Sulfometuron Resistance as a Genetic Marker for Yeast Populations in Wine Fermentations

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Winemaking with selected yeasts requires simple and cheap techniques to monitor the yeast population dynamics. We obtained new sulfometuron (smr) resistant mutants, easy to detect by replica-plate assay, from selected wine yeasts. The mutations were dominant and were located at the *ilv2* locus that encodes for acetolactate synthase enzyme. The mutants were genetically stable and maintained the killer phenotype of the parent yeast strain. They were genetically improved by elimination of recessive growth-retarding alleles followed by spore clone selection according to the must fermentation kinetics and the organoleptic quality of the wine. Some mutants were tested in industrial winemaking and were easily monitored during must fermentation using a simple plate assay. They accounted for more than 95% of the total yeasts in the must, and the resulting wine had as good a quality as those made with standard commercial wine yeasts.

KEYWORDS: Sulfometuron; resistance; yeast; fermentation; winemaking

INTRODUCTION

The use of selected yeast strains in winemaking requires simple and cheap techniques to differentiate the inoculated strain from the wild yeasts in the must. Several techniques based on molecular polymorphisms have been used to solve this problem (*1*−*11*). Unfortunately, the expense, complexity, and time required by these techniques make it difficult to apply them in most commercial wineries.

The use of yeast strains with specific genetic markers provides an alternative monitoring possibility during must fermentation. This option is particularly suitable for industrial application because of its simplicity. Approaches using natural or induced mutants resistant to certain drugs (e.g., chloramphenicol, oligomycin, diuron, erythromycin) have already been proposed. Generally, these markers confer resistance to antibiotics that inhibit yeast growth by preventing mitochondria function (*12*, *13*). Many industrial and wild yeasts are resistant to these drugs, so multiply marked strains have to be used to avoid the high background. Studies involving a doubly marked strain (diuron and erythromycin resistance) have provided an insight into the kinetics of yeast populations during fermentation (*14*, *15*), but a limitation still exists in that wine yeast strains of choice cannot be easily marked. Moreover, the petite mutants that arise in industrial vinifications would not be detectable by using mitochondrial markers. The *Escherichia coli* β-glucuronidase gene can be introduced as a nuclear marker by transformation

into *S. cerevisiae*. It provides easy assay by fluorimetry and agar plate tests (*16*, *17*). However, the procedure involves transgenic yeast and *E. coli* sequences, which mean additional difficulties in gaining consumer confidence and approval for industrial use according to the legislation of many countries (*18*).

A fast, reliable, and economic method to monitor inoculated selected yeast through must fermentation by using *Saccharomyces cerevisiae* cycloheximide-resistant mutants (*cyh2*R) has been developed (*19*). The method can be recommended to the food industry because it is simple and does not require sophisticated equipment or special personnel skills. The spontaneous *cyh2*R mutants were isolated from industrial wine yeast (*19*). The mutations were recessive, and they did not affect the fermentation kinetics, the quality of the wines, or the viability of active dry yeast made with the mutants. Some *cyh2*R selected mutants have been marketed and used to confirm their dominance during industrial grape juice fermentation for five years. While the procedure has been working excellently, we recently detected up to 10% of *cyh2*R yeasts in the spontaneous fermentations of some wineries. The mitDNA restriction pattern of these *cyh2*R yeasts matches that of the marketed strain. Therefore, we are beginning to be concerned about *cyh2*R yeasts becoming resident in the wineries that use *cyh2*R commercial yeasts.

To solve this problem, we obtained new wine autochthonous yeasts resistant to sulfometuron (smr), easy to detect by replica-plate assay, from selected diploid wine yeasts. They can be used in alternate years with other genetically marked yeasts to avoid genetic markers spreading. The mutants were tested for industrial

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winemaking and were compared to their parent strains and standard commercial wine yeasts.

MATERIALS AND METHODS

Yeast Strains, Culture Media, Sporulation, Genetic Mapping, and Phenotype Tests. JP73, JP85, JP88, and JP33 are prototrophic and homothallic *S. cerevisiae* wine yeasts (*20*). JP73, JP85, and JP88 are K2-killer, and JP33 is virus-free killer-sensitive. E7AR1 is a K2-killer cycloheximide-resistant wine yeast from the hybrid 7AR (*21*) sold by BIOTEX (Talavera la Real, Spain). The haploid laboratory yeasts YMR107w (*mat a, ho, his3, leu2, met15, ura3, ymr107::G418*[R]), YGL013C (*mat a, ho, his3Δ1; leu2Δ0;* met15Δ0; ura3Δ0, pdr1:: *G418*[R]), and YJR094C (*mat a, ho, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0,* ime1::G418[R]) used for genetic mapping were obtained from EURO-SCARF (European Saccharomyces Cerevisiae Archive for Functional Analysis). The sulfometuron resistance (*SMR*[R]) mutations were mapped by analyzing crosses of single spore clones from spontaneous SMR[R] mutants with the above genetically marked yeasts. Standard culture media were used for yeast growth and phenotype tests in the genetic mapping (*22*). Minimal medium for auxotroph analysis was Difco (Detroit, MI) Yeast Nitrogen Base (without amino acids, with ammonium sulfate). YEPD+cyh is YEPD-agar supplemented with cycloheximide (cyh, prepared in a concentrated ethanol solution) to a final concentration of 2 μg/mL (*19*). SD+smr is standard SD-agar supplemented with smr to the desired final concentration (100 μg/mL unless a different concentration is given). Sulfometurom was prepared in a concentrated dimethyl sulfoxide solution (1%) and was added to the media just before pouring into Petri dishes. YEPD+G418 is YEPD-agar supplemented with G418 (Sigma G7034, a concentrated water solution) to a final concentration of 200 μg/mL.

Standard yeast genetics procedures were used for sporulation of cultures and dissection of asci (*23*). Cells were grown on YEPD plates for 2 days at 30 °C, transferred to sporulation plates (1% potassium acetate, 0.1% Bacto-yeast extract, 0.05% glucose, 2% Bacto-agar), and incubated for 7−20 days at 25 °C until more than 50% of the cells had sporulated. Twenty-four asci from each yeast were dissected on YEPD plates and were incubated for 5 days at 30 °C at which time the percentage of viable spores was determined. The spore clones were tested for phenotypic segregation by replica-plating on the appropriate media. The phenotype test was performed only if spore viability was >95%.

Assay for killer activity was performed in low-pH (pH 4) blue plates (4MB) (*23*) seeded with 100 μL of a 48-h culture of the sensitive strain JP33. Strains being tested for killer activity were loaded (4 μL of a 48-h culture to produce a patch approximately 5 mm in diameter) or replica-plated onto the seeded 4MB plates and were incubated for 4 days at 20 °C. Killer strains produce a clear halo as a result of killing the seeded sensitive yeasts.

Virus (ScV-LA and ScV-M2) dsRNA Extraction, Purification, and Agarose Gel Electrophoresis. Rapid yeast dsRNA extraction and purification was performed as previously described (*24*). dsRNA molecules were separated in 1 × TAE-1% agarose gel for 60−75 min.

Detection of Wild Yeasts Resistant to smr in Spontaneous Must Fermentations. Samples were collected from fermenting musts supplied by several wineries located throughout southwestern Spain. All the musts were in the active fermentation stage. Each sample was diluted with sterile distilled water, plated on YEPD-agar and SD+smr (5 μg/mL of smr), and incubated at 28 °C. Most of the colonies had the typical aspect of *S. cerevisiae* colonies (white or cream color, buttery, smooth, circular, and prominent).

Isolation and Characterization of Spontaneous SMR[R] Mutants. The yeasts JP73, JP85, and JP88 were grown in YEPD broth for 48 h at 30 °C with orbital shaking. A sample of 1 mL was taken from each culture. Cells were collected by centrifugation, suspended in 0.1 mL of sterile distilled water, and plated onto SD+smr plates supplemented with 20 μg/mL of smr. In addition, a diluted sample of each culture was plated onto YEPD-agar to calculate the frequency of spontaneous SMR[R] mutants. All plates were incubated at 30 °C until the colonies became clearly visible (2−8 days). To determine the minimal inhibitory concentration (MIC), the parent yeasts and the spontaneous mutants

were plated on YEPD-agar and were incubated for 24 h at 30 °C. These plates were then replica-plated to SD+smr containing different concentrations of smr (20, 50, and 100 μg/mL) and were incubated for 2 days at 30 °C. The MIC for each mutant is the lowest concentration of smr in which it does not grow.

To measure the stability of the *smr*[R] mutations, the mutants were cultured by serial transfers onto YEPD plates at 30 °C (nonselective conditions) every 24 h until the population had undergone 100 doublings (20 transfers). Also, they were grown in sterile must at 18 °C until the end of fermentation. Thereafter, a sample of each mutant was plated on YEPD-agar to obtain from 200 to 300 single colonies. The presence of sensitive revertant yeasts was analyzed by the replica-plating method on SD+smr.

Must Fermentation Kinetics. This was carried out in 50-mL Erlenmeyer flasks with 50 mL of Pardina juice (22.4 °Brix, pH 3.5) sterilized by membrane filtration through a Millipore system (0.45-μm membrane). Yeast cells of mutants and parental strains were cultured in YEPD broth for 2 days at 30 °C, washed twice (by centrifugation) with sterile water, and suspended in the must at a concentration of 5×10^6 cells/mL. Fermentations were conducted at 18 °C for 20 days. Yeast growth (absorbance at 590 nm) and the °Brix were monitored each day.

Vinification Trials. For laboratory microvinifications, yeast cells of mutants and parental strains were obtained as above and were inoculated in Erlenmeyer flasks with 5 L of must at a concentration of 5×10^6 cells/mL. Cayetana white grape juice (22.4 °Brix, pH 3.5) and Merlot destemmed crushed grapes (25 °Brix, pH 3.5) were used. For winery vinification trials, the yeast inocula were produced in a pilot plant of the company BIOTEX by using their industrial procedure. Cells were cultured in beet molasses broth for 18 h at 30 °C with strong aeration, washed twice (by centrifugation) with sterile distilled water, and inoculated in 1000-L stainless tanks containing fresh white must (Cigüentes, 22.4 °Brix, pH 3.42 in 2002 and Macabeo, 21.2 °Brix, pH 3.29 in 2003) or destemmed crushed grapes (Cabernet-Sauvignon, 25 °Brix, pH 3.3 in 2002; Cabernet-Sauvignon, 24.4 °Brix, pH 3.25 in 2003; Tempranillo, 22 °Brix, pH 3.5 in 2002; and Tempranillo, 23 °Brix, pH 3.6 in 2003) to a final concentration of $1−2 \times 10^7$ cells/mL. The vinification process was conducted at 18 °C for white wine and at 22 °C for red wine. The density and the °Brix were monitored every day. Flasks and tanks were capped hermetically when reducing sugars reached around 1% to avoid oxidation problems. At the end of fermentation, the settled solids were discarded. An 800-mL centrifuged sample of each wine was taken for the analytical assays. The uncentrifuged wines were stored at 4 °C. After 50 days following the end of fermentation, settled solids were again discarded and wines were returned to store at 4 °C. At 85 days, settled solids were discarded once more and the wines were bottled. After 105 days following the end of fermentation, the organoleptic characteristics (flavor, color, and odor) of the wines produced were tested by a panel of 12 experts. Wines were presented in clear tulip-shaped wine glasses covered with glass Petri dishes. A sample of 50−70 mL of wine was poured into each glass immediately before being analyzed by each judge. Temperature of samples was from 10 to 13 °C for white wines and from 16 to 18 °C for red wines. The judges scored the quality of the wines on a six-point scale (0 = very poor, 1 = deficient, 2 = acceptable, 3 = good, 4 = very good, and 5 = excellent). Maximum score possible (60 points) was considered 100% of preference. Hydrogen sulfide odor was determined by the judges on a 30-unit (U) scale (0 = no odor, 10 = low odor, 20 = high odor, 30 = very high odor).

Determination of the Number of Inoculated Yeasts in the Vinification Trials. Determination of the percentage of genetically marked yeasts was done by the replica-plating method (*19*). Samples from fermenting musts were diluted and plated onto YEPD-agar to obtain 200−300 colonies per plate. The detection of the cyh[R] and SMR[R] mutants was accomplished by replica-plating these plates to either YEPD+cyh (2 μg/mL) or SD+smr (100 μg/mL) plates using sterile velvets and thereafter to other plates of YEPD-agar to detect wild yeasts sensitive to cyh or smr. The time needed to easily observe growth of resistant yeasts on YEPD+cyh or SD+smr at 30 °C varied between 1 and 3 days depending on the mutant. The numbers of SMR[R] mutants were also determined by the direct method (*19*), that is, samples from

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Table 1. Isolation and Characterization of SMR^R Spontaneous Mutants from Wine Yeasts

parent strain (MIC, μg/mL)	frequency of SMR^R spontaneous mutants isolated in SD+smr 20 μg/mL^a	number of mutants that grow at increasing smr concentration in μg/mL		
		20	50	100
JP73 (5)	$1.15 \times 10^{-7} \pm 7.10 \times 10^{-8}$	15	9	8
JP85 (5)	$2.22 \times 10^{-8} \pm 1.77 \times 10^{-8}$	15	10	10
JP88 (5)	$1.26 \times 10^{-7} \pm 9.21 \times 10^{-8}$	15	7	6

^{a} The data are the means of three independent experiments and standard errors.

fermenting musts were diluted and plated directly onto YEPD-agar and SD+smr (100 μg/mL) to obtain the same number of colonies per plate (200–300). The time needed to easily see colonies of resistant yeasts growing on SD+smr at 30 °C varied between 3 and 4 days depending on the mutant. The percentage of wild parent yeasts was determined by analyzing the mtDNA restriction pattern (*25*). Purified mtDNA was digested with *RsaI*. The fragments were separated in 0.5 × TBE–0.8% agarose gel for 75–90 min and were visualized on a UV transilluminator after ethidium bromide staining.

Analytical Methods. Density, °Brix, pH, total acidity, volatile acid, reducing sugars, alcohol, and malic acid were determined according to the EC recommended methods (*26*). Lactic acid was determined using the EEC recommended method (*27*). Major volatile compounds and polyols were quantified by gas chromatography (*28*). T15 is the time needed to ferment 15% of the total sugars present in the must, and T100 is the time needed to ferment 100% of the total sugars (*29*).

Statistical Analysis. Data were analyzed for statistical significance by a one-way analysis of variance (ANOVA) with the software package SPSS version 11.5 for Windows (Chicago, IL).

RESULTS AND DISCUSSION

Presence of Wild Yeasts Resistant to smr in Spontaneous Must Fermentations. For smr resistance to be easily used as a marker for the detection of inoculated yeasts in industrial vinifications, the colonies of *S. cerevisiae* must be distinguishable from the rest of the wild yeast species by simple observation. *S. cerevisiae* colonies are easy to identify by their aspect in YEPD-agar (white or cream color, buttery, smooth, circular, and prominent). In a previous study performed for all the wine-producing zones of southwestern Spain, we found that all the yeast colonies isolated from fermenting musts having the typical aspect of *S. cerevisiae* sensu stricto strains belonged to this species group. Similarly, all the colonies with aspects different from *S. cerevisiae* belonged to other species (unpublished data: Dr. Manuel Ramírez, Universidad de Extremadura, Spain, 1994–2003). Moreover, the frequency of wild yeasts resistant to smr in musts and wines should be low enough to avoid any major background error. As previously reported (*30*), the growth of wild *S. cerevisiae* strains was inhibited by low concentrations (3–5 μg/mL) of smr on SD medium. The frequencies of wild yeasts resistant to 20 μg/mL of smr in fermenting musts collected from different wineries during five consecutive vintages (1999–2004) were always less than 1 × 10^{-5} of total yeast cells. As expected, in no case did *S. cerevisiae* colonies resistant to 100 μg/mL of smr appear.

Isolation and Characterization of Spontaneous smr Resistant (SMR^R) Mutants. We easily isolated spontaneous SMR^R mutants from three wine yeasts (JP73, JP85, and JP88) by plating them onto SD+smr (20 μg/mL). The minimal inhibitory concentration (MIC) for all the parent strains was 5 μg/mL of smr. The frequency of spontaneous mutants depended on each yeast strain, but it was always less than 1.3×10^{-7} (**Table 1**). By using this antibiotic concentration or higher, there is therefore no risk of interference from spontaneous resistant mutants

originating from wild strains of *S. cerevisiae* in the detection of SMR^R strains inoculated in industrial fermentation.

Fifteen SMR^R mutants isolated from each parental strain were characterized by analyzing their resistance to smr concentrations higher than 20 μg/mL. As expected, all the mutants were capable of growing in the smr concentration in which they were isolated. In higher concentrations, the behavior of the mutants was variable. Around half of them resisted antibiotic concentrations higher than 100 μg/mL (**Table 1**). Twenty mutants with MIC higher than 100 μg/mL were chosen for further analyses (SMR1, SMR2, SMR3, SMR4, SMR5, SMR15, SMR16, and SMR17 from JP73; SMR6, SMR7, SMR8, SMR10, SMR18, and SMR19 from JP85; SMR11, SMR12, SMR13, SMR14, SMR20, and SMR21 from JP88).

Continued propagation of these mutants under laboratory conditions (20 transfers on YEPD plates at 30 °C) yielded no sensitive colony among 200 colonies analyzed of each mutant after replica-plating in SD+smr 100 μg/mL. That is, the frequency of revertants after approximately 100 doublings (*31*) was less than 5×10^{-3}. Therefore, the mutations were genetically stable enough to discard any possibility of interference because of the appearance of any sensitive revertants from the inoculated SMR^R mutants in the detection of wild sensitive yeasts during the must fermentation.

All the mutants maintained the same K2 killer phenotype from their respective parent strains and the ScV-M2 virus responsible for this phenotype (*20*). Therefore, neither the mutation responsible for the SMR^R phenotype nor the presence of smr in culture medium affected the replication of the viruses (ScV-LA and ScV-M2) responsible for K2 killer phenotype, contrary to previous findings for the isolation of cyh^R wine yeasts (*19, 21*).

Genetic Improvement of SMR^R Mutants. Generally, these mutants were able to ferment grape must but not as fast as the parent wine yeasts. Most of them had a little longer T15 (time needed to ferment 15% of the total sugars), T100 (time needed to ferment 100% of the total sugars), or both. As all the mutants came from diploid yeasts bearing recessive growth-retarding alleles, it is possible to eliminate these alleles by obtaining fully homozygous yeasts and simultaneously to increase fermentation vigor and even to improve wine quality (*29*). Thereby, the 20 chosen SMR^R mutants were sporulated, the spore colonies from the tetrads were analyzed, and new homozygous single-spore descendants were selected as previously reported (*29*). As expected, the segregation ratio for spore colony size was 2 large:2 small for all the mutants obtained from JP85 and JP88 and irregular segregation for those from JP73 (*29*). The segregation ratio for spore colony SMR^R phenotype was 2 resistant:2 sensitive, which indicates that the resistance phenotype is due to a dominant mutant allele. No relationship between spore colony size and SMR^R phenotype was observed, so they must be independent traits. Again, all the spore colonies maintained the K2 killer phenotype of the parent yeasts. We chose 14 spore clones among those with large colony size and strong SMR^R phenotype (SMR3-1D and SMR3-2A from SMR3, SMR6-1D and SMR6-2B from SMR6, SMR8-9D and SMR8-10C from SMR8, SMR10-1C and SMR10-11D from SMR10, SMR12-1A and SMR12-2A from SMR12, SMR16-5A and SMR16-6A from SMR16, and SMR20-1A and SMR20-2C from SMR20).

The 14 spore clone mutants and the parent strains were inoculated in sterile Cayetana grape must to analyze the fermentation performance. Generally, there were no very important differences in the evolution of the sugar consumption, all yeasts finished must fermentation properly. However, 7 out

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Table 2. Must Fermentation Parameters and Wine Analysis Results of Six Independent Winery Vinifications Made with Cigüentes, Macabeo, Cabernet-Sauvignon, and Tempranillo Grapes. ANOVA to Study the Effect of Inoculation with SMR16-5A and SMR10-11D Selected SMR^R Mutants[c]

	yeast					
parameter	control	E7AR1	JP88	SMR16-5A	SMR10-11D	p[a]
T15 (days)	3.8 ± 0.4b[d]	1.36 ± 0.2a	1.6 ± 0.2a	1.95 ± 0.3a	1.5 ± 0.2a	0.000
T100 (days)	10.1 ± 2.2a	5.9 ± 1.4a	5.7 ± 0.8a	6.8 ± 0.8a	6.7 ± 1.5a	0.258
preference (%)	66 ± 5.1a	67 ± 4.8a	67 ± 2.5a	66 ± 4.2a	72 ± 2.6a	0.877
degree of dominance (%)	8.3 ± 6a	100 ± 0.0b	100 ± 0.0b	97 ± 1.4b	98 ± 1.3b	0.000
alcohol (% v/v)	13 ± 0.4a	12.7 ± 0.5a	12.8 ± 0.4a	12.9 ± 0.4a	12.8 ± 0.4a	0.935
pH	3.3 ± 0.07a	3.3 ± 0.09a	3.4 ± 0.07a	3.4 ± 0.09a	3.3 ± 0.07a	0.993
total acidity (g/ L)	8.1 ± 0.6a	8.3 ± 0.5a	8.1 ± 0.7a	7.5 ± 0.5a	8.1 ± 0.4a	0.870
volatile acidity (g/L)	0.26 ± 0.03b	0.2 ± 0.01ab	0.18 ± 0.01a	0.20 ± 0.02ab	0.18 ± 0.02a	0.111
reducing sugars (g/L)	0.67 ± 0.42b	0.05 ± 0.03a	0.01 ± 0.00a	0.01 ± 0.00a	0.00 ± 0.00a	0.084
malic acid (g/L)	1.3 ± 0.13a	1.69 ± 0.12a	1.56 ± 0.09a	1.43 ± 0.12a	1.52 ± 0.16a	0.335
lactic acid, (g/L)	0.16 ± 0.1a	0.05 ± 0.02a	0.08 ± 0.04a	0.08 ± 0.04a	0.05 ± 0.02a	0.706
acetaldehyde (mg/L)	18.5 ± 4.0a	16.2 ± 4.1a	16.8 ± 3.9a	18.2 ± 4.4a	18.5 ± 3.8a	0.991
ethyl acetate (mg/L)	17.3 ± 6.2a	13.2 ± 4.8a	15.8 ± 5.9a	15.3 ± 6.1a	11.8 ± 4.5a	0.960
methanol (mg/L)	132 ± 32a	111 ± 29a	136 ± 41a	130 ± 21a	132 ± 28a	0.981
fusel alcohols (mg/L)	342 ± 65a	335 ± 55a	348 ± 47a	368 ± 30a	309 ± 47a	0.947
butanol-1 (mg/L)	0.83 ± 0.54a	0.56 ± 0.36a	1 ± 0.63a	2.83 ± 1.79a	1.66 ± 1.66a	0.664
butanol-2 (mg/L)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
1-propanol (mg/ L)	27 ± 4.2a	28.8 ± 3.9a	34.3 ± 8.0a	45.3 ± 7.1a	38 ± 6.3a	0.251
isobutanol (mg/ L)	55.7 ± 10.7a	56.3 ± 9.3a	53.2 ± 14.1a	39.5 ± 2.9a	32 ± 3.8a	0.256
amyl alcohols (mg/L)	259 ± 54.3a	250 ± 47.3a	260 ± 34a	280 ± 26.2a	237 ± 38.7a	0.965
H$_2$S (U[b])	3.3 ± 3.3a	3.3 ± 2.1a	5 ± 3.4a	3.3 ± 3.3a	3.3 ± 3.3a	0.994

[a] p values obtained by ANOVA for the wines made with each yeast. [b] U = arbitrary units. [c] The data are the mean values of six independent experiments and standard errors. [d] Different letters (a and b) mean significantly different groups found with the Duncan test at $p < 0.05$.
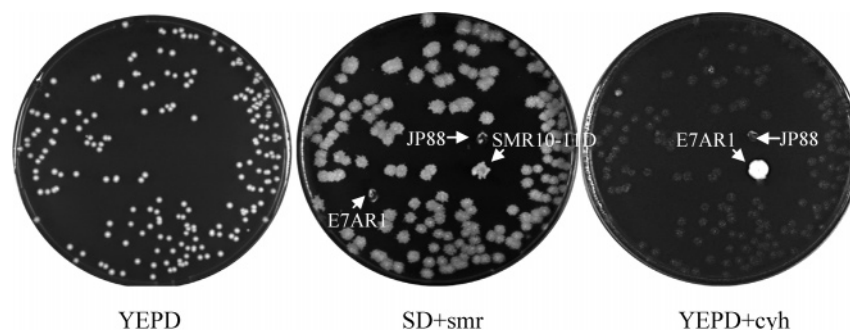
Figure 1. Replica-plating on SD+smr and YEPD+cyh of colonies isolated in YEPD from a sample (day four) of the winery vinification made with Tempranillo grapes in 2002. The arrows indicate the patches of control yeasts added with sterile toothpicks onto a colony-free part of the plate.

of the 14 spore clone mutants had a T15 even shorter than the parent and a standard commercial yeast, and four among them (SMR12-1A, SMR16-5A, SMR10-11D, and SMR3-2A) had roughly equal or lower T100 than parent and standard yeasts. Therefore, in some spore clones, the fermentation kinetics was improved by elimination of recessive growth-retarding alleles, and the SMR^R mutation did not reduce the must fermentation vigor relative to the parent and standard commercial yeasts (data not shown). No sensitive smr colony was detected among the 200 colonies analyzed from each mutant fermentation (replica-plating on SD+smr 100 μg/mL), so the frequency of spontaneous revertants was less than 5×10^{-3}. That is, the mutations are genetically stable enough to discard the possibility of interference of sensitive revertants raised from these four SMR^R spore clones in the detection of wild sensitive yeasts during the must fermentation. These four SMR^R mutants were preselected to further evaluate their usefulness for winemaking.

Genetic mapping of the SMR^R mutations of these four mutants indicated that they are ligated to *ymr107::G418*^{R}, at only around 700 base pairs from the *ilv2* gene on the right arm of chromosome XIII that encodes for acetolactate synthase enzyme (ALS, or acetohydroxyacid synthase) involved in the isoleucine-valine biosynthetic pathway. An SMR^R mutation in the *ilv2* gene (with an MIC > 30 μg/mL) had been reported in

a heterothallic haploid yeast strain (*30*). However, this is the first time that SMR^R spontaneous mutants with an MIC > 100 μg/mL have been isolated from diploid homozygous wine yeasts.

Laboratory Microvinification Trials with the SMR^R Mutants. All the SMR^R preselected mutants (SMR10-11D, SMR3-2A, SMR12-1A, and SMR16-5A) again showed similar fermentation kinetics to the parent (JP73, JP85, and JP88) and standard (E7AR1) strains, and the uninoculated control conducted in parallel was much slower than the rest: the uninoculated control fermentations had T15 and T100 values of 4.75 and 13 for the white and 6.5 and 15 for the red, compared with the respective values for the various mutants of 2.1−2.5 and 9−12 for the white and 2.75−3.7 and 11−14 for the red. They dominated the must fermentations (100%) as analyzed by the replica-plating method. The same results were obtained by directly plating the diluted samples onto SD+smr agar, because all the seeded single yeast cells grew in the presence of smr. The contrary has been reported for cycloheximide resistant wine yeast single cells, part of which did not grow in the presence of the drug (*19*). All the analyzed colonies (20 from each sample, 3 samples from each vinification) had the same mitDNA restriction pattern as the original parent strain, and this pattern was not detected in samples from uninoculated controls. In no case did we detect sensitive spontaneous revertants. No SMR^R

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yeast colonies were detected in the uninoculated controls. The must fermentation parameters (T15 and T100), the wine parameters (organoleptical quality, degree of domination, alcohol, pH, total acidity, volatile acidity, reducing sugars, malic acid, lactic acid, acetaldehyde, ethyl acetate, methanol, total fusel alcohols, butanol-1, butanol-2, 1-propanol, isobutanol, amyl alcohols, and H_2S), and the organoleptic tests revealed no significant differences (in the ANOVA) between the SMR^R mutants and the corresponding parent strains (data not shown). Therefore, the SMR^R mutations of the analyzed mutants did not significantly affect the yeast metabolism related to the production or elimination of compounds responsible for the aroma and flavor of the wines. Generally, the resulting wines had as good a quality as those made with standard commercial wine yeasts. However, in the organoleptic test, the JP88 and SMR16-5A white wines and E7AR1, SMR16-5A, and SMR10-11D red wines were the best evaluated. Therefore, we selected SMR10-11D and SMR16-5A mutants for further winery trials.

Winery Vinification Trials with the Selected SMR^R Mutants. Winery vinifications were carried out with fresh white must (Cigüentes in 2002 and Macabeo in 2003) and red grapes (Cabernet-Sauvignon and Tempranillo in 2002 and 2003) using the two selected mutants SMR10-11D and SMR16-5A, the parent strain JP88, and a standard commercial wine yeast E7AR1. A uninoculated control (spontaneous fermentation) was done in parallel for each vinification series, that is, a total of six different vinifications series were done, resulting in 30 wines. All the SMR^R mutants again showed similar fermentation parameters (T15 and T100) to the parental and standard strains, and the uninoculated control was much slower than the rest (**Table 2**). The degree of dominance of all the inoculated yeasts ranged from 97 to 100% (**Table 2** and **Figure 1**). All the SMR^R analyzed colonies (20 from each sample, 3 samples from each vinification) had the same mitDNA restriction pattern as the original parent strain (SMR10-11D), and once again, this pattern was not detected in samples from uninoculated controls. In no case did we detect sensitive spontaneous revertants. No SMR^R yeast colonies were detected in the uninoculated controls. Among all the analyzed fermentation or wine parameters, there were only significant differences between inoculated and noninoculated vinifications for the means of T15 and the degree of dominance (**Table 2**). This is because the onset of fermentation is always slow in noninoculated vinification, and obviously there is no dominance of noninoculated strains. Apart from this, there were no significant differences in the means of any of the parameters (T15, T100, organoleptical quality, degree of domination, alcohol, pH, total acidity, volatile acidity, reducing sugars, malic acid, lactic acid, acetaldehyde, ethyl acetate, methanol, total fusel alcohols, butanol-1, butanol-2, 1-propanol, isobutanol, amyl alcohols, and H_2S) between inoculated and noninoculated vinifications (**Table 2**). Although the SMR10-11D wines were the best evaluated (71.7%), the rest of the wines also had very good quality because the grapes had good sanitary quality and no incidences (such as sluggish fermentation or undesirable bacterial growth) occurred during the wine making.

In conclusion, the SMR^R mutations were dominant and genetically stable, so that they are even easier to obtain than the previously obtained recessive $cyh2^R$ mutants (*19*, *31*). The mutations do not significantly affect the yeast metabolism related to the production or elimination of the compounds responsible for the aroma and flavor of the wines. The statistical analysis showed that SMR10-11D and SMR16-5A mutants are as good as their parent or as commercial wine yeasts, plus they can be monitored by an easy and inexpensive replica-plating assay

during fermentation. Therefore, they can be used in alternate years with other genetically marked yeasts, such as the cycloheximide resistant strains, to avoid any inoculated yeast becoming resident at the wineries.

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