RESEARCH ARTICLE

Adaptive response to wine selective pressures shapes the genome of a *Saccharomyces* interspecies hybrid

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Abstract

During industrial processes, yeasts are exposed to harsh conditions, which eventually lead to adaptation of the strains. In the laboratory, it is possible to use experimental evolution to link the evolutionary biology response to these adaptation pressures for the industrial improvement of a specific yeast strain. In this work, we aimed to study the adaptation of a wine industrial yeast in stress conditions of the high ethanol concentrations present in stopped fermentations and secondary fermentations in the processes of champagne production. We used a commercial *Saccharomyces cerevisiae* × *S. uvarum* hybrid and assessed its adaptation in a modified synthetic must (M-SM) containing high ethanol, which also contained metabisulfite, a preservative that is used during wine fermentation as it converts to sulfite. After the adaptation process under these selected stressful environmental conditions, the tolerance of the adapted strain (H14A7-etoh) to sulfite and ethanol was investigated, revealing that the adapted hybrid is more resistant to sulfite compared to the original H14A7 strain, whereas ethanol tolerance improvement was slight. However, a trade-off in the adapted hybrid was found, as it had a lower capacity to ferment glucose and fructose in comparison with H14A7. Hybrid genomes are almost always unstable, and different signals of adaptation on H14A7-etoh genome were detected. Each subgenome present in the adapted strain had adapted differently. Chromosome aneuploidies were present in *S. cerevisiae* chromosome III and in *S. uvarum* chromosome VII–XVI, which had been duplicated. Moreover, *S. uvarum* chromosome I was not present in H14A7-etoh and a loss of heterozygosity (LOH) event arose on *S. cerevisiae* chromosome I. RNA-sequencing analysis showed differential gene expression between H14A7-etoh and H14A7, which can be easily correlated with the signals of adaptation that were found on the H14A7-etoh genome. Finally, we report alterations in the lipid composition of the membrane, consistent with conserved tolerance mechanisms.

DATA SUMMARY

All sequencing data generated in this study are available in the National Center for Biotechnology Information under BioProject PRJNA604709 (genome) and PRJNA604708 (transcriptomic data). Genomic data that were obtained in previous studies and which are used in the present study can be downloaded from NCBI SRA accession numbers SRP148850 and PRJNA473074.

INTRODUCTION

Winemaking is one of the fermentation practices in which yeast species play an important role, by converting sugar

present in the grape must into ethanol, CO_2 and different metabolites. Yeast cells undergo different stresses during fermentation: osmotic pressure due to the high sugar concentration in grape musts at the beginning of the process, ethanol accumulation that can represent up to 16% in the media, low pH, SO_2 presence, etc. [1–6].

Saccharomyces cerevisiae is the *Saccharomyces* species most widely used in fermentation, as it can overcome these stressful conditions, especially ethanol stress conditions [7], during the fermentation process. *Saccharomyces uvarum* is a cryotolerant species that produces more glycerol and less acetic acid than *S. cerevisiae* as well as presenting rich aroma profiles [8, 9]. *S. cerevisiae* × *S. uvarum* hybrids are found in natural habitats and mainly in alcoholic fermentation environments [10, 11]. It has been stated that they can present an advantage in winemaking, especially for white wines, which are fermented at low temperatures [12].

Hybrid genomes are known to fix mutations under selective pressure and undergo adaptive evolution through genome re-organization [13–15]. In this way, *Saccharomyces* interspecies hybrids can be used as model organisms for studying adaptation to stressful environments and better understand the interactions of their subgenomes in the adaptation to these conditions [16].

The study of how genome adaptation occurs is an interesting area of study, and in recent years, several studies on how the adaptation to a specific condition affects either a yeast clone or a yeast population have been conducted [17–19]. The use of a pool of yeast cells as the evolved population instead of the selection of a single adapted clone has the advantage of providing a more general picture of the whole evolution process.

Moreover, adaptation strategies have been carried out to improve yeasts at the industrial level. The use of sequential batch fermentations with selected strains has proved to increase the fitness of *Saccharomyces* hybrids in sulfate limitation conditions and lager-brewing conditions [15, 20].

As for wine strains, it is possible to use a medium simulating wine fermentation and its stresses to adapt strains to that particular must [21]. Adaptation to the wine must is interesting, as this medium contains high concentrations of ethanol and sulfites, which are toxic to yeast cells. In response to ethanol exposure, yeasts incorporate this molecule into the membrane, which causes an increase in the membrane fluidity and an alteration in the lipid composition of membranes [22, 23]. Sulfite (SO_3^{2-}) is usually added in the form of potassium metabisulfite ($K_2S_2O_5$) and it is used because it inhibits the presence of other undesirable microorganisms, although it also affects yeast cells [24].

In a previous study, we obtained an *S. cerevisiae* × *S. uvarum* hybrid, H14A7 [25], which showed a high glycerol production during natural must fermentations in comparison to an *S. cerevisiae* parental strain and a higher ethanol tolerance than the *S. uvarum* parental strain, which are interesting traits for wine strains. This hybrid was stabilized by vegetative growth under fermentative conditions.

Impact Statement

In the last few years, industry has implemented the use of yeast hybrids as starters in different fermentative processes. Different relevant characteristics of the fermentative processes, such as resistance to the pressures imposed by the media, can be improved by using *Saccharomyces* hybrids. How these organisms behave and adapt to stressful environmental conditions is an important question. With the advent of sequencing technologies, the study of genome adaptation and gene expression of yeasts of interest is possible. In this work, we have adapted in the laboratory a *Saccharomyces* wine commercial strain to a medium that mimics that present in wine industrial fermentations. We provide evidence that both phenotypic and genomic changes occur in this hybrid, and that these changes are correlated with the main stressing factors that are present in the wine media: high sulfite and high ethanol concentrations.

In the present work, we aimed to characterize what happens in the H14A7 hybrid genome when we perform a laboratory adaptation strategy by mimicking a must medium similar to that present in advanced stages of wine fermentations when a high sulfite content and reduced levels of sugars and increasing levels of ethanol are present, similar to a stop fermentation or a champagne second fermentation.

H14A7 has two subgenomes: *S. cerevisiae*, conferring ethanol tolerance, and *S. uvarum*, conferring higher glycerol production and capacity to grow at low temperatures [25]; so the analysis of how adaptation to this stressful medium affects each subgenome is one of the main goals of this work.

The adapted strain genome was sequenced and wine fermentations at 15 and 25 °C were performed. Analysis of the transcriptomic and the lipidomic profiles of the newly generated hybrid during the fermentation was carried out to compare the expression and the membrane composition of the adapted hybrid with the original H14A7 strain.

METHODS

Growth on modified synthetic must

Adaptation to a stressful medium of the *S. cerevisiae* AJ4 × *S. uvarum* BMV58 hybrid H14A7, obtained by Lairón Peris *et al.* [25], was performed using batch cultures in triplicate, in bottles of 100 ml with 60 ml of modified synthetic must (M-SM) [26]. Different M-SM compositions were used with different sugar and ethanol concentrations simulating a stop fermentation (low sugar and high ethanol concentration), which are specified in Table 1. In all conditions, 100 mg l^{-1} of metabisulfite, $K_2S_2O_5$, were added to M-SM. The inoculated cell population in each bottle was approximately $2×10^6$, and once the stationary phase was achieved the culture was transferred in fresh media and cultivated in the same way. The

Table 1. Composition of the modified synthetic must (M-SM) used during the adaptive laboratory evolution

Besides the compounds described previously [26] for synthetic must, different ethanol percentages were added and sugar content was modified. In all conditions, metabisulfite ($K_2S_2O_5$) at 100 mg l^{-1} was added to M-SM.

Condition	Ethanol (%, v/v)	Glucose (g l^{-1})	Fructose (g l^{-1})
0	2.5	75	75
1	5	50	50
2	6	40	45
3	6.5	35	40
4	7	30	40
5	7.5	25	35
6	8	20	35
7	9	20	35

initial ethanol concentration was 2.5% (v/v). The medium was refreshed approximately every 7 days. Ethanol concentration was increased every 2 or 3 weeks depending on the latency period and the time the cultures took to reach the stationary phase. All adaptation processes were performed at an incubation temperature of 28 °C, which is an optimal temperature for this strain to grow, with orbital continuous shaking at 100 r.p.m. The growth of the triplicates was followed by OD$_{600}$ measurements. When an ethanol concentration of 9% in the medium was reached, a pool of colonies of one of the triplicates was selected and named H14A7-etoh. This selection was based on the higher specific growth rate of the triplicate under these conditions. H14A7-etoh consists of a population of subclonal lineages which have emerged during the adaptation process. Hereafter, the term adapted strain will be used to refer to the pool of subclonal lineages of H14A7-etoh retrieved at this point. By definition, a strain is a genetically uniform microbial culture, and although H14A7-etoh is the result of an adaptation process, during both industrial processes and growth, populations accumulate genome variations from the initial clonal population, and as a consequence, the term adapted strain can be used [19, 27–30].

Yeast growth media conditions

Tolerance to ethanol was determined by growing H14A7 and H14A7-etoh strains in YNB with increasing ethanol concentrations (0, 1, 2.5, 4, 6, 8, 10, 12.5, 14, 15.5, 17, 18, 20 %) in microtitre plates (an initial OD$_{600}$ of 0.1 in 220 µl of each medium).

Overall yeast growth was estimated as the area under the OD vs. time curve using GCAT [31], and the parameters NIC and MIC, which are ethanol-tolerance indicators, were obtained as described elsewhere [7].

Tolerance to sulfite stress was evaluated by drop tests. Sulfite plates were prepared by using YEPD+TA (tartaric acid) agar plates and supplementing them with different $K_2S_2O_5$

concentrations. YEPD+TA plates were prepared as described previously [32] (YEPD: 2% glucose, 2% peptone, and 1% yeast extract; 75 mM L-tartaric acid buffered at pH 3.5). YEPD+TA+$K_2S_2O_5$, sulfite plates, were prepared by pouring and spreading freshly prepared $K_2S_2O_5$ on each YEPD+TA solid plate to reach the following concentrations of metabisulfite: 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 mM.

Yeast precultures with the pool of H14A7 and H14A7-etoh strains were grown overnight in GPY medium. Cell cultures were diluted to an OD$_{600}$ of 0.1. Then, serial dilutions of cells were transferred to the plates and incubated at 25 °C for a week.

Genome sequencing, copy number analysis and SNP fixation analysis

The sequenced reads of the H14A7 original hybrid and the genome assemblies and genome annotation files of BMV58 and AJ4 strains were available from previous work [25]. H14A7-etoh DNA was extracted according to Querol *et al.* [33], and the sequencing library was prepared using the TruSeq DNA PCR-free kit, using sonication as the fragmentation method. Sequencing was performed using the Illumina Miseq system, with paired-end 250 bp reads and were deposited in NCBI (accession number PRJNA604709). These reads were trimmed and quality-filtered to a quality of 28 and a length of 180 using Sickle [34]. H14A7-etoh reads were mapped to the concatenated sequences assemblies of *S. cerevisiae* and *S. uvarum* parentals AJ4 and BMV58 by using BOWTIE2 with default settings. Bedtools was used to obtain the coverage 'per base'. These coverage files were processed to reduce the noise using sliding windows with a mean window size of 1000 positions. As a complementary approach, CNVnator was used for the discovery of copy number variation (CNV) [35]. We used sppIDer (https://github.com/GLBRC/sppIDer) to plot chromosome coverage [36].

The gdtools command installed as part of breseq (version 0.27.1) was used to identify SNPs in the H14A7-etoh genome which were present in neither of the parental genomes (AJ4 and BMV58) nor in the hybrid H14A7. We used H14A7-etoh read files and the annotation files of AJ4 and BMV58 as a reference, with option-p --polymorphism-frequency-cutoff of 0.20. The same procedure was performed with H14A7, AJ4 and BMV58 reads to retain only variants that are only present in H14A7-etoh. We manually curated the SNPs present in non-synonymous positions using the software Tablet [37], by visualizing the reads of H14A7 and H14A7-etoh against the assemblies of AJ4 and BMV58 parentals, to only take into account SNPs that were fixed in the adapted hybrid. Only indels and SNPs with a coverage of >19 were kept.

Flow cytometry analysis

The DNA content of the adapted hybrid was assessed by flow cytometry using a FACSVerse flow cytometer (BD Biosciences). Cells were grown overnight in GPY and 1 OD$_{600}$ of each culture was harvested by centrifugation. DNA staining was performed using SYTOX Green dye as previously

described [38]. Haploid (S288c) and diploid (FY1679) reference *S. cerevisiae* strains were used to compare the fluorescence intensity.

Microfermentations in Verdejo must and transcriptomic analysis

Microvinifications were conducted in triplicate in Verdejo must with the adapted strain H14A7-etoh at two different temperatures: 25 and 15 °C, as described previously [25] for *S. cerevisiae* AJ4, *S. uvarum* BMV58 and the hybrid H14A7 strains. Final metabolites were measured by HPLC in the last stage of fermentation. Weight loss data were followed during the fermentations and corrected to the percentage of consumed sugar as described previously [39]. Data on the percentage of consumed sugars were fitted to the Gompertz equation [40]. Kinetic parameters D, maximum sugar consumption value reached (the asymptotic maximum, %), m (maximum sugar consumption rate, g L^{-1} h^{-1}), nd l (lag phase period, h) were calculated. These data were tested to find significant differences among them by using the one-way ANOVA module of the Statistica 7.0 software (StatSoft). Means were grouped using the Tukey HSD test (α=0.05).

Samples for RNA-sequencing (RNA-seq) were collected at two different time points: lag phase (which corresponded to 4 h of fermentation at both temperatures) and mid-exponential growth phase (which corresponded to 24 h of fermentation at 25 °C and 48 h of fermentation at 15 °C respectively) and were analysed as previously described [25]. Reads were sequenced using the Illumina Hiseq 2000, and paired-end reads of 75 bases long were generated and submitted to NCBI SRA (accession number PRJNA604708). These reads were quality-trimmed using sickle (length 50, quality 23) and aligned to the fasta reference using bowtie2. We used HTSeq-count [41] with both annotated files and the mapping files ordered by names and generated the counts table. The mapping reads with a quality score <2 and with more than one alignment were discarded. Data were analysed using the EdgeR package to look for differential expression genes [42]. We calculated normalization factors to scale the raw library sizes and then we tested for differential expression between two groups of count libraries. Differential expression levels (relative RNA counts) between the different conditions were considered significantly different with a false discovery rate (FDR) [43] at a threshold of 5%. Gene Ontology (GO) terms were attributed to the lists of differentially expressed genes by using YeastMine from the SGD Database (https://yeastmine.yeastgenome.org/). GO terms enrichment were retrieved with *P*-values <0.05 after computing the Benjamini and Hochberg correction for multiple hypotheses.

MS of lipids present in the membrane of the strains

Four yeast strains (AJ4, BMV58, H14A7 and H14A7-etoh) were grown in 25 ml GPY media, with five flasks set up per strain. After 4 h of growth, the cultures were harvested and total lipids were extracted using a modified Bligh and Dyer protocol [44]. The lipids were reconstituted in 100 μl chloroform and then diluted 1 in 50 in solvent A [50:50 acetonitrile/ H$_2$O, 5 mM ammonium formate, and 0.1% (v/v) formic acid]. Analysis of 10 μl samples was performed by LCMS. LC was performed on a U3000 UPLC system (Thermo Scientific) using a Kinetex C18 reversed-phase column (Phenomenex, 2.6 μm particle size, 2.1×150 mm), at a flow rate of 200 μl min^{-1} with a gradient from 10% solvent A to 100 % solvent B [85:10:5 isopropanol/acetonitrile/H$_2$O, 5 mM ammonium formate and 0.1% (v/v) formic acid] with the following profile: t=0 10% A, t=20 86%A, t=22 96%A, t=26 95%A. MS analysis was carried out in positive and negative ionization mode on a Sciex 5600 Triple TOF device. Source parameters were optimized on infused standards. Survey scans were collected in the mass range 250–1250 Da for 250 ms. MSMS data were collected using top five information-dependent acquisition and dynamic exclusion for 5 s, using a fixed collision energy of 35 V and a collision energy spread of 10 V for 200 ms per scan. ProgenesisQI was used for quantification and LipidBlast for identification. All data were manually verified and curated.

Lipid quantification by ammonium ferrothiocyanate assay

To quantify the lipids, a 10 μl sample was taken from the above 100 μl reconstituted lipids in chloroform and added to 2 ml chloroform with 1 ml of assay reagent (0.1 M FeCl$_3$,6H$_2$O, 0.4 M ammonium thiocyanate) in a 15 ml glass tube. Samples were vortexed for 1 min and centrifuged at 14500 **g** for 5 min. The lower layer was collected into quartz cuvettes. The absorbance was measured at 488 nm, and the concentration of lipid was determined by comparison with a standard curve of a mixture of phospholipid standards (POPC, POPE, and POPG; Sigma).

TLC analysis

Yeast lipids extracted as above after 24 h growth were analysed by TLC. Briefly, 20 μg of lipid sample and 10 μg phospholipid lipid standards (POPC, POPE, and POPS; Sigma) were loaded onto silica gel TLC plates (Sigma) and separated using chloroform/methanol/acetic acid/water (25:15:4:2). The plates were air dried and either sprayed with molybdenum blue reagent (1.3% molybdenum oxide in 4.2 M sulphuric acid; Sigma), or *sp* ninhydrin reagent (0.2% ninhydrin in ethanol; Sigma) and charred at 100 °C for 5 min. Spot intensity was determined using ImageJ software.

Laurdan membrane fluidity assay

Yeast precultures of each one of the four selected strains (AJ4, BMV58, H14A7 and H14A7-etoh) were first propagated overnight in 25 ml of GPY medium at 200 r.p.m. and 28 °C. Then, 10 ml of GPY medium in 15 ml Falcon tubes was inoculated to an OD$_{595}$ of 0.4 and incubated at 200 r.p.m. and 28 °C. Samples were taken after 24 h and live yeast were diluted to an OD$_{595}$ of 0.4 in GPY and incubated with 5 μM laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) for 1 h. Fluorescence emission of these cells stained with laurdan was taken using a microplate reader (Mithras, Berthold) with the following filters; λ_{ex}=350 and λ_{em}=460 and 535. Generalized polarization (GP), derived from fluorescence intensities at critical
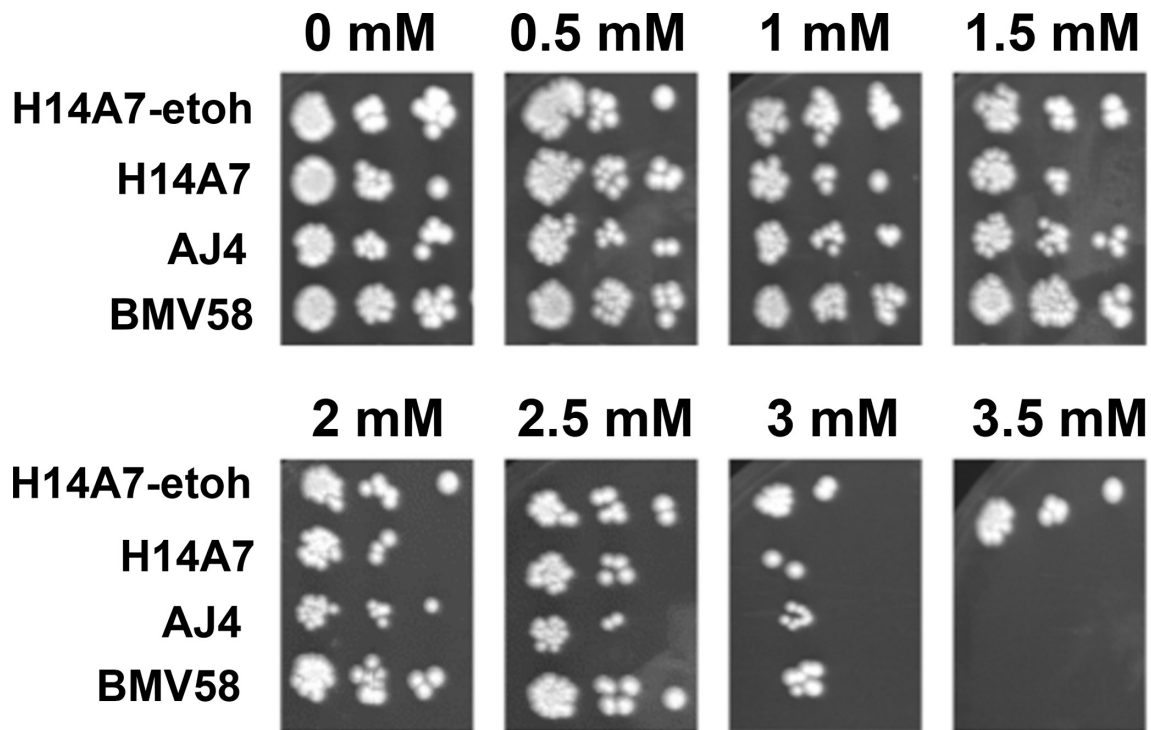
Fig. 1. Sulfite tolerance analysis of the strains H14A7-etoh, H14A7, BMV58 and AJ4 in YEPD+TA+different $K_2S_2O_5$ concentrations. Images were taken after 7 days of growth at 25 °C.

wavelengths, can be considered as an index of membrane fluidity and is calculated as $GP=(I_{460}-I_{535})/(I_{460}+I_{535})$.

RESULTS

Characterization of the hybrid after the adaptation process

Hybrid H14A7 was subjected to adaptation to ethanol in liquid media. A series of synthetic musts with increasing ethanol content mimicking different stages of the fermentation process were used to that end, maintaining a high concentration of metabisulfite in all cases and increasing the ethanol concentration (Table 1). H14A7 was exposed to these media conditions for approximately 200 generations and the obtained adapted strain was named H14A7-etoh. The term 'strain is used during the text, but H14A7-etoh consists of a population of subclonal lineages.

After that process, we carried out drop tests in plates containing different concentrations of sulfite to see if the presence of metabisulfite in our adaptation media affected sulfite tolerance in the adapted strain. Interestingly, the phenotypes of the different tested strains (H14A7, H14A7-etoh, BMV58, and AJ4) showed remarkable differences, with H14A7-etoh being the most resistant (Fig. 1).

We tested the ethanol tolerance of strains H14A7-etoh and H14A7 to see if the addition of ethanol also had an impact on the phenotype. The NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration), which are two parameters that respectively indicate which ethanol concentration affects a strain and at which ethanol concentration the strain is not able to grow, were calculated. H14A7 NIC and MIC values were 8.51±0.27 and 14.5±0.354 %; whereas H14A7-etoh NIC and MIC values were 7.93±0.26 and 15.3±0.143 %. The adapted hybrid showed slightly greater ethanol tolerance than H14A7 in its MIC value ($P<0.1$, ANOVA and Tukey's test), NIC values being non-significant ($P<0.05$, ANOVA and Tukey's test).

H14A7-etoh shows different signals of adaptation to the selection media on its genome: CCNVs (chromosome copy number variations)

This part of the study aimed to detect genomic differences of the adapted hybrid in comparison to the original strain after the different rounds of adaptation. After obtaining the sequenced reads of the H14A7-etoh genome, they were quality-trimmed and reduced to a total number of 6767268 reads, which represents a coverage of approximately 67.5×. With these reads, we performed analyses to identify differences from the original H14A7 hybrid genome.

From previous work [25], we knew that H14A7 had two copies of each *S. cerevisiae* chromosome, except for chromosome III, which was present only in one copy; and one copy of each *S. uvarum* chromosome. After this adaptation process, we expected that the hybrid would present changes in its genome, as the accumulation of aneuploidies in hybrids is frequent even under non-stress conditions [45]. We performed an

analysis with sppIDer and CNVnator and noted large modifications in the chromosomes of the adapted strain H14A7-etoh compared to the hybrid H14A7 genome; *S. cerevisiae* (III-cer) and *S. uvarum* chromosome VII–XVI (VII-XVI-uva) had been duplicated. Moreover, a chromosomal loss of *S. uvarum* chromosome I (I-uva) had taken place.

Analysis by flow cytometry revealed that the ploidy of H14A7-etoh was 3.27±0.1, whereas H14A7 had a ploidy of 2.98±0.02 [25]. This increased ploidy can be explained because of the aneuploidies mentioned above, especially the VII-XVI-uva duplication. III-cer and I-uva are small chromosomes, and their contribution to the ploidy is smaller than that of VII-XVI-uva. In Fig. 2a, b, a representation of the chromosome copy number of H14A7 and H14A7-etoh is presented.

III-cer and VII-XVI-uva aneuploidies could have a relevant role in the adapted hybrid. VII-XVI-uva is a chromosome with a translocation in parental strain BMV58 that confers sulfite resistance to strain BMV58, as it recombines the *FZF1t* transcription factor (present in chromosome VII) with the *SSU1* gene involved in sulfite metabolism (present in chromosome XVI) (Macias *et al.* unpublished data). The presence of an extra copy of this chromosome in H14A7-etoh is the most reasonable explanation of the strain's high resistance to sulfite. III-cer aneuploidies have been correlated with ethanol tolerance in *S. cerevisiae* strains [46].

H14A7-etoh shows different signals of adaptation to the selection media on its genome: SNPs, duplications and deletions

To better understand genetic variation in the adapted strain, we retrieved SNPs in H14A7-etoh. The total number of SNPs present in coding positions of H14A7-etoh that were not present in H14A7 were: 200 in non-synonymous positions and 256 in synonymous positions of genes. Of these SNPs, we manually retrieved those in which the adapted hybrid has fixed a variant, that is, they were present in the hybrid as a heterozygous nucleotidic base and their frequency is now 1 or they have changed the nucleotidic base present in the H14A7 genome. There are four positions in *S. cerevisiae* chromosome I with fixed positions: they are in genes YAL016C-A (dubious ORF), YAL010C (a subunit of both the ERMES and the SAM complex), YAR019C (a protein kinase of the mitotic exit network) and YAR035W (an outer mitochondrial carnitine acetyltransferase) (Fig. 2c). None of these changes is generating loss of functional genes. Moreover, we observed that the heterozygosity present in *S. cerevisiae* chromosome I is lost all over this chromosome, as a loss of heterozygosity (LOH) event took place during the adaptation and no CNVs were found among the genes of this chromosome.

The possible duplications and deletions of different chromosome regions that were obtained by using CNVnator on H14A7-etoh were compared with H14A7 CNVnator coverage values and were visualized by using the mapped reads of H14A7 and H14A7-etoh against AJ4-BMV58 parental genomes. CNVnator normalizes the coverage values to 1,

and if the resulting coverage number has deviated from these values, there is a putative deletion or duplication in the region.

In H14A7-etoh *S. cerevisiae* chromosome I there is a region that comprises 6.2 kB whose coverage value is 0.2983 instead of 1. In this region, two genes are deleted in the adapted hybrid: YAR028W and YAR027W, putative integral membrane proteins of unknown function, and members of the DUP240 gene family. This region has two flanking Ty1 elements in the original hybrid genome, so a Ty1–Ty1 recombination event could have taken place and provoked a deletion (Fig. 2c).

In the H14A7-etoh *S. uvarum* subgenome the gene *TDH1*/YJL052W seems to be duplicated [coverage ratio (cr) of H14A7-etoh/H14A7 is 2.14). This is a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) involved in glycolysis and gluconeogenesis which is located next to the ARS1011 replication origin. In the H14A7-etoh AJ4 subgenome there are four genes that showed a cr H14A7-etoh/H14A7 >1.5, indicating four possible duplications: *GAD1*/YMR250W (cr=1.65); *GUT1*/YHL032C (cr=1.58); *STR3*/YGL184C (cr=1.52) and *HPF1*/YOL155C (cr=1.68) (Fig. 2d).

GAD1/YMR250W is a glutamate decarboxylase that converts glutamate to gamma-aminobutyric acid (GABA) during glutamate catabolism and that is involved in response to oxidative stress [47]. It is located between the Ty2 LTR and ARS1328. *GUT1*/YHL032C is a glycerol kinase; it converts glycerol to glycerol 3-phosphate; *STR3*/YGL184C is a peroxisomal cystathionine beta-lyase that converts cystathionine to homocysteine; and *HPF1*/ YOL155C is a haze-protective mannoprotein. None of the SNPs, nor small duplications and deletions detected seem to have a particular role in adaptation of the hybrid adapted yeast to the M-SM used. Instead, it is important to point out that detected CNVs are near ARS and Ty elements.

H14A7-etoh performance during Verdejo fermentations

H14A7 and H14A7-etoh were used as starters of fermentations in Verdejo at 15 and 25 °C, conditions that mimic wine industrial conditions. The fermentation kinetics were similar between strains H14A7 and H14A7-etoh and showed no statistical differences in the calculated parameters except the maximum sugar consumption rate value, which was higher for H14A7 at 15 °C (Table 2). Final wine composition varied between H14A7 and H14A7-etoh (Table 3). H14A7-etoh left fructose in the fermentations at both temperatures. The amounts of fructose left behind were significantly higher than that of H14A7 (5.55 g l^{-1} at 25 °C and 5.44 g l^{-1} at 15 °C; whereas H14A7 left 0.77 and 1.44 g l^{-1}, respectively) (Table 3). One of the three biological replicas of the H14A7-etoh fermentation at 15 °C was slightly delayed in comparison with the other two biological replicas (data not shown).

Ethanol and glycerol percentages were similar for both strains if we compare the final must concentrations for these compounds at the same temperature. Surprisingly, glycerol production was higher at 25 °C than at 15 °C. Acetic acid
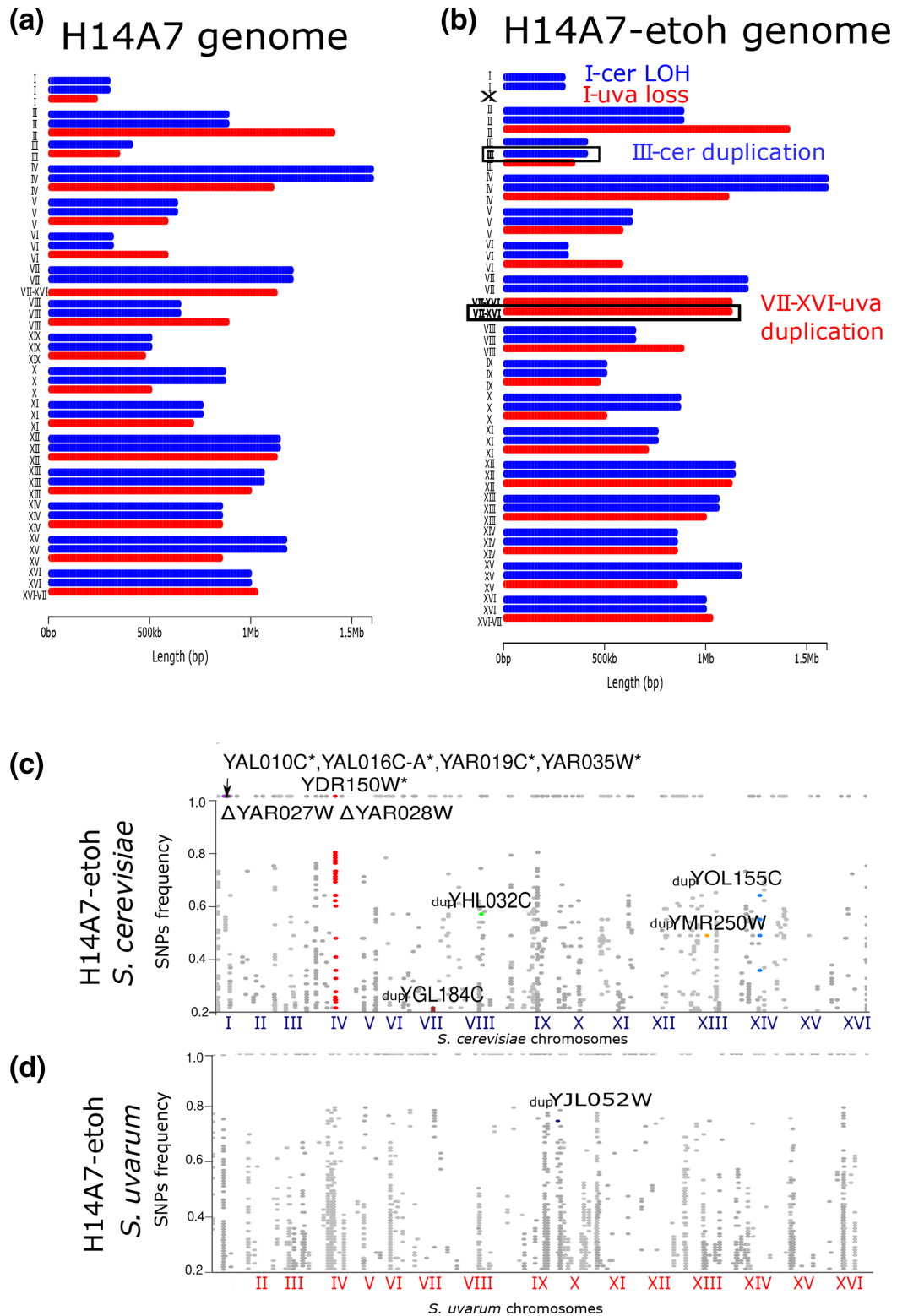
(a) H14A7 genome

(b) H14A7-etoh genome

Fig. 2. Genome-wide representation of strain H14A7-etoh. The chromosomes of H14A7 (a) and the H14A7-etoh adapted strain (b) were represented after analysis with spplDer and CNVnator by using the chromoMap R package. Chromosome length is based on AJ4 *S. cerevisiae* and BMV58 *S. uvarum* reference genomes. SNPs present in *S. cerevisiae* (c) and *S. uvarum* (d) chromosomes of the H14A7-etoh adapted strain are represented. SNPs whose frequency is 1 and whose change affects a non-synonymous position of a gene are marked with an asterisk, while confirmed duplications are marked as dup and confirmed deletions as Δ.

Table 2. Kinetic parameters of the fermentations performed at 25 and 15 °C in Verdejo must

Parameters were obtained through an adjustment to the Gompertz equation [40]. *D* represents he maximum sugar consumption value reached (%), *m* the maximum sugar consumption rate (g l^{-1} h^{-1}) and *l* the lag phase period (h). Values are given as mean±SD of three biological replicates. An ANOVA was carried out and values followed by different superscript letters are significantly different according to the Tukey HSD test (α=0.05).

	D (%)	*m* (g l^{-1} h^{-1})	*l* (h)
H14A7-25 °C	97.51±0.28 [a]	1.761±0.0985 [a]	9.84±0.080 [a]
H14A7-etoh-25 °C	98.02±0.49 [a]	1.79±0.028 [a]	11.30±0.95 [a]
H14A7-15 °C	96.96±0.78 [a]	0.78±0.026 [a]	23.96±2.20 [a]
H14A7-etoh-15 °C	94.65±0.64 [b]	0.77±0.069 [a]	25.08±5.10 [a]

production was higher for the H14A7-etoh adapted strain at both temperatures and the remaining acids (tartaric, malic, citric and L-lactic) showed no statistical differences in their content between the two strains.

Transcriptomic analysis of the adapted hybrid correlates with its phenotype

To better understand the properties acquired in the adapted hybrid (H14A7-etoh) compared to the initial strain (H14A7), we performed a comparative study of gene expression of the adapted hybrid and H14A7 during Verdejo fermentations. We retrieved a total of 24 samples (2 strains × 2 times × 2 temperatures × 3 replicates) that were obtained during the Verdejo fermentations and processed to obtain RNA and transcriptomic data. We first subdivided these samples into 48 subfiles with gene counts for each species' subgenome of the two strains (a file with the expression of *S. uvarum* alleles and a file with the expression of *S. cerevisiae* alleles). We observed that samples belonging to the third replicate of H14A7-etoh fermentation at 15 °C – whose growth was delayed during the fermentation – were outliers, so we excluded them.

The first step of our analyses consisted of carrying a principal component analysis (PCA) that clustered the remaining 44 subfiles depending on the variance among their gene expression (Fig. S1a). This plot showed that the most important condition that separates samples is the stage of growth (58% of the variance is explained by this variable), and the second component, PC2, depended on the species subgenome analysed (*S. cerevisiae* or *S. uvarum* part) (it explained 26% of the variance).

As we wanted to use the total number of ORFs annotated in both H14A7 and H14A7-etoh (expression of *S. cerevisiae* and *S. uvarum* alleles), we constructed another PCA based on 22 samples corresponding to H14A7 and H14A7-etoh fermentations (Fig. S1b). PC1 depends on the stage phase and temperature (it explained 82% of the variance). PC2 accounts only for 9% of the sample variability and it corresponds to variation between strains (H14A7 or H14A7-etoh).

We therefore performed one differential expression (DE) test for each of the four conditions: exponential 25 °C, latency 25 °C; exponential 15 °C and latency 15 °C, to compare differential gene expression of H14A7-etoh against H14A7. We first retrieved the ORFs with less than 0.5 counts per

million (CPM) in one of the conditions in order not to use not expressed genes or lowly expressed genes. The H14A7-etoh adapted strain has an aneuploidy in I-uva – the copy of this chromosome was lost during the adaptive process – and, consequently, no transcription of H14A7-etoh I-uva genes took place. After excluding the genes with low expression, a total number of 10589 ORFs were used in the differential analysis, with 5411 *S. cerevisiae* ORFs and 5177 *S. uvarum* ORFs.

We performed the comparison between H14A7-etoh and H14A7 by using the *edgeR* R package and kept the DE genes whose Benjamini–Hochberg corrected *P*-value was <0.05. The list of DE genes, with the log$_2$ fold change (FC), log CPM and *P*-values for each one of the four conditions, as well as the enriched GO biological process terms that were retrieved from these gene lists, is given in Table S1.

Given that VII-XVI-uva and III-cer chromosomes have two copies in H14A7-etoh and one copy in H14A7, we first plotted the logFC, which indicates if a gene is more expressed in H14A7 or H14A7-etoh, for each of the genes (not just the DE genes) against its calculated gene ratio (H14A7-etoh vs H14A7) (Fig. 3). The calculated gene ratios are based on the coverage files that were obtained for each of the strains using the sliding windows approach. In Fig. 3, it can be observed that gene expression is significantly related to the number of copies of each gene. Genes belonging to III-cer and VII-XVI-uva have mainly negative logFC for each of the four conditions, which in our comparison (H14A7 vs H14A7-etoh) indicated that these genes are more expressed in the adapted hybrid than in the hybrid. This tendency can be observed in particular in the exponential stage at 25 °C. We further analysed if any other chromosome had DE genes in the H14A7 or H14A7-etoh strains under any of the experimental conditions (Table S2, Fig. S2). In the case of the exponential stage at 25 °C, apart from VII-XVI-uva and III-cer, *S. uvarum* chromosome III (III-uva) genes seem to be more expressed than the genes of the remaining chromosomes (Wilcoxon test, *P*<0.0001).

If we analyse the number of statistically DE genes for each condition and strain in comparison with the other, and identify which of the genes belong to the *S. uvarum* and *S. cerevisiae* subgenome and which genes belong to III-cer or VII-XVI-uva, we can confirm that in the four conditions, H14A7-etoh overexpressed more genes that belong to III-cer

Table 3. Final chemical composition of the Verdejo fermentations at 25 and 15 °C

The amount of each component was measured by HPLC. Values are given as mean±SD of three biological replicates. An ANOVA was carried out and values followed by different superscript letters are significantly different according to the Tukey HSD test (α=0.05).

	Glucose (g l⁻¹)	Fructose (g l⁻¹)	Glycerol (g l⁻¹)	Ethanol (%)	Acetic acid (mg l⁻¹)	Citric acid (mg l⁻¹)	Tartaric acid (mg l⁻¹)	Malic acid (mg l⁻¹)	L-Lactic acid (mg l⁻¹)
H14A7-25 °C	0.02±0.02ᵃ	0.77±0.16ᵃ	11.23±0.13ᵃ	12.72±0.36ᵃ	0.46±0.07ᵃ	0.39±0.01ᵃ	2.4±0.12ᵃ	1.96±0.14ᵃ	1.02±0.14ᵃ
H14A7-etoh-25 °C	0.02±0.00ᵃ	5.55±1.04ᵇ	11.11±0.54ᵃ	12.78±0.58ᵃ	0.907±0.07ᵇ	0.39±0.05ᵃ	2.36±0.17ᵃ	2.36±0.24ᵃ	0.79±0.06ᵃ
H14A7-15 °C	0.00±0.00ᵃ	1.41±0.53ᵃ	8.70±0.09ᵃ	12.86±0.12ᵃ	0.58±0.07ᵃ	0.28±0.05ᵃ	1.92±0.09ᵃ	1.79±0.07ᵃ	0.38±0.03ᵃ
H14A7-etoh-15 °C	0.03±0.01ᵇ	5.44±0.57ᵇ	8.82±0.53ᵃ	12.53±0.18ᵃ	1.06±0.15ᵇ	0.24±0.02ᵃ	2.19±0.18ᵃ	1.77±0.06ᵃ	0.40±0.15ᵃ

and VII-XVI-uva than H14A7 (Fig. S3). The number of up-regulated genes in H14A7-etoh compared to H14A7 is higher than the number of H14A7 compared to H14A7-etoh at 25 °C. At this temperature at both time points (latency and exponential growth phase), the overexpressed genes in H14A7-etoh belong to the *S. uvarum* subgenome of the adapted hybrid, and especially to chromosomes VII–XVI.

In the latency stage at 25 °C, the H14A7-etoh *S. cerevisiae* subgenome overexpressed genes related to protein folding and catabolic process, and in the exponential stage at 25 °C, the H14A7-etoh *S. cerevisiae* subgenome overexpressed genes related to the GO term alpha-amino acid metabolic process (BH correction with $P<0.05$).

At 15 °C, for both time points the number of DE genes between the strains was lower than at 25 °C, and the number of up-regulated genes in H14A7-etoh and the number of up-regulated genes in H14A7 was very similar. One interesting fact is that at low temperature, 15 °C, *S. uvarum* alleles show more up-regulation in H14A7 compared to H14A7-etoh than the *S. cerevisiae* alleles and in the latency stage at 15 °C H14A7-etoh overexpressed more *S. cerevisiae* than *S. uvarum* alleles. With PheNetic [48], we further explored the regulation of the down-regulated *S. uvarum* alleles in H14A7-etoh in the latency stage at 15 °C (upstream mode, number of best paths=20, pathlength=4, cost=0.1). We obtained a regulatory network with nine central regulatory nodes (Fig. S4). Among them, the transcription factors *CIN5* (YOR028C) and *YAP6* (YDR259C), which are paralogues involved in carbohydrate metabolism, are present. Moreover, *ADR1* (YDR216W), which is a carbon source-responsive transcription factor; required for transcription of the glucose-repressed gene *ADH2*, may be involved in the down-regulation of these genes. *SKN7* (YHR206W), a transcription factor required for optimal induction of heat-shock genes in response to oxidative stress, is down-regulated too.

These differences in gene expression could be of interest as *S. cerevisiae* and *S. uvarum* species show differential adaptation at low temperatures, *S. uvarum* being better adapted than *S. cerevisiae* to low temperatures. H14A7-etoh showed a delayed lag phase in one fermentation carried at 15 °C and the maximum sugar consumption rate of H14A7-etoh was lower than that of H14A7. This decrease in the capcity of H14A7-etoh to perform fermentation at 15 °C could be related to the lower expression of *S. uvarum* alleles.

The five GO terms that were significantly enriched (BH correction with $P<0.05$) in this list of overexpressed *S. cerevisiae* alleles in H14A7-etoh are protein folding (GO:0006457), protein refolding (GO:0042026), regulation of ATPase activity (GO:0043462), positive regulation of ATPase activity (GO:0032781) and response to heat (GO:0009408), with genes related to growth at low temperatures being *HSP30*, *AHA1* and *HSC82* on its *S. cerevisiae* allele.

Many GO terms that were overrepresented in H14A7 in comparison with H14A7-etoh at the 15 °C latency stage could be identified. The *S. uvarum* GO terms are secondary
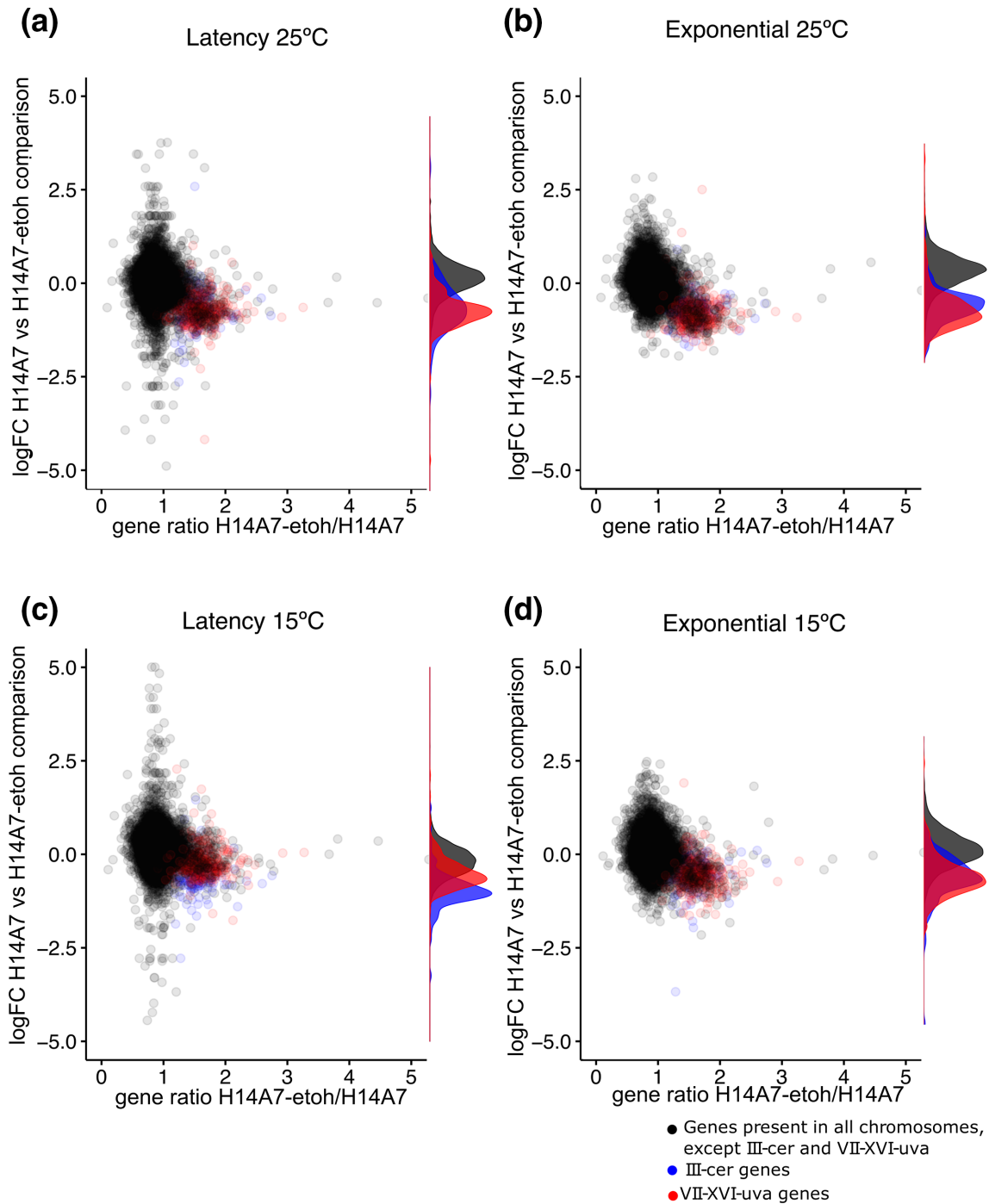
Fig. 3. Representation of the logFC ($\log_2$ FC) of the H14A7 vs H14A7-etoh transcriptomic comparison against the H14A7-etoh vs H14A7 gene coverage for every gene present in both strains. Negative values indicate that the genes are more expressed in H14A7-etoh and positive values that these genes are more expressed in H14A7. Genes belonging to *S. cerevisiae* chromosome III are coloured in blue, genes belonging to *S. uvarum* chromosome VII-XVI are coloured in red, and genes belonging to the rest of the chromosomes are coloured in black.

alcohol biosynthesis (GO:1902653), ergosterol metabolism (GO:0008204) and cellular alcohol metabolism (GO:0044107). Thus, the adaptation of strain H14A7 to low temperatures may be due to the expression of *S. uvarum* genes, which codify for

different secondary alcohols, and ergosterol, which may play a role in membrane composition. *S. cerevisiae* specific GO terms (BH correction with *P*<0.05) are related to energy reserve metabolic processes: glycogen biosynthesis (GO:0005978),

glycogen metabolism (GO:0005977) and oxidation–reduction (GO:0055114).

Since we determined that H14A7-etoh was more sulfite-resistant than H14A7, we examined the expression of genes YGL254W (transcription factor *FZF1*) and YPL092W (*SSU1* sulfite pump) in the four conditions and species alleles expression. The *SSU1 S. uvarum* allele is overexpressed in H14A7-etoh for three out of four conditions whilst *S. uvarum* transcription factor *FZF1* is overexpressed in two out of four conditions. None of the *S. cerevisiae FZF1* and *SSU1* alleles showed differential expression. Therefore, we identified a tendency for the overexpression of *S. uvarum SSU1* and *FZF1* alleles in H14A7-etoh when compared with strain H14A7 at 25 °C latency and exponential stages. We also explored the expression of genes involved in sulfite consumption by sulfite reductase [*MET1* (YKR069W), *MET5* (YJR137C), *MET8* (YBR213W), *MET10* (YFR030W)], and sulfitolysis of glutathione [*GLR1* (YPL091W)]. Of them, the *MET5 S. cerevisiae* allele was overexpressed in H14A7-etoh at the latency phase at 15 °C, the *MET10 S. cerevisiae* allele was overexpressed in H14A7-etoh at the latency phase at 15 °C and at the latency phase at 25 °C and the *GLR1 S. uvarum* allele was overexpressed in H14A7-etoh at the exponential phase at 25 °C. In the remaining conditions and genes not mentioned, no overexpression occurred.

As the ability of H14A7-etoh to uptake glucose and fructose from the medium was worse than in strain H14A7, we also examined the expression of hexose transporters in H14A7-etoh and H14A7 to search for the genetic basis for this different behaviour. The main hexose carriers of both glucose and fructose during wine fermentation are *HXT*1, *HXT2*, *HXT3*, *HXT4*, *HXT5*, *HXT6* and *HXT7* [49], and we found that in the different conditions most of them were up-regulated in H14A7 alleles in comparison with H14A7-etoh (Fig. S5).

Membrane lipid composition of the strains

Modulation of membrane lipid composition is a key mechanism by which yeast increase ethanol tolerance [50–52]. However, the homeoviscous response is complex [53] and the effect of altered gene expression on membrane composition may not be intuitive. Therefore, we compared the membrane properties of the adapted strain with the initial strains. We used MS and TLC to characterize the membrane composition of strains AJ4, BMV58, H14A7 and H14A7-etoh, and a Laurdan dye assay as an indication of the relative fluidity of the membranes. The lipid species identified using LipidBlast are shown in Table S3. As a surrogate for the general abundance of lipid classes, the number of species for each class of lipid between the strains is shown in Fig. 4a; there were significant differences for glycerophosphocholine (GPCho), with more species observed for both AJ4 and H14A7-etoh compared to BMV58 (*P*<0.05 and *P*<0.01 respectively, ANOVA and Tukey's multiple comparison test). For phosphatidylserine species (GPSer), there were significantly more species identified in H14A7-etoh than in BMV58 (*P*<0.01).

Membrane fluidity is affected by the presence of short chain alcohols, and two key lipid characteristics that influence membrane fluidity are acyl chain length and saturation. The average number of carbons in the acyl chains was not significantly different between the different strains (Figs S6 and S7). The two main genes related to sphingolipids synthesis are *LCB1* and *ELO2*. Since we have the list of DE genes among H14A7-etoh and H14A7 (Table S1), we inspected this table and found that *ELO2* (YCR034W) is more expressed in H14A7-etoh under two conditions: latency at 15 °C (the *S. cerevisiae* allele of the adapted hybrid) and exponential at 25 °C (the *S. uvarum* allele of the adapted hybrid). *ELO2* is involved in the biosynthesis of very long chain fatty acids but we saw no evidence for an increase in average chain length, suggesting a complex phenotype.

Fig. 4(b) illustrates the lipid species in which significant changes to saturation between the strains were observed. For phosphatidylethanolamine (GPEth), a lower percentage of saturated species was observed for BMV58 than for H14A7 and H14A7-etoh (*P*>0.01 and *P*<0.05). For phosphatidylglycerol (GPGro), higher percentages of monounsaturated lipids were seen in AJ4 and H14A7-etoh compared to BMV58 (*P*<0.05). Significantly greater percentages of monounsaturated species were observed for GPSer in BMV58 compared to AJ4, H14A7 and H14A7-etoh (*P*<0.5, *P*<0.5 and *P*<0.001) and fewer saturated species in BMV58 compared to H14A7 and H14A7-etoh (*P*<0.05 and *P*<0.01). For TG, the percentage of saturated species was greater for BMV58 compared to H14A7-etoh (*P* <0.01), while a higher percentage of poly-unsaturated species was observed for AJ4 compared to both H14A7 and H14A7-etoh (*P*<0.05).

A further important contributor to membrane characteristics is the nature of the phospholipid head group. Quantitative TLC analysis of the abundance of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) in the H14A7 and H14A7-etoh samples (Fig. 5a) showed that there was significantly less PE in the H14A7-etoh adapted strain, while the abundance of PC and PS was not significantly different.

Ethanol has been demonstrated to affect membrane fluidity, resulting in toxicity. Laurdan is sensitive to the polarity of the membrane environment and has been used to study membrane fluidity [54]. We utilized this to compare the state of the membrane for each of the strains (Fig. 5b). The data show that H14A7-etoh has a significantly lower GP compared to H14A7 (*P*<0.01) and AJ4 (*P*<0.05). This indicates that the membrane is less ordered and more fluid in H14A7-etoh, while H14A7 possessed the most ordered membrane.

DISCUSSION

In previous work, an *S. cerevisiae* × *S. uvarum* hybrid, H14A7, was obtained in our laboratory [25]. The objective of that initial work was to improve the ethanol tolerance of BMV58 (*S. uvarum* strain) by hybridization with a high ethanol-tolerant *S. cerevisiae* strain (AJ4). Indeed, we improved the
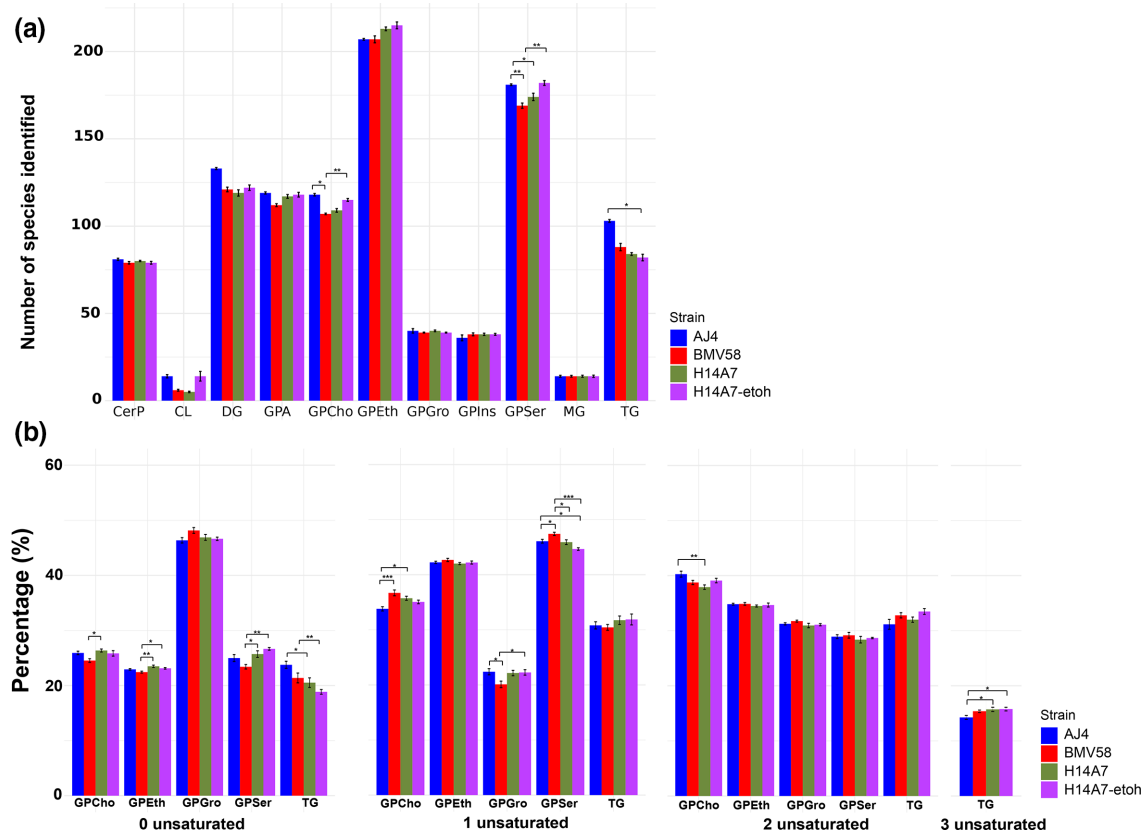
Fig. 4. Number of species identified by lipid class for strains AJ4, BMV58, H14A7 and H14A7-etoh. Lipids were extracted in quintuplicate and analysed by LC-MS in positive and negative ion mode (a). Percentage of saturated, monounsaturated and polyunsaturated chains by lipid class showing significant changes for AJ4, BMV58, H14A7, and H14A7-etoh (b). Significant differences among the strains are indicated by asterisks at $*P < 0.05$, $**P < 0.005$ and $***P < 0.001$, using ANOVA and Tukey's multiple comparison test.

ethanol tolerance of the *S. uvarum* parental, as well as other fermentative properties.

In this work, we wanted to study if this interspecific hybrid, H14A7, shows genomic instability after its growth in stressful wine media conditions and if the possible genomic changes affect its phenotype. We carried an adaptation strategy in a medium that mimics the ethanol and metabisulfite conditions during industrial wine fermentations at late stages. This medium contained a high sulfite concentration and increasing ethanol concentrations but decreasing sugar concentrations. The obtained adapted strain was named H14A7-etoh and both a physiological and a genomics characterization on this strain was performed. Industrial strains, especially hybrids, are highly prone to genome rearrangements, aneuploidies, even without stress. Different subclone lineages may be highly different. Recently, we proved that an *S. cerevisiae* × *S. kudriavzevii* hybrid showed genomic instability [45] and the aim of this work was to investigate what happened with an *S. cerevisiae* × *S. uvarum* hybrid of interest for the wine industry.

Using this adaptation strategy, ethanol tolerance is only slightly improved. However, we improved H14A7-etoh sulfite tolerance with respect to H14A7. The added compound, metabisulfite, is not stable in aqueous solutions and quickly converts to sulfite, so the adaptation of H14A7 was directed to sulfite resistance [55]. The adapted hybrid proved to be more sulfite-tolerant than both BMV58 and H14A7. This phenotype improvement can be correlated with the genomic composition of H14A7-etoh. H14A7-etoh has duplicated *S. uvarum* chromosome VII-XVI. *S. uvarum* chromosome VII-XVI carries the *FZF1-SSU1* recombination whose gene expression confers sulfite resistance (Macias *et al.,* unpublished data). Different chromosomal rearrangements affecting expression of the *SSU1* gene have been observed in different wine yeast strains. Some examples are the reciprocal translocation of chromosomes VIII and XVI that led to the regulation of the *SSU1* gene by the *ECM34* gene [56], the XV–XVI translocation that involved the promoter region of the *ADH1* and *SSU1* genes [57], and the inversion in chromosome XVI which involved *SSU1* and *GCR1* [58].

SSU1 is a gene involved in the sulfite efflux from the cell by the membrane pump, which is one of the strategies that yeasts use to cope with sulfite toxicity [59–61]. It has been reported that *SSU1* gene expression is generally constitutive and that its expression level is strain-dependent and is not regulated by the presence of sulfite [61–63]. In the fermentation media from which we retrieved the transcriptomic samples, no
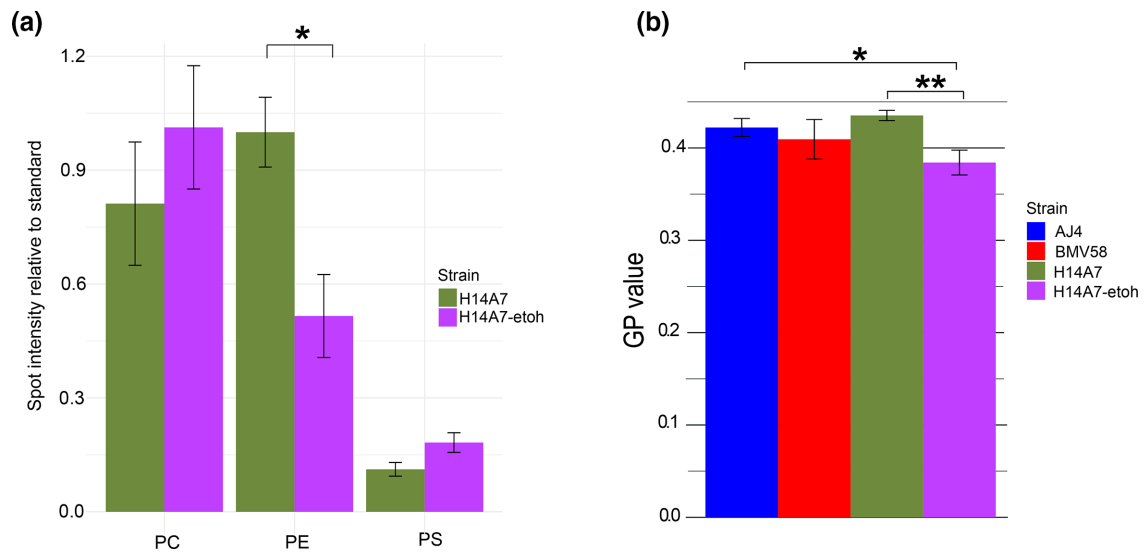
Fig. 5. TLC analysis of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) abundance for strains H14A7 and H14A7-etoh. Samples were loaded in triplicate and spot intensity was analysed using ImageJ. Spot intensity is plotted relative to phospholipid standards loaded onto each plate (a). Laurdan assay to compare the state of the membranes of strains AJ4, BMV58, H14A7 and H14A7-etoh. Relative GP was determined after 24 h of growth in GPY media (b). Significant differences among strains are indicated by asterisks at *$P<0.05$, **$P<0.005$ and ***$P<0.001$, using ANOVA and Tukey's multiple comparison test.

metabisulfite was added, and in three out of four conditions, H14A7-etoh expressed more of the *S. uvarum-SSU1* gene compared with H14A7, indicating the constitutive expression of this allele.

Wineries widely use sulfite (SO_2) as a preservative to avoid contamination by spoil microorganisms [64], but it also can result in toxicity to *Saccharomyces* yeasts [63, 65]. Thus, sulfite tolerance improvement of strain H14A7 is interesting for the wine industry.

However, the adapted hybrid showed a trade-off in its behaviour, as it left more fructose in Verdejo must fermentation than the original strain. The modified synthetic must has a lower sugar concentration than a natural must at the moment of inoculation in the winery because it was designed to simulate more advanced stages of fermentation. Thus, the hybrid could have lost fermenting capacity, as it was not obliged to ferment the regular amount of sugars during that adaptation process, but rather to cope with the ethanol and sulfites present in the media. Despite this, the obtained end-point sugar concentration was still within the limits that wineries consider acceptable in the final product. In other studies that used experimental evolution on *Saccharomyces* strains, fitness trade-offs were also found [66–68], demonstrating that when applying adaptive evolution strategies to generate new microbial strains with desirable traits, side effects may also appear.

Adaptation during evolution experiments generates structural variants, as deletions, amplifications and translocations in different yeast populations [69–71]. It is interesting to note that when we first obtained the hybrid [25], its genome seemed to be stable, and no significant deletions, duplications or rearrangements were reported, except for some SNPs in

S. cerevisiae chromosome III. Here, we have concluded that under adaptation to a stressful environment, as well as SNP fixation, deletions and duplications occurred in the H14A7-etoh genome due to the selective media employed during the experiment. Hybridization could generate genetic instability, especially under the selective pressures present in fermentation environments, that generate variability [45].

Subgenomes of the yeast interspecies hybrid H14A7 adapted differently during the process. A small region of the *S. cerevisiae* genome was deleted, which contains two genes coding for putative integral membrane proteins of the DUP240 family. This region could be eliminated from the genome as it is surrounded by Ty1-Ty1 retrotransposon sites, and it has been described that a recombination event under environmental stress can take place between these two elements [72]. Moreover, some fixation of SNPs and small duplications in concrete genes may have taken place in this part of the *S. cerevisiae* genome, as well as an LOH event in *S. cerevisiae* chromosome I. LOH events are usual during adaptive selection processes in *S. cerevisiae* yeasts [73] and these events also drive adaptation in hybrid yeasts [74]. Nevertheless, one large aneuploidy occurred in the *S. cerevisiae* subgenome: the duplication of chromosome III. As H14A7 was an aneuploid allotriploid with one *S. uvarum* genome copy, and two heterozygous copies of each *S. cerevisiae* chromosome except for a single copy of chromosome III, *S. cerevisiae* chromosome III duplication could be the result to a restoration of diploidy in all *S. cerevisiae* chromosomes, or because chromosome III affects ethanol tolerance. Previously, it was reported that yeast cells favour restoration of euploidy for chromosomes [75]. Moreover, it has been hypothesized that tolerance to aneuploidy occurs at the chromosome level,

perhaps through the action of DNA *cis*-acting elements, or selection for the restoration of euploidy of the previously aneuploid chromosome.

It has also been reported that chromosome III is one of the chromosomes which undergoes gains in strains under stress conditions, such as ethanol present in the media [76]. Morard *et al.* [46] also observed that *S. cerevisiae* chromosome III aneuploidy appears frequently in the most ethanol-tolerant strains. *S. cerevisiae* chromosome III duplication may be a result of an adaptation of strain H14A7 to the ethanol media present during adaptation, as this chromosome III duplication confers an advantage when growing at high ethanol concentrations.

The *S. uvarum* H14A7-etoh subgenome only seems to have one gene duplicated, YJL052W, but this subgenome was modified in the form of chromosomal losses (chromosome I) and gains (chromosome VII–XVI). *S. uvarum* chromosome I is the smallest chromosome, and it has been reported that chromosome losses often affect the smaller chromosomes [77].

The most interesting changes in H14A7-etoh compared with H14A7 are these whole chromosome duplications and losses. Yeasts have the ability to increase and maintain individual chromosomal copy number, as these aneuploidies are well tolerated and stable [75]. Previous studies growing *S. cerevisiae* yeasts under stress conditions have demonstrated that hyperploidy of concrete chromosomes can occur spontaneously. In Whittaker *et al.* [78], an *S. cerevisiae* culture was grown in a copper-rich environment. These yeasts increased the copy number of chromosome VIII, which carries *CUP1-1* and *CUP1-2* genes, related to resistance to high copper concentrations. The duplication of *S. uvarum* chromosome VII–XVI in H14A7-etoh may be related to strain adaptation to a medium with an elevated concentration of sulfites.

The change in the copy number of chromosomes is one accessible way to change the expression levels of specific key genes [79–81], although there are groups of genes subject to dosage compensation whose expression does not vary in response to their copy number [82, 83]. In the case of H14A7-etoh, the expression of most of the genes that changed their copy number appears to change, as transcriptomic analysis revealed that, in general terms, III-cer and VII-XVI-uva genes are up-regulated in H14A7-etoh in comparison with strain H14A7 under the same condition.

Transcriptomic analysis of both H14A7 and H14A7-etoh strains also revealed that H14A7 was more efficient in fermenting wine must at low temperatures than the H14A7-etoh adapted strain. Enrichment in GO terms related to secondary alcohol biosynthetic process (GO:1902653) and ergosterol metabolic process (GO:0008204) was found for H14A7 in the latency stage at 15 °C. An increase in ergosterol metabolism has been previously associated with low temperature tolerance in *Saccharomyces* [84, 85] and higher alcohol production is correlated with the use of *S. uvarum* strains and low temperatures at fermentations [86–88].

These same GO terms were obtained in our previous work [25] when comparing the H14A7 hybrid with its parental strains. Thus, the capability for growth and fermentation under low temperature conditions could be related to these processes, and the lack of expression of the genes related to them could be caused by the absence of that selective pressure during H14A7-etoh development. That would also explain why the adapted strain shows poorer complete sugar consumption.

Improvements to ethanol tolerance were observed for H14A7-etoh, and we investigated changes to the membrane which may have occurred as a mechanism of ethanol tolerance. The differences in the lipidome of the yeast strains, such as a number of species identified for each class and the unsaturation status of the acyl chains, appear to be complex, and the overall effect upon the membrane is difficult to predict. Several studies have found a correlation between chain length, membrane fluidity and ethanol tolerance, with the incorporation of longer chains at the expense of short chains to counteract the fluidizing effect of ethanol upon the membrane [89, 90]. The ability of cells to change the unsaturation index has been suggested as an ethanol adaptation response. Furthermore, cholesterol acts to modulate membrane fluidity and it is possible that the transcriptomic changes seen within the ergosterol metabolic process genes are responsible for the increased fluidity of H14A7-etoh membranes.

S. cerevisiae has been demonstrated to increase unsaturated lipids in response to ethanol [50, 51, 89], and this has been associated with more tolerant strains [51]. However, another study found that unsaturation did not correlate with membrane fluidity and ethanol tolerance [91].

It has been suggested that membrane fluidity alone cannot fully account for ethanol tolerance in some microorganisms, and that mechanisms of adaptation vary between strains and between organisms [51, 91]. Our analyses suggest that, whilst changes in saturation may occur within the hybrid strains, this alone is unlikely to fully account for the observed increase in ethanol tolerance.

In our study, we observed a significantly lower abundance of PE in the H14A7-etoh adapted strain compared to H14A7; this may be an adaptive response to ethanol stress. PE is known to play a role in the regulation of membrane fluidity [92], and membranes containing PE have been demonstrated to be less fluid than those containing PC alone, possibly because PE increases lipid packing [93].

The Laurdan experiments suggested that the membranes of H14A7-etoh were more fluid compared to those of H14A7; this is consistent with the TLC data and a decrease of PE, which was expected to result in an increase in membrane fluidity. A study by Chi and Arneborg [89] compared two yeast strains with different abilities to tolerate ethanol, and found that the more tolerant strain contained a greater proportion of PC and a lower proportion of PE. Another study demonstrated increased mass fractions of PC and less PE in recycled yeast exposed to fermentation stress compared to non-stressed starter yeast cultures [94].

These results are consistent with our findings, suggesting one possible conserved mechanism of increasing membrane tolerance to ethanol. Reported membrane changes upon ethanol production/exposure remain conflicting [52]. This is probably due to differences in the experimental conditions. Yeasts are known to incorporate exogenous polyunsaturated fatty acids [95, 96], and this can be influenced by the composition of the growth media. In addition, there may be multiple alternative cellular strategies for mitigating ethanol tolerance. Due to the sampling in our experiments, we are likely looking at the 'basal' membrane condition before significant ethanol challenge and further remodelling may occur with increased ethanol concentrations.

Overall, our results show that when an *S. uvarum* × *S. cerevisiae* strain is adapted under a medium which mimics wine pressures during fermentation – ethanol and sulfites – its genome is unstable and shows different genomic changes that have an effect on its phenotype. Both subgenomes adapt differently to this medium, and the characteristic that was improved was the sulfite tolerance. The way to improve sulfite tolerance was based on the duplication of *S. uvarum* chromosome VII–XVI, which has an impact on gene expression of this entire chromosome. Ethanol tolerance seems to be improved too, and we hypothesize that *S. cerevisiae* chromosome III duplication is the cause of this improvement. Membrane fluidity of the adapted hybrid is increased and this seems a potential mechanism by which the ethanol tolerance is higher for H14A7-etoh. A trade-off is present in this adapted hybrid, as its speed to ferment sugars is reduced.

Author contributions
E. B. and A. Q., conceived and designed the study. G. C., obtained the adapted hybrid. M. L., performed the phenotypic assays, the genome sequence, and the transcriptome analyses under J. A., A. Q. and E. B., supervision. S. R. and J. L., performed and analysed the lipidomic assays under A. P. and A. G. supervision. M. L., wrote the first versions of the article and J. A. and A. Q., the final version. The other co-authors revised the manuscript and added comments.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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