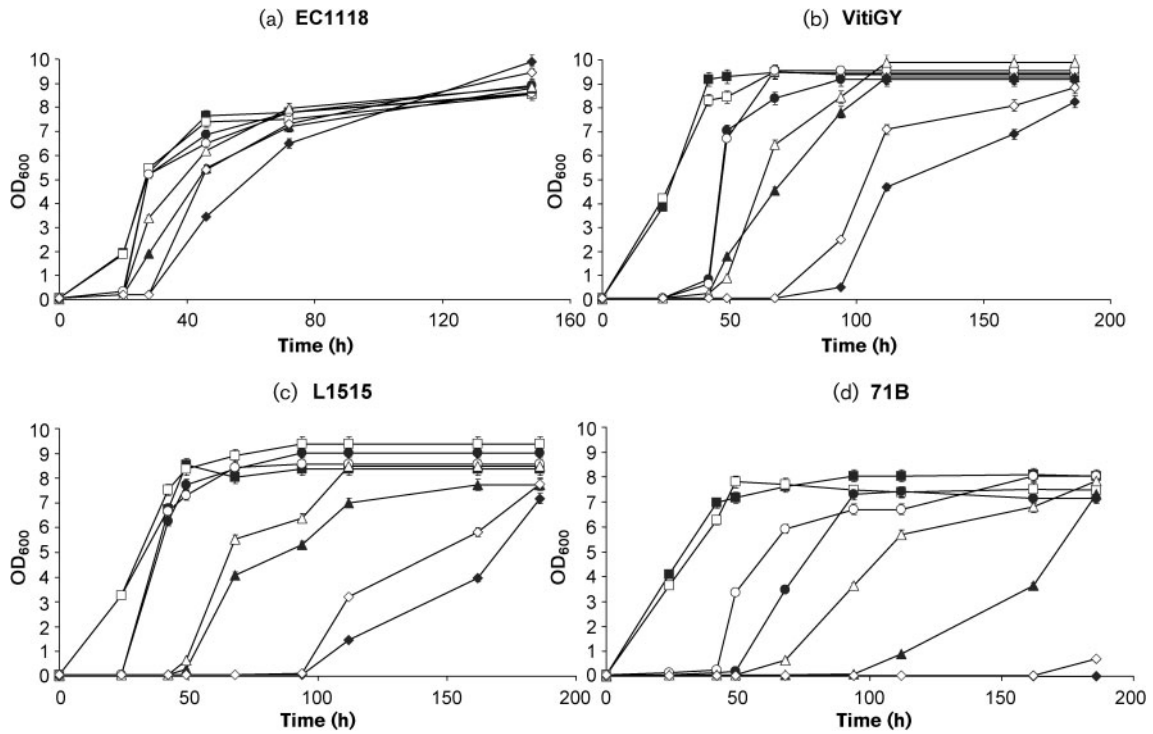


Fig. 5. Impact of sulphite pre-adaptation on the growth properties. Growth of cells: pre-adapted (open symbols) and non-pre-adapted (filled symbols) to sulphite (100 mg l^{-1} in YPD) in synthetic must supplemented with 0 (squares), 25 (circles), 50 (triangles) and 75 (diamonds) $\text{mg SO}_2 \text{ l}^{-1}$. Mean \pm sd cell populations were determined from data from two independent cultures.

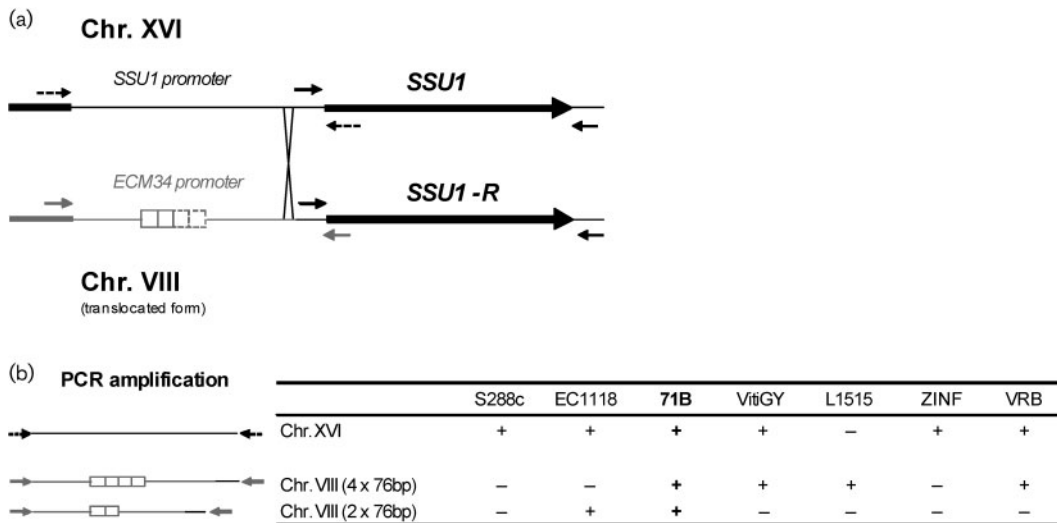


Fig. 6. (a) Diagrams representing the organization of *SSU1* and *SSU1-R* loci, respectively on chromosome XVI (wild-type) and on chromosome VIII (translocated). Within the *ECM34* promoter (grey) squares show the 76 bp enhancer sequence repetitions (2–4). (b) Summary of PCR amplification results of the *SSU1* promoter region on chromosome XVI (primers used: *SSU1*pr16) and *SSU1-R* promoter region on chromosome VIII (primers used: *SSU1*pr8). +, Band corresponding to the amplified fragment was visualized after gel electrophoresis of PCR products (Supplementary Fig. S1). Arrows represent primers used in this study: *SSU1*pr8 (grey) used for PCR amplification (a); *SSU1*pr16 (dotted) used for PCR amplification (b); *SSU1*seq (black) used for sequencing of the *SSU1* open reading frame.

71B sequence (Table 2), suggesting that the coding sequences for *SSU1* in this strain may have at least two different origins. These findings have not been described previously (Aa *et al.*, 2006) and were not observed for EC1118. We also amplified, sequenced and aligned the untranslocated *SSU1* promoter from both strains. This sequence also displayed a notable degree of polymorphism (Table 2), but all the point mutations found had been detected previously in other strains (Aa *et al.*, 2006; Liti *et al.*, 2009). Furthermore, no double peaks were observed, indicating a lack of heterozygosity in the promoter region.

Finally, we amplified and sequenced the *FZF1* ORF for both strains (see Supplementary Fig. S2 for full alignments), finding almost the same level of polymorphism in both strains; all the point mutations had been previously detected (Aa *et al.*, 2006). Moreover, the most well-known mutations affecting protein activity in *FZF1* (Park & Bakalinsky, 2000) were not detected in either EC1118 or 71B.

DISCUSSION

Most previous studies of *SSU1* have described a constitutively expressed gene, the expression level of which varies substantially between different wine strains (Goto-Yamamoto *et al.*, 1998; Perez-Ortin *et al.*, 2002; Townsend *et al.*, 2003). Indeed, although *SSU1* is a target of two signal-dependent stress responses [low oxygen response (Yuasa *et al.*, 2005) and nitrosative stress response (Sarver & DeRisi, 2005)], its expression level has never been found to be affected by sulphite. Previous studies have clearly demonstrated that this gene is not regulated by sulphite in the medium nor by sulphite accumulation in the cell (Yuasa *et al.*, 2005), and that, although *SSU1* is

regulated by transcriptional control, sulphite itself is not a powerful inducer of *SSU1* transcription (Aranda *et al.*, 2006). Indeed, *SSU1* was not part of a set of genes identified as being upregulated by sulphite exposure (Park & Hwang, 2008).

In this study, we show that the strain 71B harbours a sulphite-inducible *SSU1* gene. This wine yeast was the only strain studied that displayed strong overexpression of *SSU1* in the presence of sulphite, providing, for the first time to our knowledge, evidence of sulphite-dependent activation of this gene. We observed this induction in various culture conditions: anaerobic and aerobic. Thus, the induction mechanism triggered by sulphite appears to be mostly independent from other factors known to affect *SSU1* expression. However, *SSU1* expression was always maximal at the stationary phase of alcoholic fermentation, possibly due to a coupling of anaerobic fermentation conditions and/or growth arrest and the effect of sulphite on *SSU1* expression. A delay has been observed, in all conditions, between exposure to sulphite and *SSU1* induction; this could be consistent with several explanations, including secondary effects of the chemical or the possibility that non-growing cells are more inducible. The late induction may correspond to the accumulation of a secondary compound derived from sulphite, which may be the real inducer. Given the high chemical reactivity of sulphite with carbonyl functions, secondary yeast metabolites with such reactivity must be considered, particularly acetaldehyde, which can accumulate during fermentation. Slight upregulation of *SSU1* was observed during the stationary phase in the absence of sulphite, consistent with previous reports (Gasch *et al.*, 2000).

Analysing the effect of pre-adaptation to sulphite on yeast growth revealed a unique response of 71B: a reduced

Table 2. Polymorphic sites at the *SSU1* locus of EC1118 and 71B

Sequences are also available in GenBank, accession nos GQ995455–GQ995463.

| Strain | Locus*/mutation | | | | | | | | | | | | | |
|-------------------------------|-----------------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| | 55 | 99 | 154 | 180 | 269 | 357 | 364 | 469 | 490 | 570 | 571 | 630 | 1031 | 1034 |
| <i>SSU1</i> ORF | | | | | | | | | | | | | | |
| S288c | A | C | G | G | A | C | G | C | T | C | G | C | G | A |
| Previously observed mutation† | G | T | A | A | G | T | T | T | C | T | A | T | A | G |
| EC1118 | G | – | A | – | G | – | – | T | – | – | – | – | – | – |
| 71B | – | – | G/A | – | A/G | – | G/T | C/T | C/T | – | – | C/T | – | – |
| <i>SSU1</i> promoter | –469 | –442 | –394 | –374 | –371 | –355 | –271 | –243 | –233 | –222 | –181 | –166 | –152 | –119 |
| S288c | A | T | A | T | T | T | T | C | A | T | T | T | T | G |
| Previously observed mutation† | T | C | G | A | A | G | C | T | T | G | A | C | G | A |
| EC1118 | – | – | – | – | – | – | C | – | T | – | – | – | – | A |
| 71B | – | – | G | A | – | – | C | – | T | – | – | – | G | A |

*Sites are designated 1 and above from the first nucleotide of the start codon, and –1 and below from the nucleotide before the first nucleotide of the start codon.

†Among 30 wine strains (Aa *et al.*, 2006) and among 36 strains from different sources (Liti *et al.*, 2009).

exponential phase, which could be explained by the regulation of *SSU1*. Indeed, other general adaptation mechanisms could take place with all strains (such as physiological changes affecting acetaldehyde or sulphur metabolism) but they seem poorly effective in reducing the exponential phase, as suggested by the singularity of the behaviour of 71B. This is in line with the key role of the *SSU1* pump in the detoxification process. The sulphite-inducible nature of *SSU1* expression and its effect on sulphite resistance could explain the apparently conflicting observations for the sensitivity of 71B to sulphite. Indeed, in the absence of pre-adaptation and *SSU1* induction, 71B cells display a low resistance to sulphite. However, it is notable that although the *SSU1* expression level in 71B can be much higher after induction than in EC1118, the strain always exhibited a lower resistance than EC1118. This suggests that additional post-transcriptional factors (secretion or control of pump activity) limit the impact of *SSU1* overexpression.

Sequencing of the 71B *SSU1* gene promoters showed the existence of three different alleles, one standard and two translocated, for *SSU1-R*. Given that strain 71B is not known to be aneuploid for any chromosome (Bradbury *et al.*, 2006), the additional copy of *SSU1-R* may result from the duplication of a chromosomal region. The promoter sequences themselves are similar to those that have been described previously and shown to trigger strong constitutive expression of *SSU1*. Although strain 71B harbours two *SSU1-R* copies with repetition of the 76 bp enhancer region, this strain displays a low level of constitutive *SSU1* expression. This suggests that the regulation of basal *SSU1* expression levels is significantly altered in this strain. However, this strain seems to have gained a new regulatory system and has become sulphite-inducible. The mechanisms of *SSU1* induction are unclear. The main regulator of *SSU1* expression, *FZF1*, does not seem to be involved, since its expression was unaltered and no specific sequence variation was detected. However, we cannot rule out a modification of a post-transcriptional control of Fzf1p in 71B. The involvement of additional regulatory elements lying outside the sequenced promoter region (950 bp upstream of ATG) cannot be ruled out either. Further, more targeted, experiments are required to clarify these observations.

This unique regulation of *SSU1* may be consistent with the particular genetic history of the 71B strain. Sequencing of the *SSU1* ORF revealed polymorphisms suggesting that at least two different origins exist for the coding sequences of *SSU1*. This has not been described in other wine yeasts. In a large survey of the genetic diversity of *S. cerevisiae* (Legras *et al.*, 2007), this wine yeast was shown to belong to a group of bread yeasts, suggesting that 71B was a former baking yeast, which was later isolated in vineyards for wine making. However, the alleles detected in 71B have already been described in wine yeasts. The two strains VitiGY and L1515, which are phylogenetically closely related to 71B, do not display sulphite-inducible expression of *SSU1*. Thus,

the gain of a sulphite-inducible form of *SSU1* by 71B may correspond to an isolated and rare event.

Overall, our findings show that strain 71B displays a new sulphite-inducible system for the regulation of *SSU1* expression. Elucidation of the underlying control mechanisms could lead to the identification of new protein partners involved in sulphite sensing and signalling. Sulphite resistance is an important property for yeasts in winemaking, as it allows them to compete with wild microflora in grape musts. Until now, yeast sulphite resistance was considered to involve a constitutive mechanism and the possibility of adapting cells has thus not been investigated. The results obtained here suggest that the specific processing of 71B yeast cells may be of particular interest to reduce the exponential phase in sulphited musts. A more general evaluation of sulphite-inducible properties in wine yeast should be carried out to determine whether such adaptation techniques may be of interest for other yeasts.

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