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Copper-based grape pest management has impacted wine aroma

Irene De Guidi, Virginie Galeote, Bruno Blondin & Jean-Luc Legras✉

Despite the high energetic cost of the reduction of sulfate to H_2S , required for the synthesis of sulfur-containing amino acids, some wine *Saccharomyces cerevisiae* strains have been reported to produce excessive amounts of H_2S during alcoholic fermentation, which is detrimental to wine quality. Surprisingly, in the presence of sulfite, used as a preservative, wine strains produce more H_2S than wild (oak) or wine velum (*flor*) isolates during fermentation. Since copper resistance caused by the amplification of the sulfur rich protein Cup1p is a specific adaptation trait of wine strains, we analyzed the link between copper resistance mechanism, sulfur metabolism and H_2S production. We show that a higher content of copper in the must increases the production of H_2S , and that SO_2 increases the resistance to copper. Using a set of 51 strains we observed a positive and then negative relation between the number of copies of *CUP1* and H_2S production during fermentation. This complex pattern could be mimicked using a multicopy plasmid carrying *CUP1*, confirming the relation between copper resistance and H_2S production. The massive use of copper for vine sanitary management has led to the selection of resistant strains at the cost of a metabolic tradeoff: the overproduction of H_2S , resulting in a decrease in wine quality.

The most ancient traces of wine making have been discovered in Georgia¹ and have been dated as 6000 BC. Since that ancient time, cultivation of grapevine and winemaking knowledge spread progressively all over the world². All along this period, winemaking practices have evolved, especially with the discovery of the use of sulfite to limit the growth of undesired microorganisms to protect wine from oxygen and to preserve aroma profile. Similarly, the cultivation of *Vitis vinifera* has faced changes, especially with the development of grafting and the spray of chemical compounds required to face the import in Europe of three major pests for vine: phylloxera, powdery mildew and downy mildew. Among chemicals sprayed on vines, copper has been intensively used in vineyards to control the development of *Plasmopara viticola*. This intensive use of copper in vineyards has translated into high copper in grape musts³.

Wine fermentation is mainly achieved by the yeast species *Saccharomyces cerevisiae* which is found also in many fermented products: sake, bread, cheese and more^{4–7}, as well as in natural biotopes such as forests^{8,9}. *S. cerevisiae* strains display specific physiological properties associated to the different ecological niches they live in, as result of several domestication events^{4,10–12}.

One of the most remarkable and contrasting adaptation events can be seen in fermenting wine strains and in wine velum isolates (*flor* yeasts). *S. cerevisiae* velum strains have developed a specialized aerobic lifestyle, highly different from the one of fermenting wine strains¹³. Since they colonize the wine when fermentation is concluded, velum strains develop the ability to grow in media depleted for nitrogen, vitamins, glucose and fructose.

Wine fermentation poses a challenging environment for *S. cerevisiae*. Different genomic features have been identified as traces of adaptation to the wine environment, in line with its domestication¹⁴. The first and best described adaptation of *S. cerevisiae* to the wine environment is the resistance to sulfite, obtained from several translocation events resulting in a high expression of the sulfite export pump Ssu1^{15–19}. Another example of adaptation to the grape and must environments can be seen in the selection of strains carrying multiple copies of the *CUP1* gene. This gene amplification leads to an enhanced protein abundance/synthesis, providing resistance to high concentrations of copper in the grape must, resulting from the massive use of copper as fungicide²⁰. Cup1p is among the ten sulfur richest yeast proteins²¹ and some *S. cerevisiae* strains can harbor up to 79 copies^{18,20,22}. Therefore, the high synthesis of Cup1p caused by its amplification requires a high availability of sulfur containing amino acids methionine and cysteine that are scarce in grape musts. These amino acids can be synthesized by yeast through the sulfur assimilation pathway (SAP), which reduces inorganic sulfate into hydrogen sulfide (H_2S) with the consumption of 7 mol of NADPH and 4 of ATP per mole of S-amino acid²³. Consequently, the

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biosynthesis of the sulfur amino acids has a significant impact on the yeast redox and energy balances. A high diversity in the production of H_2S during alcoholic fermentation has been described for wine strains²⁴, and because its content is detrimental to wine aroma, different studies have deciphered its genetic bases and found allelic variations in *MET10*, *SKP2*, *MET2*, *TUM1*^{25–28}, genes involved in the sulfur assimilation pathway or its regulation. Some of these findings have been patented and have led to the improvement of industrial winemaking starters. Surprisingly, no investigation has been carried out to understand the biological meaning of such overproduction, nor to evaluate a potential relation with different ecological niches. Interestingly, for wine *S. cerevisiae*, SO_2 and copper tolerance have been found negatively associated²⁹. Transcriptional and proteomic analysis in sulfur-limited medium, demonstrated that *SSU1* over-expression induced sulfur limitation during exposure to copper and provoked an increased sensitivity to copper³⁰.

Because the production of H_2S is so costly to the cell²³, we wondered why some wine strains were overproducing it. Comparing three groups of strains: isolated from velum and wine, two contrasted anthropogenic environments, and oak, as a natural environment, we show that the total content of H_2S produced during alcoholic fermentation depends on the ecological niche, and that exposure of yeast cells to copper enhances H_2S production. We evaluated how the amplification of *CUP1* may explain such variation, using a set of strains with variable number of copies of *CUP1* or strains carrying a plasmid overproducing *CUP1*. Last, we measured the impact of sulfites availability in the media on copper resistance.

Results

Strain variability in H_2S production during alcoholic fermentation

To assess the variability of the production of H_2S during alcoholic fermentation of *Saccharomyces cerevisiae*, we evaluated 33 strains isolated from three ecological niches: wine (n = 10), wine velum (n = 14), and from oak trees (n = 9), as a wild reference. Because SO_2 is an intermediate of the sulfur assimilation pathway, and used in most wine fermentations as an additive for its antimicrobial, antioxidant and anti-oxidizing activities, we compared the H_2S production of the mentioned ecological groups in a synthetic grape must in the absence or presence of sulfites. The variability in H_2S production among strains of these three groups is presented in Fig. 1 and Supplementary Fig. 1. A two-way ANOVA revealed that the origin of the strain has a significant effect on H_2S produced ($F_{2,128} = 36.31$, $p_value = 3.24 \times 10^{-13}$) as well as the addition of SO_2 to the must ($F_{1,128} = 59.19$, $p_value = 3.36 \times 10^{-12}$). A significant interaction between the effects of the two factors (i.e. SO_2 and origin) on H_2S produced during alcoholic fermentation ($F_{2,128} = 14.5$, $p_value = 2.20 \times 10^{-6}$) was detected.

Tukey multiple comparisons of means at 95% family-wise confidence level showed that H_2S production between the independent origins was significant when the must contained sulfite (Fig. 1b). Wine strains produced more H_2S compared to velum and oak ($p_value = 1.50 \times 10^{-6}$ and 0.014 respectively). Oak isolates also produced more H_2S than velum strains in the presence of sulfite ($p_value = 1.50 \times 10^{-6}$). This difference was not noticeable when the must did not contain sulfites (Fig. 1a).

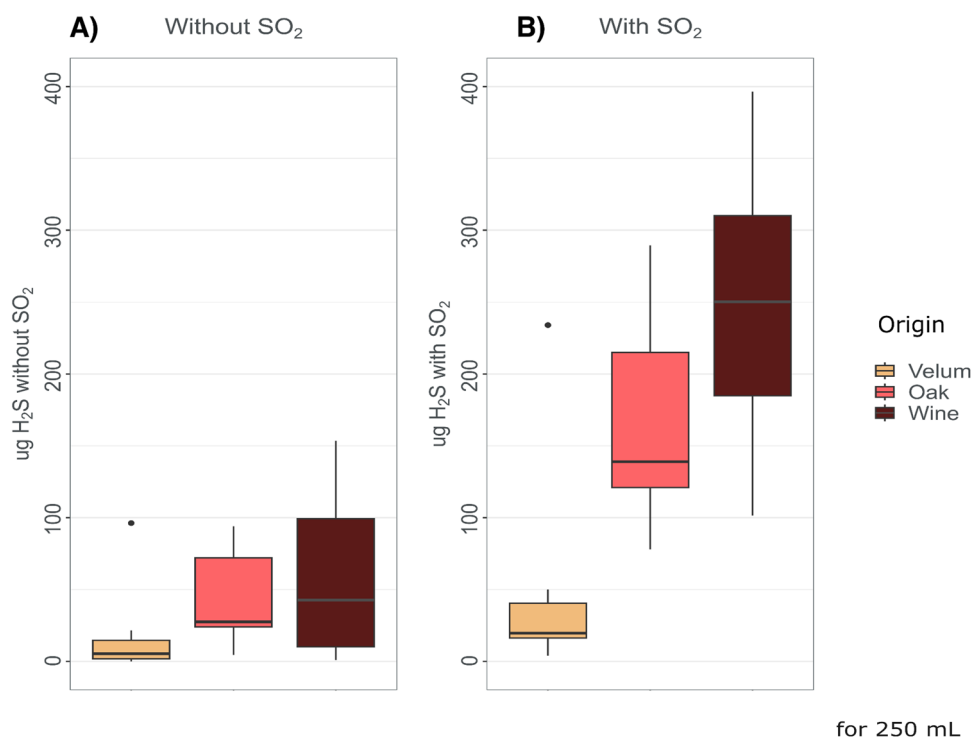


Figure 1. Difference in the average of cumulate H_2S production during alcoholic fermentation of velum, oak and wine strains in absence (A) or presence (B) of SO_2 .

Comparing Fig. 1a,b, it was clear that strains isolated from different origins did not respond with the same amplitude to the sulfite treatment. Velum strains displayed a remarkable low H₂S production even in the presence of sulfite, in comparison to wine and oak strains ($p_{\text{value}} = 1.50 \times 10^{-6}$ and 3.34×10^{-5} respectively). This explains the significant interaction detected by the model.

Influence of copper content on H₂S production

The copper content of the grape must or wine can result from the traces left with sanitary treatment performed on vine, and also from treatments aimed at reducing H₂S production. Indeed, thiols functions reduce Cu²⁺ and produce a Cu⁺ that binds to -SH functions³¹. We evaluated the effect of copper concentrations of the must on H₂S production of two industrial winemaking starters: VL1, a wine strain, identified as low H₂S producer in the first experiment, and LMD17, a high H₂S producer wine strain³². The analysis of variance revealed a significant effect of both factors: strain ($F_{1,14} = 57$, $p_{\text{value}} = 2.7 \times 10^{-6}$), and copper ($F_{2,14} = 10.6$, $p_{\text{value}} = 0.002$). The results presented in Fig. 2 show a clear increase of H₂S production with an increase in copper content in the synthetic grape must at concentrations compatible with those encountered in winemaking³. For both strains, higher concentrations provoke the formation of a black precipitate that hampers H₂S measurement with our method, and suggests higher production (Supplementary Fig. 2).

Evaluating the relation between H₂S production and *CUP1* copy number

Besides inducing H₂S production, the copper content in the growth medium controls the expression of *CUP1*³³ that is involved in its detoxification. In addition, we observed that *CUP1* is one of the proteins with the highest sulfur containing aminoacid content (21.31%), just after *MNC1* (25.76%), another membrane protein that is upregulated by toxic concentrations of heavy metal ions³⁴.

In a first approach aimed at exploring the effect of *CUP1* copy number on H₂S production in strains of the same three niches analysed above, we increased the number of strains to test (+ 18 wine isolates, total $n = 51$), in order to include strains with 2 to 71 *CUP1* copy number. Surprisingly, we observed a non-linear relation between *CUP1* copy number and total H₂S production.

As shown in Fig. 3A, strains with 1 to 10 *CUP1* copies exhibit an increasing total H₂S production, whereas for more copies, it progressively decreases until it reaches almost null values. A 3rd degree polynomial model described well the H₂S production in relation to the *CUP1* copy number of the strains (black line in Fig. 3), displaying a bell shape, that remained even after the removal of the two highest values for H₂S production (red line in Fig. 3A). A complex polynomial relation between *CUP1* copy number and H₂S production is noticeable when the model was built with wine strains only (Fig. 3C).

This bell shape is conserved and amplified in the presence of sulfite in the media, except for strains with a high copy number of *CUP1* (Fig. 3B,D).

Notably, the increase in H₂S concentration with the copy number of *CUP1* within the range 1–10 copies is similar to the response caused by the increase in copper content of the grape must observed for VL1 and LMD17.

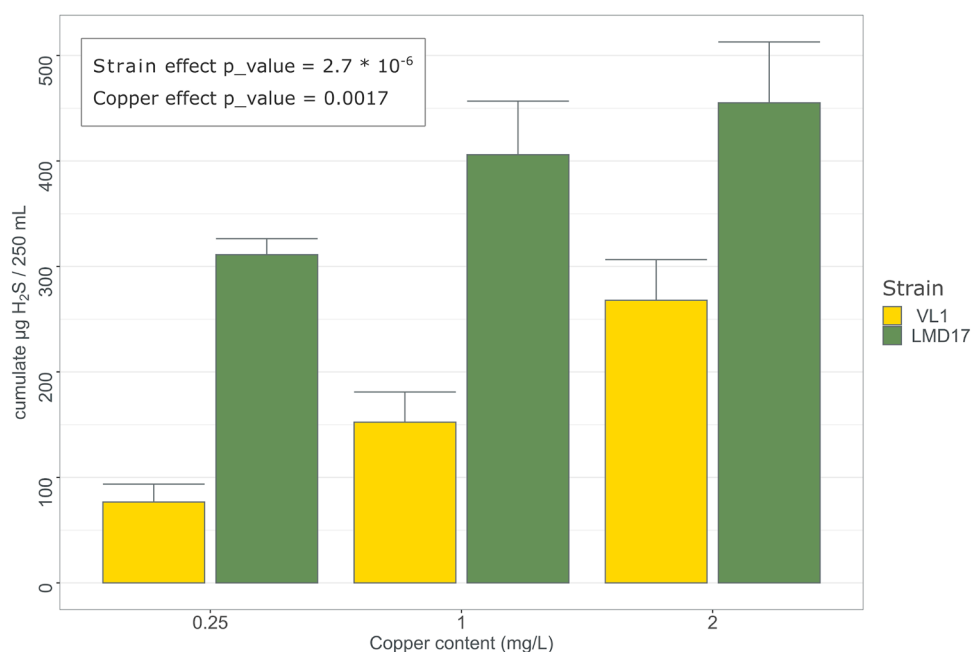


Figure 2. Effect of copper content in synthetic must without SO₂ on total H₂S production during alcoholic fermentation by wine strains VL1 and LMD17. p values refer to two-way ANOVA.

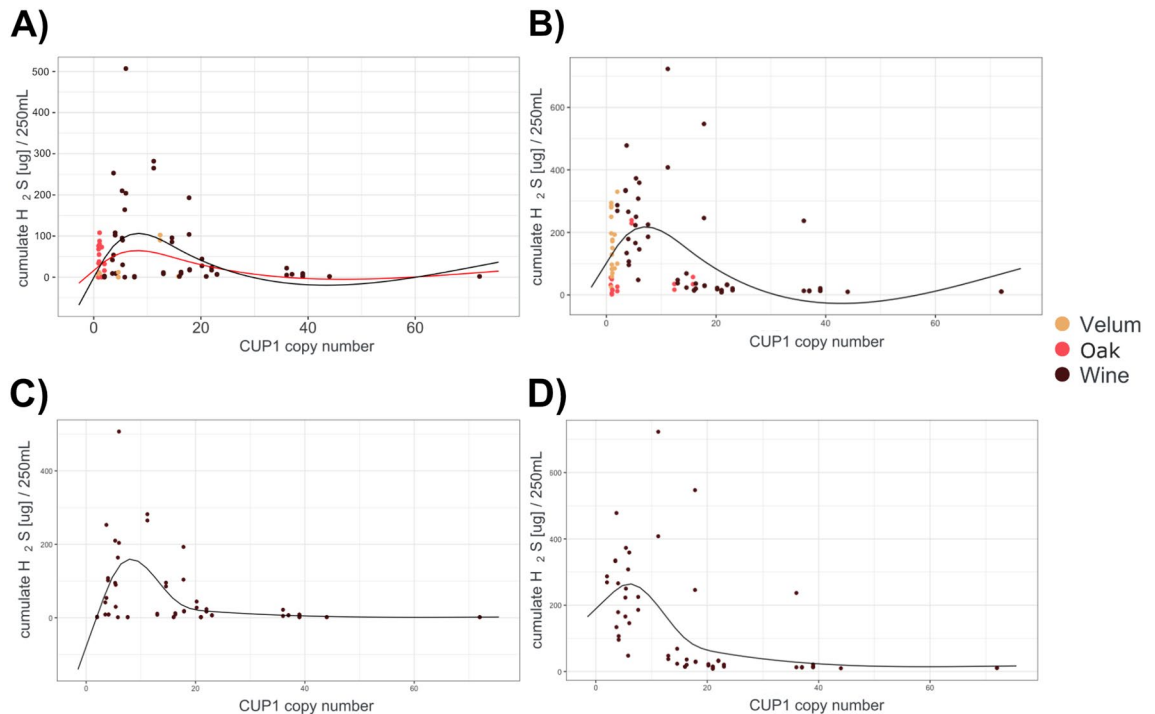


Figure 3. H_2S production (in synthetic must without SO_2) distribution as function of *CUP1* copy number of each strain. (A) Whole set of studied strains, in synthetic must without SO_2 ; (B) whole set of studied strains, in synthetic must supplemented with SO_2 ; (C) wine strains, in synthetic must without SO_2 ; (D) wine strains in synthetic must supplemented with SO_2 ; Isolation origins are described by colours as in Fig. 1. Black solid line: polynomial model describing the relation between H_2S production and *CUP1* copy number; red line: same model excluding the two highest H_2S producers.

Impact of the modulation of *CUP1* copy number on H_2S production

In order to validate the effect of *CUP1* copy number on H_2S production, we tried to manipulate the number of *CUP1* copies per cell. With this aim, we built a multicopy yeast episomal plasmid (YE_p) expressing *CUP1* under the control of the strong promoter from the translational elongation factor EF-1 alpha (*TEF1*). Three strains with different number of genomic copies of *CUP1* were transformed with this plasmid and tested in a media containing a low copper concentration (0.25 mg/L).

First, the overexpression of *CUP1* in the oak strain OAK-Rom 3_2, a low H_2S producer with one copy of *CUP1*, led to a significant increase of H_2S production ($F_{2,6} = 9.61$, $p_value = 0.013$, Fig. 4), without affecting the growth. In contrast to the oak strain, the overexpression of *CUP1* in the wine strain LMD17, a high H_2S producer, with 11 copies of *CUP1*, decreased H_2S production by half ($F_{2,5} = 17.49$, $p_value = 0.006$, Fig. 4), with no impact on fermentation kinetic. Last, the overexpression of *CUP1* in L1374, which carries 36 copies of *CUP1* and was ranked among the lowest H_2S producers, did not change its production ($F_{2,6} = 0.2$, $p_value = 0.824$, Fig. 4). The responses displayed by these three constructions are in agreement to the experimental data presented in Fig. 3, reproducing the “bell-shape” trend of H_2S production.

Impact of sulfite addition in the culture media on copper resistance

Because sulfite is required for the synthesis of sulfur containing amino acids essential for *CUP1* synthesis, it was logical to test how the deregulation of the pathway by exogenous sulfite might affect copper resistance. Three strains were tested: oak strain Oakrom 3.2, and wine strains LMD17 and L1374, that have 1, 10 and 34 copies of *CUP1* respectively. The overexpression of *CUP1* in Oakrom 3.2, increased the resistance to copper in the control media, which was not the case for wine strains LMD17 and L1374. However, yeast growth was improved for all strains overexpressing *CUP1* on copper-supplemented media when SO_2 was present (Fig. 5A,B).

Discussion

Our findings, obtained under conventional winemaking conditions, involving the presence of sulfites, demonstrate for the first time that the production of hydrogen sulfide (H_2S) by *Saccharomyces cerevisiae* during alcoholic fermentation varies among natural and two distinct groups of domesticated strains. Surprisingly, wine populations exhibited the highest H_2S production levels when sulfites are added to the grape must. This observation is unexpected, given the widely acknowledged undesirability of H_2S in winemaking processes, caused by its unpleasant smell of rotten egg. Indeed, high residual concentrations of H_2S require specific treatment to eliminate this off-flavour. The increase in H_2S production induced by sulfite addition is explicable due to its role as an intermediate metabolite in the sulfur assimilation pathway. However, the significant differences we observed between wine and oak strains are intriguing, especially considering that wine strains harbour several

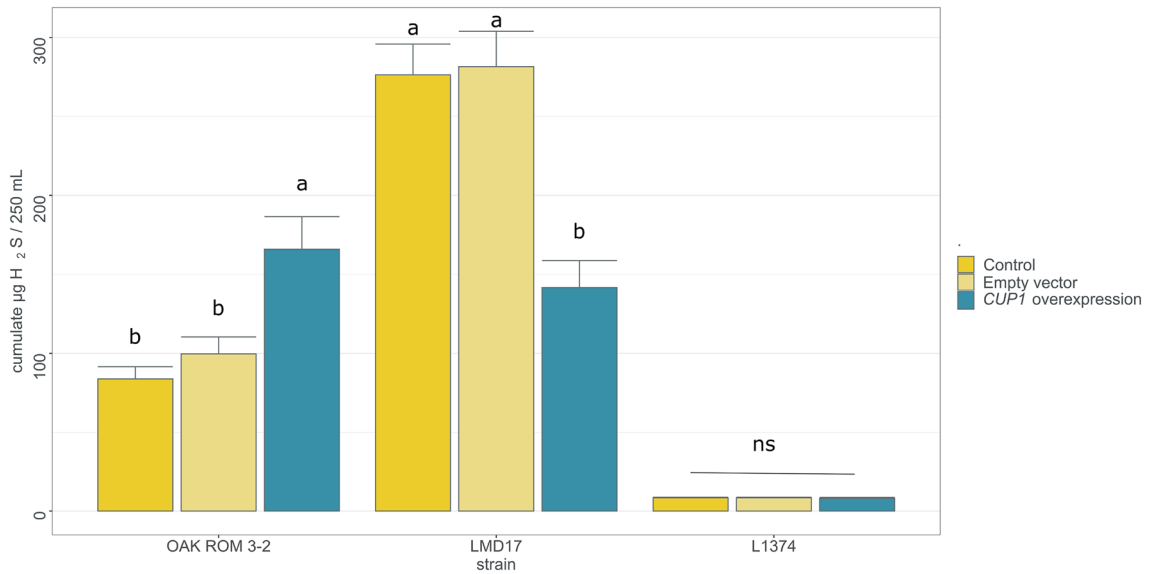
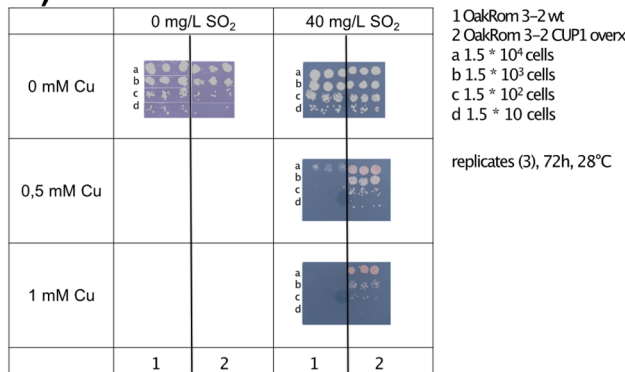


Figure 4. Effect of *CUP1* overexpression on total H₂S released during alcoholic fermentation (in synthetic must without SO₂) in three *S. cerevisiae* strains. Different lowercase letters indicate statistically significant differences between the molecular modifications (control wild-type strain, empty vector or *CUP1* overexpressing vector) for each strain separately, after Tukey multiple comparison of means at 95% family-wise confidence level.

A)



B)

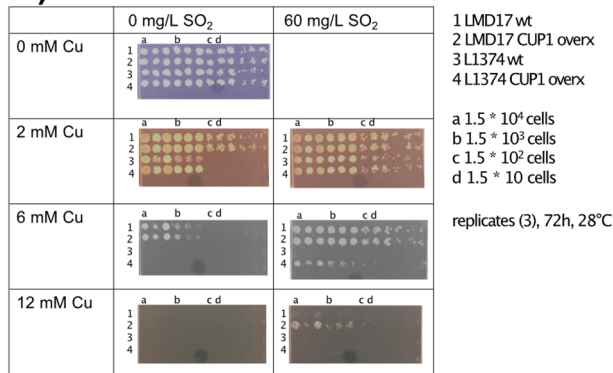


Figure 5. Effect of SO₂ addition in the media on copper resistance. (A) Resistance of oak strain Oak-Rom 3.2 to copper (0 to 1 mM) in the absence (control) and presence of 40 mg/L SO₂. (B) Resistance of wine strain LMD17 and L1374 to copper (0 to 12 mM) in the absence (control) and presence of SO₂ (60 mg/L).

types of translocations leading to a higher expression of the sulfite efflux pump *SSU1*. This apparently contradicts the antagonistic role of *SSU1* in copper resistance³⁰, suggesting a limitation in the sulfur assimilation pathway. However, it should be noted that Onetto et al.'s study was conducted in the absence of added sulfites and indeed, we could show that the introduction of sulfites into the grape must, as commonly practiced by winemakers, increases copper resistance, including for strains with a high *CUP1* copy number. We therefore propose that exogenous sulfite may exceed the expulsion capacity of the sulfite export transporter, thereby increasing H₂S, and hence sulfur containing amino acids and *CUP1* synthesis in wine strains.

We also demonstrate that the presence of copper in grape must increases H₂S production. Our results align with previous expression data^{35,36}, which revealed that copper exposure triggers a higher expression of genes encoding the two subunits of the sulfite reductase *MET5*, *MET10* (i.e., the main enzyme of the sulfur assimilation pathway), and the protein responsible for copper resistance/detoxification *CUP1* following. Furthermore, the metallothionein protein Cup1p, which has one of the highest contents of sulfur-containing amino acids methionine and cysteine in the *S. cerevisiae* proteome, requires the availability of these amino acids for its synthesis. The differences in H₂S production among oak, wine, and velum yeast strains may be attributed to variations in the number of *CUP1* copies in their genomes. However, we describe a complex relationship between the number of *CUP1* copies and H₂S production. Moderate amplification of *CUP1* (up to approximately 10 copies) leads to an increase in H₂S production, whereas higher copy numbers result in a lower fraction of H₂S being stripped by the CO₂ generated during fermentation. One possibility of this curve could result from an increasing activation of SAP to support *CUP1* production when up to at least 10 gene copies are present, whereas for higher number of copies, the higher requirement of sulfur amino acids could exceed maximum activity of the SAP, leading to an increased use of H₂S for the synthesis and a lower release. The increased resistance obtained in the presence of SO₂ supports this hypothesis. In this case, we propose that this reflects a higher utilization of H₂S for amino acid synthesis.

Lastly, our results also shed light on the specific behaviour of flor strains. Unlike wine strains, velum strains exhibit very low H₂S production, and have a lower number of *CUP1* copies. Velum strains grow at the surface of wine, after alcoholic fermentation, which significantly reduces the copper content of wine. It is likely that the selection pressure for copper-resistant strains has been less intense for flor strains compared to wine yeast. Another possible explanation for the lower H₂S production in velum strains is the reduced activity of the pentose phosphate pathway, which provides NADPH, in comparison to oak, bread and wine strains³⁷. This observation elucidates the divergent domestication trajectories of wine and flor strains, reflecting their distinct lifestyles¹³.

Conclusion

The long-term exposure of yeast to copper, used for vine pest management over 150 years, has led to their adaptation by selecting strains with multiple copies of *CUP1*. Our results suggest that this adaptation involves a significant trade-off: increased resistance to copper, but also high H₂S production by the yeast, which is detrimental to wine quality. This increased H₂S production is further exacerbated in the presence of sulfite, another common additive in winemaking. Given the energetic cost of H₂S production, its impact on the global yeast metabolism should be evaluated. Although many projects and techniques have been dedicated to understanding and limiting H₂S production^{26,27,28}, none have investigated the potential role of copper use in causing this phenotype. Therefore, the yeast *CUP1* background should be considered when selecting wine yeast for low H₂S production. However, diversity data suggests that the amplification of *CUP1* likely is not the sole mechanism explaining variations in H₂S production, which requires further investigations.

Materials and methods

Strains

Fifty-one *Saccharomyces cerevisiae* from different geographical areas were characterized for their H₂S production during alcoholic fermentation. The genetic group, identified in previous works indicated in the references of Supplementary Table 1, reflected the colonized ecological niche: 28 belong to the “wine” clade, 14 to “velum” group and 9 to the “oak” one. Strains were selected from our laboratory collection and maintained on solid medium (agar YPD: 2% glucose, 1% yeast extract, 2% bactopectone, 2% agar) at 4 °C.

Fermentation conditions and H₂S quantification

Fermentation experiments were conducted using synthetic must (SM), designed to mimic the characteristics of a natural grape must³⁸. It contained a 200 g/L equimolar glucose and fructose content, and 200 mg/L assimilable nitrogen, 3.8 mg/L phytosterol, and 0.25 mg/L of Cu²⁺. The pH was adjusted to 3.3 with sodium hydroxide solution.

One colony of each strain was grown in 5 ml of liquid YPD at 28 °C for 24 h and then diluted 100 times in SM. After 24 h at 28 °C, cells were counted with an electronic particle counter (Multisizer 3 counter; Beckman Coulter) and 250 mL of SM, supplemented with 60 mg/L of SO₂ when the impact of sulfite was evaluated, were inoculated to 1 × 10⁶ cells/mL. Fermentations were carried out at 28 °C, under permanent stirring (280 rpm) and they were followed daily by weight loss, until the theoretical percentage of sugar consumed reached 95% (87.4 g CO₂/L produced). Total H₂S produced during alcoholic fermentation was collected with a zinc-based trap system and quantified with sulfide specific fluorescent probe, as described before³².

When the impact of the overexpression of *CUP1* was in study, SM was supplemented with Geneticin (G418—Sigma A1720-5G) to maintain the plasmid allowing the overexpression itself. Suitable antibiotic concentrations were defined for each strain (100 µg/mL for wine strains, 40 µg/mL for the oak one), to simultaneously allow the maintenance of the plasmid and a good fermentation rate, but prevent the growth of the sensitive strain (i.e. the wild-type strain without the plasmid).

When assessing the impact of copper concentration on H₂S production, SM was supplemented with copper sulfate to reach 1 or 2 mg/L of copper; control copper concentration was 0.25 mg/L in all the experiments. More details about the experiments are given in the “[Experimental design and statistical analyses](#)” section.

Drop test on copper and sulfite supplemented media

Copper resistance in presence or absence of SO₂, was assessed by a drop-test for three wild type strains with different *CUP1* copy number in their genome (Oak-Rom 3-2, LMD17 and L1374), and their counterpart engineered to over-express *CUP1* (see below). Triplicates of these strains were grown overnight at 28 °C in 5 mL of YPD. Cells were then counted with an electronic particle counter (Multisizer 3 counter; Beckman Coulter), washed with PBS and resuspended in sterile PBS to obtain 10⁷ cells/mL. Three successive 1/10 dilutions were prepared and 1.5 µL of each dilution was spotted on synthetic must having the same composition of the one used for the fermentations, gelled with 20 g/L agar. According to the tested modalities, copper (0, 0.5, 1, 6, 12 mM) and sulfite (0, 40, 60 mg/L SO₂) were added to the media to evaluate their effect. Agar plates were incubated at 28 °C for 72 h and growth was assessed by visual examination.

CUP1 copy number evaluation

For most of the strains, *CUP1* copy number was estimated from their genome sequence, obtained from previous works or from sequencing performed in this study. To obtain the values, the median sequencing depth measured at SNPs encountered between coordinates 212,500 and 213,000, and between 214,500 and 215,000 on Chromosome VIII was divided by the median sequencing depth over the entire genome (excluding mitochondria and 2 microns). For Italian strains, *CUP1* copy number data had been already quantified by Real Time PCR¹⁸.

Genomic DNA extraction for sequencing

Genomic DNA was isolated from liquid yeast cultures in stationary phase, with a classical phenol–chloroform method, as described before³⁹, with an additional purification step based on the use of silica-coated magnetic beads (GMG-252-A-100 mL—PerkinElmer), as follows. Cells were broken mechanically by shaking them in the presence of 600 µm diameter glass beads, lysis buffer (Tris 50 mM pH 8, EDTA 50 mM, NaCl 100 mM, Triton 2%, SDS 1.25%) and phenol chloroform isoamyl alcohol 25:24:1. DNA was precipitated with isopropanol and ethanol, dried, resuspended in TE (Tris 10 mM, EDTA 1 mM) and treated with RNase A. Samples were mixed with the DNA absorption solution (for one sample: 50 µL 5 M NaCl, 15 µL magnetic beads (GMG-252-A-100 mL—PerkinElmer), 250 µL 7.8 M guanidium chloride, 800 µL isopropanol), after which metal beads with DNA absorbed on their silica surface were recovered using the DynaMag™-2 Magnet tube holder (12321D-DynaMag-2—Invitrogen) and washed twice with AMMLAV/E buffer (10 mM Tris pH 8, 0.1 mM EDTA, 60 mM potassium acetate, 65% ethanol) and twice with ethanol 75%. DNA was then desorbed and in aqueous solution.

DNA purity was checked from the 260 nm/280 nm and 260 nm/230 nm OD ratio measured with NanoDrop 1000 (ThermoScientific). The DNA was quantified by fluorescence using the QuantiFluor kit, dsDNA system (Promega) and then stored at –20 °C.

Genome sequence and analysis

DNA samples were processed to generate libraries of 500 bp inserts. After passing quality control, the libraries were sequenced with DNBseq technology using BGISEQ-500 platform, generating paired-end reads of 2 × 150 bp.

For each library, low-quality reads were processed and filtered using the FASTX Toolkit v0.0.13.2 and TRIM-MOMATIC v0.36⁴⁰ with the following parameters (LEADING:10 HEADCROP:5 SLIDINGWINDOW:4:15 MINLEN:50).

Reads were then mapped to the S288C reference genome with BWA v0.6.2 with default parameters⁴¹ and genotyping made with samtools v1.11 to obtain a variant file including the sequencing depth of each variant position. Sequence positions were afterwards filtered for quality criteria: sufficient coverage position as well as genotyping and mapping quality (MQ > –20) were kept.

Plasmid construction and yeast transformation

CUP1 was inserted via Gibson assembly method⁴² between TEF promoter and terminator in a high copy Yeast Episomal plasmid (YEp352), modified to confer geneticin resistance (YEp352-G418) to the host cell. In detail, the backbone was amplified with primers P1 and P2, designed to replace the original *URA3* copy of YEp with *CUP1*, since the strains used were not auxotrophic and the selection had been made by antibiotic. Therefore, the backbone contained a 2 um replication origin (multicopy), AmpR, ColE1, pPGK and G418 resistance cassette. *CUP1* was amplified from OakGri7_1, a strain previously sequenced by our laboratory¹⁰, with a single metallothionein copy and the same sequence as laboratory reference strain S288C, used to design primers (P5–P6). TEF promoter and terminator were amplified from pCfB2312⁴³ with primers P3–P4 and P7–P8, respectively. Primer sequences are listed in Table 1.

Proper fragment insertion was verified by enzymatic digestion (NarI, ClaI, PacI—New England Biolabs). To assure that the phenotype was related to the overexpression of *CUP1*, a Yep352-G418 plasmid without *CUP1* was used as control. PCRs were performed with Phusion™ High-Fidelity DNA Polymerase and validated by gel electrophoresis. *Escherichia coli* strain DH5α was used to maintain and amplify the plasmid; cells were selected on LB medium with ampicillin (100 µg/mL) and grown at 37 °C. Yeasts (Oak-Rom 3-2, LMD17 and L1374) were transformed with the lithium acetate method⁴⁴ and strains containing the recombinant plasmids were selected on YPD agar with 200 µg/mL geneticin (G418—Sigma A1720-5G).

Primer name	Primer sequence (uppercase letters are homologous to the template to amplify, lowercase letters are homologous to the adjacent fragment to assemble)
P1	acgccgcatccagtgctgaACATATGCGTATATATACCAATCTAAG
P2	cggcgggacaaggcaagctAGGAGAAAATACCGCATCAG
P3	ctgatgctgtattttctctAGCTTGCCTGTCCCCGCCG
P4	ttaattaatcgctgaacatGGTTGTTTATGTTCCGGATGTGATGTGAGAA
P5	acatccgaacataacaaccATGTTTCAGCGAATTAATTAACCTC
P6	ctttttatgtcagtactgaTCATTTCCAGAGCAGCATG
P7	catgctgctctgggaatgaTCAGTACTGACAATAAAAAGATTCTGTGTTTCAAGA
P8	tggtatataatcgcatgtTCGACACTGGATGGCGCGTTA

Table 1. Primers used in this work.

Experimental design and statistical analyses

Experiment 1: impact of the origin of the isolate and sulfites on H₂S production

33 strains were selected randomly from our laboratory collection (Supplementary Table 1, Dataset 1). Alcoholic fermentations were performed in absence or presence of SO₂, in duplicate for each strain and each condition.

The factors accounting for the variation of H₂S were analyzed with the following analysis of variance model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ijk},$$

where Y_{ijk} is the H₂S production, μ the overall grand mean, α_i is the fixed strain group effect, β_j is the fixed SO₂ effect, γ_{ij} is their interaction effect, and ε_{ijk} the residual error.

The analysis of the residuals showed that three values were distant from the global distribution. Since results of the statistical analysis did not change after removing all the observations of the three outlier strains, the complete dataset was kept as the method is sufficiently robust to mild deviations.

Experiment 2: impact of copper content of the media on H₂S production

Fermentations were performed without SO₂ in triplicate, for each strain (VL1 and LMD17) and each condition (0.25–1 and 2 mg/L of copper).

To evaluate the effect of copper and strains on H₂S production, ANOVA was performed, after checking for the equality of variance with a Levene test. The most parsimonious model was kept after checking of the absence of interaction between strain and the copper content:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk},$$

where Y_{ijk} is the H₂S production, μ the overall grand mean, α_i is the fixed strain effect, β_j is the fixed copper effect and ε_{ijk} the residual error.

Experiment 3: impact of CUP1 copy number on H₂S production

To the strains evaluated in experiment 1, we added 18 wine strains (total strains analyzed = 55), some known to harbor a high number of copies of *CUP1*, and some commercial strains known to be high H₂S producers (Supplementary Table 1, Dataset 2). Alcoholic fermentations were performed in absence of SO₂, in duplicate for each strain.

Different polynomial models were used to describe the interaction between H₂S production and *CUP1* copy number (first-, second- and third-degree polynomial models); ANOVA was used to assess the significance of these models.

Experiment 4: impact of the overexpression of CUP1 on H₂S production

Fermentations were performed without SO₂ and with standard copper content (0.25 mg/L) in triplicate, for each strain (OAK_ROM 1–3, LMD17 and L1374) and each condition (wild-type strain, strain with the empty vector, strain with the *CUP1* overexpressing vector).

ANOVA was performed to test the effect of the genetic modification in each strain. The model used was:

$$Y_{ij} = \mu + \alpha_i \cdot \varepsilon_{ijk},$$

where Y_{ij} is the H₂S production, μ the overall grand mean, α_i is the fixed genetic modification effect, and ε_{ijk} the residual error.

Figure 6 summarizes the experimental design.

For all the experiments, when the impact of one (or more) factor was significant, differences between modalities were evaluated by post-hoc testing (Tukey's HSD multiple-comparison test, $p < 0.05$).

Statistical analyses were performed in the R environment (R version 4.0.2 (2020-06-22)⁴⁵).

Compliance with international and national regulation

Yeast strains were available from culture collection, or gifted by other authors, or provided by the company Lal-lemend. The yeast collection and use was in accordance with all the relevant guidelines.

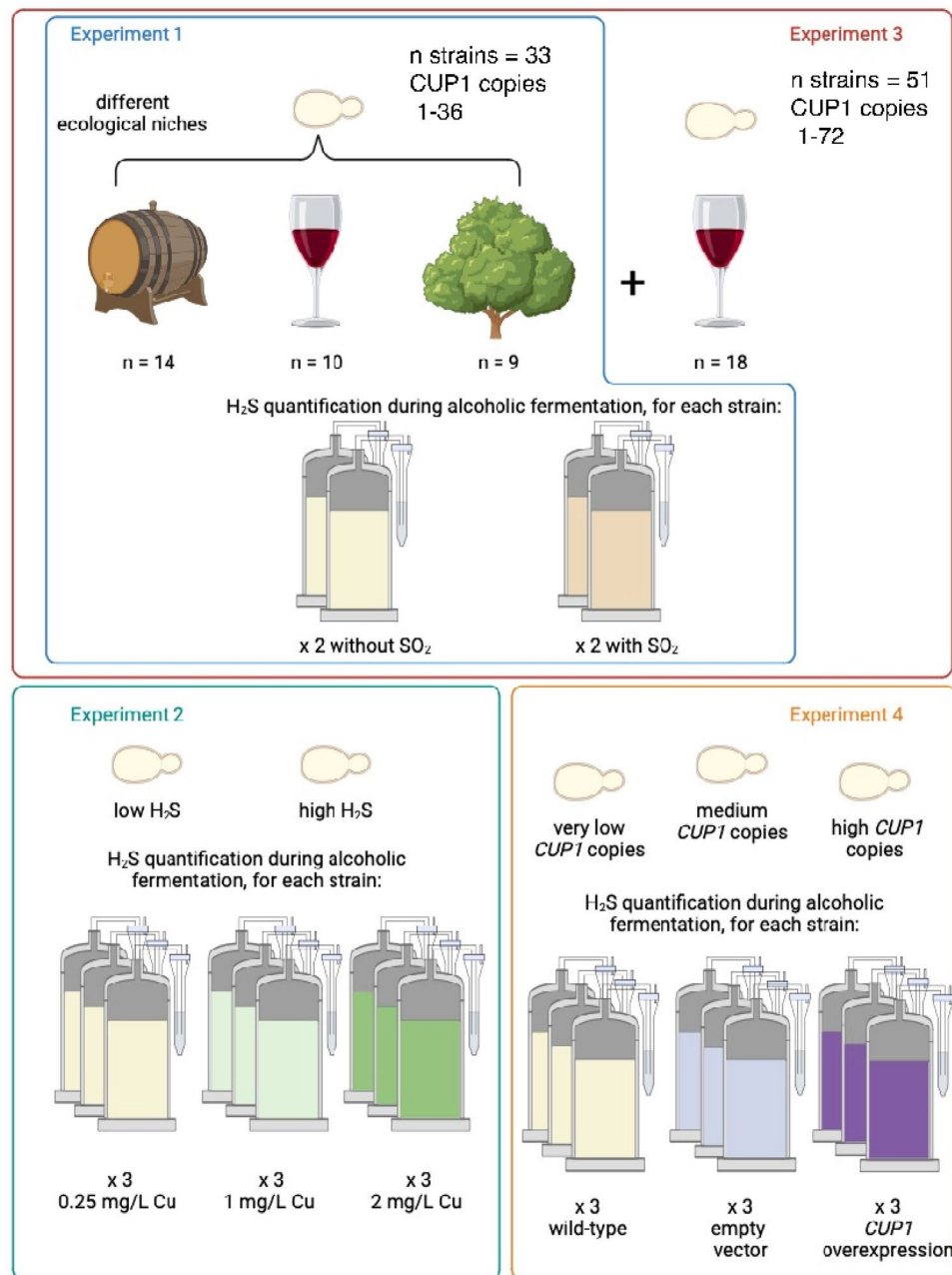


Figure 6. Experimental design.

Data availability

The datasets generated and analysed during the current study are available at Data Gouv (<https://entrepot.reche.reche.data.gouv.fr/>) with the following <https://doi.org/10.57745/5ECVDJ>. Genome sequences were deposited at EBI (<https://www.ebi.ac.uk/>) and corresponding accession numbers are given in Supplementary Table 1 Strain-dataset.xlsx.

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Author contributions

BB and JLL conceived the study and supervised the work; IDG carried out the experiments and wrote the manuscript with support from JLL and BB; VG and IDG conceived the molecular biology experiments. All authors discussed the results and contributed to the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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