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Rhodamine-Pink as a Genetic Marker for Yeast Populations in Wine Fermentation

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Winemaking with selected yeasts requires simple techniques to monitor the inoculated yeast. New high-concentration rhodamine-resistant mutants and low-concentration rhodamine-pink mutants, easy to detect by replica-plate assay, were obtained from selected wine yeasts. The rhodamine-pink mutations were dominant and were located at the *pdr5* locus that encodes for the Pdr5 ATP-binding cassette multidrug resistance transporter. The mutants were genetically stable but had lost the killer phenotype of the parent yeast strain. They were genetically improved by elimination of recessive growth-retarding alleles followed by crossing with selected killer wine yeasts. Several spore-clones were selected according to their must fermentation kinetics and the organoleptic quality of the wine. Some spore-clones were tested in industrial winemaking, and they were easily monitored during must fermentation using a simple color-plate assay. They accounted for >96% of the total yeasts in the must, and the resulting wine had as good a quality as those made with standard commercial wine yeasts. The rhodamine-pink yeasts may also be detected by direct seeding onto rhodamine agar or by observation under fluorescence microscopy. These possibilities greatly reduce the time of analysis and make the monitoring procedure for rhodamine-pink yeasts faster, easier, and cheaper than for the genetically marked wine yeasts obtained previously.

KEYWORDS: Rhodamine-pink; yeast; wine; fermentation; killer phenotype

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*−*23*). Unfortunately, the expense, complexity, and time required by these techniques make them difficult to apply 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, and erythromycin) have already been proposed. Generally, these markers confer resistance to antibiotics that inhibit yeast growth by preventing mitochondria function (*24*, *25*). 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 (*26*, *27*), 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 *Saccharomyces cerevisiae*. It provides easy assay by fluorometry and agar plate tests (*28*, *29*). However, the procedure involves transgenic yeast and *Escherichia coli* sequences, which mean additional difficulties in gaining consumer confidence and approval for industrial use according to the legislation of many countries (*30*).

A fast, reliable, and economic method to monitor inoculated selected yeast through must fermentation by using *S. cerevisiae* cycloheximide-resistant mutants (*cyh2*^{R}) has been developed (*31*). 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 (*31*). 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 5 years. Although the procedure

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has been working excellently, we recently detected up to 10% of *cyh2*^{R} yeasts in the spontaneous fermentations of some wineries. The mtDNA restriction pattern of these *cyh2*^{R} yeasts matches that of the marketed strain. We were therefore concerned about *cyh2*^{R} yeasts becoming resident in the wineries after repeated use of *cyh2*^{R} commercial yeasts. To solve this problem, we obtained new sulfometuron-resistant (SMR^R) wine yeasts (*32*), which are now on the market and are being used in alternate years with other genetically marked yeasts to avoid the genetic markers' spreading.

As a further advance along this line, our aim was to provide a new alternative choice of genetic marker for the future, which would be easy to detect by replica-plate assay, direct seeding on agar plates, or observation under fluorescence microscopy. We obtained new rhodamine-pink (RHOD^{PC}) wine yeasts that are even faster, easier, and cheaper to monitor than the previously obtained *cyh2*^{R} or SMR^R yeasts. These mutants were tested for industrial winemaking and compared to their rhodamine-red parent strains and standard commercial wine yeasts.

MATERIALS AND METHODS

Yeast Strains and Culture Media. JP73, JP85, JP88, and JP33 are prototrophic and homothallic *S. cerevisiae* wine yeasts previously isolated from Spanish wineries and selected for winemaking (*33*). 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 (*34*) sold by BIOTEX (Talavera la Real, Spain). 88P1A is a homozygous cycloheximide-sensitive K2-killer spore-clone from JP88 (*34*). The haploid laboratory yeasts YGL013C (*mat a, ho, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0, pdr1::G418*^{R}) and YOR153w (*mat a, ho, his3, leu2, met15, ura3, pdr5::G418*^{R}) used for genetic mapping were obtained from EUROSCARF (European Saccharomyces Cerevisiae Archive for Functional Analysis). The rhodamine-pink cycloheximide-resistance (*RHOD*^{PC}) mutations were mapped by analyzing crosses of single spore-clones from spontaneous RHOD^{PC} mutants with the above genetically marked yeasts; 48 tetrads from each cross were analyzed. Standard culture media were used for yeast growth and phenotype tests in the genetic mapping (*35*). Synthetic minimal medium (SD) for auxotroph analysis contained 0.67% yeast nitrogen base (without amino acids, with ammonium sulfate; Difco, Detroit, MI), 2% glucose, and 2% Bacto-agar. Uracil (20 mg/L), L-leucine (30 mg/L), L-histidine−HCl (20 mg/L), and L-methionine (20 mg/L) were added when necessary. YEPD + cyh is YEPD-agar (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose, and 2% Bacto-agar) supplemented with cycloheximide (cyh; prepared in a concentrated ethanol solution) to a final concentration of 2 μg/mL (*31*). YEPD + rhod is YEPD-agar supplemented with rhodamine 6G (rhod) to the desired final concentration (5 μg/mL unless a different concentration is given). The rhodamine 6G was prepared in water (1%) and added to the medium just before this was poured into Petri dishes. YEPD + G418 is YEPD-agar supplemented with G418 (which is the antibiotic geneticin, from Sigma, catalog no. G7034, presented as a concentrated water solution) to a final concentration of 200 μg/mL. YEPD + cyh + rho is YEPD-agar supplemented with cycloheximide (2 μg/mL) and rhodamine 6G (5 μg/mL).

Sporulation and Genetic Mapping. Standard yeast genetics procedures were used for sporulation of cultures and dissection of asci (*36*). 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, and 2% Bacto-agar), and incubated for 7−20 days at 25 °C until >50% of the cells had sporulated. Twenty-four to 48 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 phenotype segregation by replica-plating on the appropriate media. The phenotype test was performed only if spore viability was >95%.

Determination of Killer Activity. Assay for killer activity was performed in low-pH (pH 4) 4-methylene blue plates (4MB) (*36*) 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 ∼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 were performed as previously described (*37*) for all of the isolated RHOD^{PC} spontaneous mutants and for the 18 preselected homozygous RHOD^{PC} K2-killer cycloheximide-resistant spore-clones. dsRNA molecules were separated in 1× TAE−1% agarose gel for 60−75 min.

Detection of Wild Rhodamine-Pink Yeasts in Spontaneous Must Fermentations. Samples were collected from fermenting musts supplied by several wineries located throughout southwestern Spain. All of the musts were in the tumultuous fermentation stage. Each sample was diluted with sterile distilled water, plated on YEPD-agar and YEPD + rhod, 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 Rhodamine-Pink 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 on YEPD + cyh + rhod. In addition, a diluted sample of each culture was plated onto YEPD-agar to calculate the frequency of spontaneous rhodamine-pink 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 onto YEPD-agar and were incubated for 24 h at 30 °C. These plates were then replica-plated onto YEPD + cyh and YEPD + rhod containing different concentrations of cyh (2, 5, 10, 50, and 100 μg/mL) and rhodamine 6G (5, 50, 100, 500, and 1000 μg/mL) and were incubated for 2 days at 30 °C. The MIC for each mutant is the lowest concentration of cyh or rhodamine 6G in which it does not grow.

To measure the stability of the rhodamine-pink 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 YEPD + rhod.

Genetic Improvement of Rhodamine-Pink Mutants. The elimination of recessive growth-retarding alleles consists of performing successive cycles of sporulation and asci dissection until we obtained single-spore fast-growing clones free from recessive growth-retarding mutations and homozygous for all of the genes except the locus *MAT*. Then, those with the best technical characteristics for the production of quality wines were selected (*38*). Thereafter, the killer phenotype was transferred to the new single-spore clones by crossing them with selected killer wine yeasts (*34*).

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 nonsterile 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 obtained in a pilot plant of the company BIOTEX using their industrial procedure. Cells were cultured in beet molasses broth [5% beet molasses, 0.2% Bacto-yeast extract, 0.075% $(NH_4)_2HPO_4$, and 0.1%

Table 1. Isolation and Characterization of $RHOD^{PC}$ Spontaneous Mutants from Wine Yeasts

parent strain (MIC μg/mL of cyh, rhod)	frequency of $RHOD^{PC}$ spontaneous mutants isolated in YEPD + cyh 2 μg/mL + rhod 5 μg/mL	mutant phenotype		
		MIC of cyh	MIC of rhod	killer
JP73 (2, 300)	4×10^{-9}	5	1000	
JP85 (2, 100)	3.57×10^{-9}	10	500	
JP88 (2, 100)	1.26×10^{-8}	5	1000	

$MgSO_4 \cdot 7H_2O$, adjusted to pH 3.5 with HCl] for 18 h at 30 °C with strong aeration, washed twice (by centrifugation) with sterile distilled water, and inoculated in 1000-L stainless steel tanks with 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 ~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 storage 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. The 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). The 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 Amount of Inoculated Yeasts in the Vinification Trials. Determination of the percentage of genetically marked yeasts was done by the replica-plating method (*31*). 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 rhodamine-pink mutants was accomplished by replica-plating these plates to either YEPD + cyh (2 μg/mL) or YEPD + rhod (5 μg/mL) plates using sterile velvets and thereafter to other plates of YEPD-agar to detect wild yeasts sensitive to cyh or rhodamine-red. The time needed to easily observe growth of resistant yeasts on YEPD + cyh or YEPD + rhod at 30 °C varied between 1 and 3 days depending on the mutant. The amounts of rhodamine-pink mutants were also determined by the direct method (*31*); that is, samples from fermenting musts were diluted and plated directly onto YEPD-agar and YEPD + rhod to obtain the same number of colonies per plate (200−300). The time needed to easily see yeast colonies growing on YEPD + rhod at 30 °C varied between 2 and 3 days depending on the mutant. The detection of the rhodamine-pink mutants by fluorescence microscopy was as follows: the cells (from 1-mL samples of YEPD culture or fermenting must) were spun in a microcentrifuge, resuspended in 1 mL of YEPD + rhod (1 μg/mL) broth, incubated at 30 °C with shaking for 30 min, washed once with YEPD broth, and viewed with a Nikon Eclipse 600 microscope equipped with a 40× objective, a 100-W mercury lamp light source, and a rhodamine Nikon filter set (G-2A). The images were obtained rapidly to avoid fluorescence extinction, using a Nikon Coolpix 950 digital camera. Photo conditions were kept constant for each experiment so that the fluorescence levels were comparable. The proportion of fluorescent yeasts was calculated from an analysis of the images.

The percentage of wild parent yeasts was determined by analyzing the mtDNA restriction pattern (*39*). Purified mtDNA was digested with *Rsa*I. The fragments were separated in 0.5× TBE−0.8% agarose gels 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 (*40*). Lactic acid was determined using the EEC recommended method (*41*). Major volatile compounds and polyols were quantified by gas chromatography (*42*). t_{15} is the time needed to ferment 15% of the total sugars present in the must, and t_{100} is the time needed to ferment 100% of the total sugars (*38*).

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 Rhodamine-Pink Yeasts in Spontaneous Must Fermentions. For rhodamine-pink to be easily used as a marker for the detection of inoculated *S. cerevisiae* yeasts in industrial vinifications, the colonies of *Saccharomyces* sensu stricto yeasts must be distinguishable from the rest of the wild yeast species by simple observation. *Saccharomyces* colonies are easy to distinguish from the other yeasts species present in fermenting grape must by their aspect in YEPD-agar (white or cream color, buttery, smooth, circular, and prominent) and by their ability to sporulate, producing typical asci (tetrads). In a previous study (unpublished data) performed for all of the wine-producing zones of southwestern Spain, we found that all of the yeast colonies isolated from fermenting musts having the typical aspect of *Saccharomyces* sensu stricto yeasts belonged to this species group. Similarly, all of the colonies with aspects different from *S. cerevisiae* belonged to other species (unpublished data). Moreover, the frequency of wild rhodamine-pink yeasts in musts and wines should be low enough to avoid any major background error, and indeed we never detected any wild rhodamine-pink *Saccharomyces*-like colonies in fermenting musts collected from different wineries in five consecutive vintages (from 1999 to 2004)—all *Saccharomyces* yeasts were rhodamine-red. When any wild rhodamine-pink yeasts were detected, which occurred only earlier than 2 days in some spontaneous must fermentations, they were easily distinguished as non-*Saccharomyces*, and their appearance rate was always <10%. Therefore, no interference is expected in monitoring inoculated rhodamine-pink *Saccharomyces* mutants in must fermentation.

Isolation and Characterization of Rhodamine-Pink Mutants. We easily isolated spontaneous rhodamine-pink cycloheximide-resistant ($RHOD^{PC}$) mutants from three wine yeasts (JP73, JP85, and JP88) by plating them onto YEPD + cyh + rhod. We previously attempted to isolate rhodamine-pink ($RHOD^W$) mutants from the same yeast strain by plating then onto YEPD + rhod (2, 5, 50, 100, 200, 300, and 500 μg/mL). We obtained rhodamine-resistant mutants, but none of them were pink mutants, although some of them were pale red, contrasting with the red colonies from wild-type strains. The frequency of $RHOD^{PC}$ spontaneous mutants depended on each yeast strain, but it was always $< 1.26 \times 10^{-8}$ (**Table 1**). By using this mutant phenotype, there is therefore minimal risk of

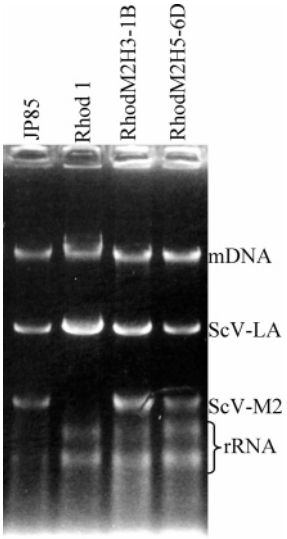

Figure 1. Agarose gel electrophoresis of virus dsRNA molecules. JP85, original parent K2-killer strain; Rhod 1, killer-sensitive genetically improved RHOD^{PC} spontaneous mutant (from JP85); RhodM2H3-1B and RhodM2H5-6D, RHOD^{PC} K2-killer cycloheximide-resistant selected spore-clones from the cross Rhod 1 × 88P1A; mDNA, mitochondrial DNA; rRNA, ribosomal RNA.

interference from spontaneous resistant mutants originating from wild strains of *S. cerevisiae* in the detection of RHOD^{PC} strains inoculated in industrial fermentation.

Ten RHOD^{PC} mutants isolated from each parental strain were characterized by analyzing their resistance to increasing concentrations of cyh and rhod. All of the mutants were capable of growing in the cyh and rhod concentration in which they were isolated. In higher concentrations the behavior of the mutants was variable. Mutants from JP85 showed higher resistance to cyh and lower resistance to rhod than those from JP73 and JP85, and some mutants from JP85 were the ones with the clearest rhodamine-pink colony phenotype (**Table 1**). As previously found for the isolation of cyh^{R} wine yeasts (*31*, *34*), the RHOD^{PC} mutants lost the parent killer phenotype (*33*) because they retained ScV-LA but lost the ScV-M2 virus that encodes for the killer K2 toxin (**Figure 1**).

Continued propagation of these mutants under laboratory conditions (20 transfers on YEPD plates at 30 °C) yielded no red colony among 200 colonies analyzed of each mutant after replica-plating on YEPD + rhod. That is, the frequency of revertants after approximately 100 doublings (*43*) was $<5 \times 10^{-3}$. Therefore, the mutations were genetically stable enough to discard any possibility of interference because of the appearance of any sensitive revertants from the inoculated RHOD^{PC} mutants in the detection of wild sensitive yeasts during the must fermentation.

Genetic Improvement of RHOD^{PC} Mutants. Generally, these mutants were able to ferment grape must but not as quickly as the parent wine yeasts. Most of them had a slightly longer t_{15} (time needed to ferment 15% of the total sugars), t_{100} (time needed to ferment 100% of the total sugars), or both. As all of 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 (*38*). Hence, eight of the best RHOD^{PC} mutants (those with the lowest t_{15} and t_{100}) were sporulated, the spore colonies from the tetrads were analyzed, and new homozygous single-spore descendants were selected as previously reported (*38*), and, as in that previous paper, the segregation ratio for spore colony size was found to be 2 large/2 small for all of the mutants obtained from JP85 and JP88 and irregular segregation for those from JP73. The segregation ratio for spore colony phenotype was 2 RHOD^{PC}/2 rhod^{+}, which indicates that the resistance phenotype is due to a dominant mutant allele. No relationship between spore colony size and RHOD^{PC} phenotype was observed, so that they must be independent traits. We chose 14 spore clones among those with higher MIC for rhodamine (>100 μg/mL) and clearer pink colony phenotype in YEPD + rhod plate (Rhod 6-11C and Rhod 6-14D from Rhod 6; Rhod 7-2A and Rhod 7-4D from Rhod 7; Rhod 8-1D and Rhod 8-3A from Rhod 8; Rhod 9-1A and Rhod 9-1D from Rhod 9; Rhod 12-2C and Rhod 12-4B from Rhod 12; and Rhod 1, Rhod 2, Rhod 3, and Rhod 4 from Rhod 85).

Table 2. Fermentation Vigor of the Best Improved RHOD^{PC} Spore Clones[a]

yeast	t_{15} (days)	t_{100} (days)
Rhod 6-11C	1.1 ± 0.19	6.0 ± 1.1
Rhod 9-1A	1.3 ± 0.29	6.0 ± 1.1
Rhod 12-4B	1.1 ± 0.24	6.0 ± 1.3
Rhod 1	1.5 ± 0.20	9.5 ± 0.8
E7AR1	1.5 ± 0.35	9.5 ± 0.9
JP73	1.5 ± 0.39	9.5 ± 1.9
JP85	1.5 ± 0.44	9.5 ± 1.7
JP88	1.5 ± 0.25	9.5 ± 0.3

[a] t_{15} is the time needed to ferment 15% of the total sugars present in the must, and t_{100} is the time needed to ferment 100% of the total sugars. The data are the mean values of three independent experiments and standard errors.

The 14 spore-clone mutants and the parent strains were inoculated in sterile Pardina grape must to analyze the fermentation performance. All yeasts finished must fermentation properly. However, there were important differences in the evolution of the sugar consumption. Seven of the 14 spore-clone mutants had a t_{15} even shorter than that of the parent yeast, and 3 among them (Rhod 6-11C, Rhod 9-1A, and Rhod 12-4B) had a lower t_{100} than the parent yeasts. The Rhod1 mutant had the same fermentation kinetics as the parent yeasts, but it showed the clearest RHOD^{PC} colony phenotype in YEPD + rhod during the must fermentation. Therefore, in some spore clones, the fermentation kinetics was improved by elimination of recessive growth-retarding alleles, and then the *RHOD*^{PC} mutation did not reduce the must fermentation vigor relative to the parent and standard commercial yeasts (**Table 2**). No red colony was detected among the 200 colonies analyzed from each mutant fermentation (replica-plating on YEPD + rhod), so the frequency of spontaneous revertants was $<5 \times 10^{-3}$. That is, the mutations are genetically stable enough to discard the possibility of interference of sensitive revertants raised from these four RHOD^{PC} spore clones in the detection of wild sensitive yeasts during the must fermentation. These four RHOD^{PC} mutants were preselected to further evaluate their usefulness for winemaking.

The tetrad analysis (for genetic mapping) of the hybrids from the cross Rhod 1 × YOR153w gave the tetrad type proportion 48 parental ditype (PD) : 0 nonparental ditype (NPD) : 0 tetratype (TT). The tetrad type proportion of the hybrids from the cross Rhod 1 × YGL013C was 18 PD : 9 NPD : 21 TT. Roughly the same results were obtained for the hybrids from the crosses of Rhod 6-11C, Rhod 9-1A, and Rhod 12-4B with YOR153w and YGL013C. Therefore, the *RHOD*^{PC} mutations of these four mutants were not linked to *pdr1*, and they mapped exactly to the *pdr5* gene on the right arm of chromosome XV that encodes

Table 3. Fermentation Parameters of Fresh White Must (Cayetana) and Red Grape (Merlot) Microvinifications Inoculated with the Preselected RHOD^{PC} Mutants

	Cayetana		Merlot	
yeast	t_{15} (days)	t_{100} (days)	t_{15} (days)	t_{100} (days)
control	4.75	13	6.5	15
Rhod 6-11	2.2	9	3.1	14
Rhod 9-1A	2.2	9	2.4	14
Rhod 12-4B	2.2	9	3.2	14
Rhod 1	2.2	9	3.1	14
E7AR1	2.1	12	3.5	14
JP73	2.3	12	3.7	14
JP85	2.2	11	3.1	13
JP88	2.1	10	2.75	14

for the Pdr5 ATP-binding cassette transporter involved in the multidrug resistance mechanism of *S. cerevisiae* (*44*, *45*). The PDR5 protein was shown to pump rhodamine 6G in intact yeast cells in an energy-dependent manner (*46*). However, this is the first time that RHOD^{PC} spontaneous mutants have been isolated from diploid homozygous wine yeasts.

Laboratory Microvinification Trials with the RHOD^{PC} Mutants. All of the RHOD^{PC} preselected mutants (Rhod 6-11C, Rhod 9-1A, Rhod 12-4B, and Rhod 1) again showed fermentation kinetics similar to those of the parent (JP73, JP85, and JP88) and standard (E7AR1) strains, and the control (spontaneous fermentation) conducted in parallel was much slower than the rest (**Table 3**). These mutants dominated the must fermentations (100%) as analyzed by the replica-plating method. The same results were obtained by directly plating the diluted samples onto YEPD + rhod agar, because all of the seeded single yeast cells grew in the presence of rhod. The contrary has been reported for cycloheximide-resistant wine yeast single cells, part of which did not grow in the presence of the drug (*31*). All of the analyzed colonies (20 from each sample, 3 samples from each vinification) had the same mtDNA restriction pattern as the original parent strain, and this pattern was not detected in samples from noninoculated controls (**Figure 2**). In no case did we detect red spontaneous revertants. No RHOD^{PC} yeast colonies were detected in the noninoculated controls. The must fermentation parameters (t_{15} and t_{100}), the wine parameters (organoleptic 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 RHOD^{PC} mutants and the corresponding parent strains (data not shown). Therefore, the *RHOD*^{PC} 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 parent and standard commercial wine yeasts. However, in the organoleptic test, the JP88, Rhod 12-4B, and Rhod 1 white wines and the E7AR1 and Rhod 1 red wines were the best evaluated. Therefore, we selected Rhod 1 mutant for further experiments.

Yeast Hybridization To Obtain K2-Killer RHOD^{PC} Mutants. The killer phenotype is an interesting property in wine yeast (*33*, *34*). As RHOD^{PC} mutants had lost the parent K2-killer phenotype, there is a need to restore it in the newly obtained mutants. Haploid spores from the Rhod 1 mutant were crossed with the homozygous cycloheximide-sensitive K2-killer spore-clone 88P1A from JP88 as previously described (*36*).

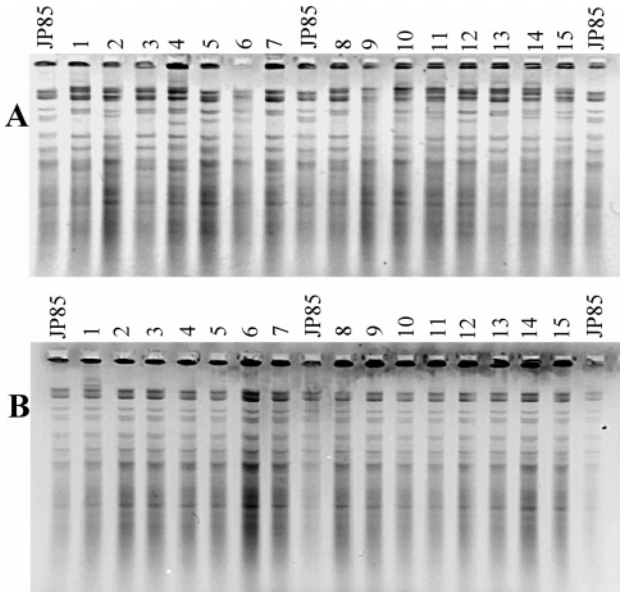

Figure 2. Mitochondrial DNA restriction analysis with restriction endonuclease *Rsa*I: (**A**) noninoculated control (all were rhodamine-red *Saccharomyces*-like colonies); (**B**) inoculated with the selected spore-clone RhodM2H5-6D (all were rhodamine-pink *Saccharomyces*-like colonies). Single colonies (from 1 to 15) were isolated from the Tempranillo grape vinification (2002). JP85, original parent strain.

Heterozygous K2-killer cycloheximide-resistant hybrids were obtained. After sporulation and tetrad dissection (*38*), all hybrids segregated 2 *cyh*^{R} *RHOD*^{PC} : 2 *CYH*^{S} *rhod*$^{+}$, and the spore viability was 100%, as was to be expected because both parent yeasts were already genetically improved by elimination of recessive growth-retarding alleles (*38*). RHOD^{PC} and cycloheximide-resistant phenotypes were genetically linked, so both should belong to the same mutation in *pdr5* gene, as previously reported (*47*−*49*). Eighteen homozygous RHOD^{PC} K2-killer cycloheximide-resistant spore-clones were preselected; all of them were confirmed to contain ScV-LA and ScV-M2 viruses (see **Figure 1** for RhodM2H3-1B and RhodM2H5-6D).

Two independent laboratory microvinification trials (with Cayetana and Merlot musts) were done with the 18 preselected spore-clones. The inoculated RHOD^{PC} mutants were monitored during the fermentation by replica-plating onto YEPD + rhod and by mtDNA restriction pattern analysis for the replica test validation. They accounted for 100% of the total yeasts in the must. All of the mutants again showed fermentation kinetics similar to those of the parent (JP73, JP85, and JP88) and standard (E7AR1) strains, and the noninoculated control conducted in parallel was much slower than the rest (data not shown). Two spore-clones, RhodM2H3-1B and RhodM2H5-6D, were selected for further winery vinification, as above, because of their better must fermentation kinetics and the organoleptic quality of the wine.

Winery Vinification Trials with the Selected K2-Killer RHOD^{PC} 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 RhodM2H3-1B and RhodM2H5-6D, the parent strain JP88, and a standard commercial wine yeast E7AR1. A noninoculated control (spontaneous fermentation) was done in parallel for each vinification series. That is, a total of 6 different vinifications series were done, resulting in 30 wines. All of the RHOD^{PC} mutants again showed fermentation parameters (t_{15} and t_{100}) similar to those

Table 4. Must Fermentation Parameters and Wine Analysis Results of Six Independent Winery Vinifications Made with Cigüentes, Macabeo, Cabernet-Sauvignon, and Tempranillo Grapes[a]

	yeast					
parameter	control	E7AR1	JP88	RhodM2H3-1B	RhodM2H5-6D	p^b
t_{15} (days)	3.8 ± 0.39b	1.36 ± 0.17a	1.6 ± 0.21a	1.7 ± 0.26a	1.47 ± 0.26a	0.000
t_{100} (days)	10.1 ± 2.24a	5.9 ± 1.45a	5.7 ± 0.82a	6.01 ± 1.49a	7.85 ± 1.86a	0.304
preference (%)	68 ± 5.09a	66.9 ± 4.8a	67 ± 2.51a	61.3 ± 6.33a	73 ± 3.76a	0.559
frequency (%)	0 ± 0a	100 ± 0b	100 ± 0b	98 ± 1.62b	96.6 ± 3.33b	0.000
alcohol (% v/v)	13 ± 0.36a	12.7 ± 0.49a	12.8 ± 0.41a	12.9 ± 0.37a	12.7 ± 0.49a	0.940
pH	3.3 ± 0.07a	3.3 ± 0.08a	3.4 ± 0.07a	3.3 ± 0.07a	3.4 ± 0.08a	0.999
total acidity (g/L)	8.1 ± 0.63a	8.3 ± 0.5a	8.1 ± 0.66a	8.7 ± 0.55a	8.6 ± 0.51a	0.899
volatile acidity (g/L)	0.26 ± 0.03ab	0.2 ± 0.01a	0.18 ± 0.01a	0.26 ± 0.04ab	0.4 ± 0.06b	0.017
reducing sugars (g/L)	0.67 ± 0.42b	0.05 ± 0.03ab	0.01 ± 0a	0.08 ± 0.08ab	0.2 ± 0.11ab	0.161
malic acid (g/L)	1.3 ± 0.13a	1.69 ± 0.12b	1.56 ± 0.09ab	1.8 ± 0.06b	1.86 ± 0.12b	0.016
lactic acid (g/L)	0.16 ± 0.1a	0.05 ± 0.02a	0.08 ± 0.04a	0.07 ± 0.03a	0.05 ± 0.02a	0.649
acetaldehyde (mg/L)	18.5 ± 4.04a	16.2 ± 4.15a	16.8 ± 3.97a	17 ± 3.47a	19.5 ± 3.6a	0.972
ethyl acetate (mg/L)	17.3 ± 6.18a	13.2 ± 4.86a	15.8 ± 5.99a	12 ± 5a	10.5 ± 4.21a	0.890
methanol (mg/L)	132 ± 32.5a	111 ± 29.1a	136 ± 41.2a	98 ± 26.6a	125 ± 30.8a	0.914
fusel alcohols (mg/L)	342 ± 65.5a	335 ± 55.2a	348 ± 47.4a	266 ± 39.8a	285 ± 51.2a	0.735
butanol-1 (mg/L)	0.83 ± 0.54a	0.56 ± 0.36a	1 ± 0.63a	0.16 ± 0.16a	0.41 ± 0.41a	0.715
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. ± 4.19a	28.8 ± 3.92a	34.3 ± 8.04a	30.3 ± 4.75a	30.6 ± 5.46a	0.910
isobutanol (mg/L)	55.7 ± 10.7a	56.3 ± 9.3a	53.2 ± 14.1a	37.8 ± 7.1a	42 ± 5.4a	0.567
amyl alcohols (mg/L)	259 ± 54.3a	250 ± 47.3a	260 ± 34a	197 ± 31.6a	228 ± 43.1a	0.823
H_2S (U^c)	3.33 ± 3.3a	3.33 ± 2.1a	5 ± 3.4a	11.6 ± 4.7a	6.6 ± 4.9a	0.535

[a] ANOVA to study the effect of inoculation with RhodM2H3-1B and RhodM2H5-6D selected $RHOD^{PC}$ mutants. The data are the mean values of six independent experiments and standard errors. Different letters (a, b) mean significantly different groups found with the Duncan test at $p < 0.05$. [b] p values obtained by ANOVA for the wines made with each yeast. [c] U, arbitrary units.

of the parental and standard strains, and the control was much slower than the rest (**Table 4**). The frequencies of all the inoculated yeasts ranged from 96.6 to 100% (**Table 4**). All of the $RHOD^{PC}$ colonies analyzed (20 from each sample, 3 samples from each vinification) had the same mtDNA restriction pattern as the original parent strain, and once again, this pattern was not detected in samples from noninoculated controls (data not shown). In no case did we detect red spontaneous revertants. No $RHOD^{PC}$ yeast colonies were detected in the noninoculated controls. Among all of the fermentation and wine parameters analyzed, there were significant differences only between inoculated and noninoculated vinifications for the means of t_{15}, the frequency, and malic acid (**Table 4**). This is because the onset of fermentation is always slow in noninoculated vinification, and obviously there is no dominance of noninoculated strains. The lowest amount of malic acid in the control wines could be due to malate degradation by lactic acid bacteria, which are more frequent in slow fermenting noninoculated fermentations than in the inoculated cases. Apart from this, there were no significant differences in the means of any of the parameters (t_{100}, organoleptic quality, degree of domination, alcohol, pH, total acidity, volatile acidity, reducing sugars, 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 4**). There was a significantly lower amount of volatile acidity in RhodM2H5-6D wines than in the rest. Although the RhodM2H5-6D wines were the best evaluated (73%), the rest of the wines also had very good quality because the grapes were of good sanitary quality and no incidences (such as sluggish fermentation or undesirable bacterial growth) occurred during the winemaking.

Optimization of $RHOD^{PC}$ Mutant Monitoring. Colony replica-plating onto YEPD + rhod is a very reliable test to monitor $RHOD^{PC}$ mutants. However, it takes 3−5 days to get the final results (2 days for colony isolation and 1−3 days for replicated yeast to grow and for phenotype observation). As the rhod concentration used (5 μg/mL) is not at all toxic for the yeast, the samples from fermenting must can be properly diluted and directly seeded onto YEPD + rhod. The time needed to easily see red wild type or pink mutant colonies varied between 2 and 3 days depending on the mutant, and the results were most often as reliable as those from the replica-plating test (**Figure 3A**). Moreover, as $RHOD^{PC}$ mutants do not emit much fluorescence (they do not take rhod), they can be monitored by direct real time fluorescence microscopy (**Figure 3B**), which greatly reduces the time of analysis. Unfortunately, there were some mutant yeasts that showed fluorescence (they were probably somehow damaged in the hostile fermentation environment and did not pump rhod), and some wild yeasts that did not show fluorescence [probably because the fluorescence staining also depends on the physiological state of individual cells (*50*)]. Therefore, $RHOD^{PC}$ mutants can be monitored by microscopy assuming an error of from 5 to 15% (depending on the mutant), and the test time can be reduced from 4−5 days of replica-plating, or 2−3 days of direct seeding, to just 45 min.

In conclusion, the $RHOD^{PC}$ mutations were dominant and genetically stable, so that they are even easier to obtain than the previously obtained recessive *cyh2*^{R} mutants (*31*, *43*). The mutations did 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 the RhodM2H3-1B and RhodM2H5-6D mutants are as good as their parent or commercial wine yeasts, plus they can be monitored by an easy and inexpensive replica-plating procedure or by direct seeding onto YEPD + rhod assay during fermentation. They may also be detected (allowing for some error) by observation under fluorescence microscopy, which greatly reduces the time of analysis compared to the previously obtained cyh^R (*31*) or SMR^R yeasts (*32*). They therefore make an ideal option for use in alternate years with other genetically marked yeasts to avoid any inoculated yeast becoming resident in the wineries.

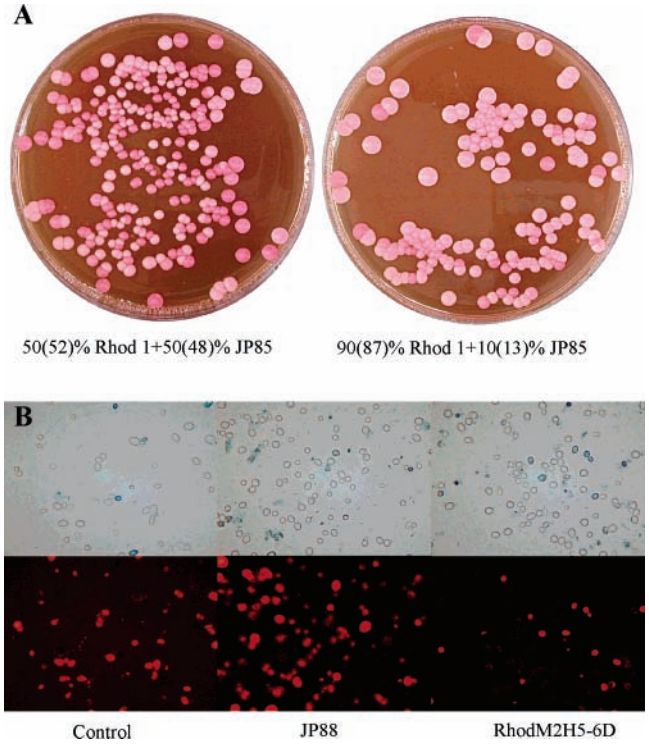

Figure 3. (**A**) Determination of the percentage of RHOD^{PC} mutants by direct seeding onto YEPD-rhod (5 μg/mL). The left plate was inoculated with 100 μL of a 50% Rhod 1 + 50% JP85 culture mix and the right plate with a 90% Rhod 1 + 10% JP85 culture mix. Starting cultures were properly diluted to get equal numbers of cells per milliliter. Actual colony count for each phenotype is in parentheses. (**B**) Microscopic observation with brightfield (above) and fluorescence (below) microscopy of yeast samples from three Pardina juice vinifications: noninoculated control, inoculated with JP88, and inoculated with the selected spore-clone RhodM2H5-6D.

The same procedure we used to obtain these mutants could be applied to get new RHOD^{PC} mutants from any previously selected wine yeast. Alternatively, the RHOD^{PC} mutations already obtained can be transferred to any previously selected yeast by a breeding program (*34*).

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