Optimized Method To Obtain Stable Food-Safe Recombinant Wine Yeast Strains

Sergi Puig,[†] Daniel Ramón,[†] and José E. Pérez-Ortín*[,†,‡]

Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Polígono La Coma s/n, Paterna, Spain, and Departamento de Bioquímica y Biología Molecular, Universitat de València, Dr. Moliner 50, Burjassot, Spain

Pure *Saccharomyces cerevisiae* cultures are commonly used to inoculate fresh musts. The added active dry yeasts dominate the fermentation and produce wine of more reproducible quality. This microbiological simplification of the vinification process opens the way for the genetic modification of active dry yeasts. Industrial yeast strains have been successfully transformed, selecting for G418 resistance. The use of this method for integrative transformation facilitates the construction of food-safe recombinant yeast strains with high efficiency. To demonstrate the potential of the method, the two *URA3* alleles of the wine yeast T_{73} strain were disrupted in two consecutive steps, obtaining a stable recombinant strain that retains all of its useful wine-making properties.

Keywords: *Yeast; wine; genetic transformation; food-safe*

INTRODUCTION

Many winemakers inoculate pure *Saccharomyces cerevisiae* cultures into fresh must to perform controlled fermentations and thus produce wine of more reproducible quality (Snow, 1983). We have previously isolated a wine yeast strain, named T_{73}, from Alicante musts (Querol et al., 1992a) and, using molecular markers, have demonstrated its imposition in the vinification process (Querol et al., 1992b). From the microbiological point of view the imposition of the inoculated strain is a simplification of the fermentation process that opens the way for the genetic modification of active dry yeasts. During recent years various laboratories have reported the construction of recombinant wine yeast strains that express metabolic activities with consistent effects on the characteristics of the wines obtained [for a review see Querol and Ramón (1996)].

To apply genetic engineering techniques, an efficient transformation system is needed to introduce recombinant DNA into the host organism. Whereas transformation of laboratory *S. cerevisiae* strains is now a common procedure, industrial strains are generally recalcitrant to transformation with exogenous DNA. A case in point is the T_{73} strain, for which only an inefficient transformation system based on cycloheximide resistance has been developed (Pérez-González et al., 1993). An ideal gene transfer system for industrial yeasts requires, among other things, a selectable marker and high transformation efficiency to allow the construction of stable strains by chromosomal integration. For commercial reasons, transformants should also (1) maintain the same useful properties of the parental strain, (2) be free of nonessential foreign DNA (for example, antibiotic resistance genes), (3) have the minimal amount of synthetic DNA linkers, and (4) not express any fusion protein that may arise as a consequence of the genomic integration events (Linko et al., 1997).

Wach et al. (1994) have designed a yeast transformation protocol for the integration of linear DNA fragments into desired sites of the yeast genome. The integrative cassette includes a selectable marker (the kan^R gene that confers kanamycin or G418 resistance) together with the fragment to be integrated (if any), flanked by two "arms" complementary to the desired integration site. There is also a version of the cassette (pFA6-kanMX3) that permits excision of the kan^R gene by homologous recombination between two direct repeats that flank the marker. The method allows a yeast gene to be disrupted or an exogenous "clean" gene to be inserted into the chosen site. To carry out this operation, it is only necessary to know the target DNA sequence. Given that the complete yeast DNA sequence for *S. cerevisiae* strain S288c is already known (Goffeau et al., 1997) and is easily accessible over the Internet, it is theoretically feasible to insert additional DNA into any site in the yeast genome.

In this paper we have applied the protocol developed by Wach et al. (1994) to construct stable, food-safe recombinant T_{73} yeast strains at high transformation efficiency. Using this method we have disrupted both copies of the *URA3* gene in two consecutive steps, obtaining a stable *ura3* yeast strain that maintains all of its useful wine-making abilities.

MATERIALS AND METHODS

Strains and Culture Conditions. The *S. cerevisiae* industrial wine yeast strain T_{73} (CECT1894) (Querol et al., 1992a), commercialized by Lallemand Inc. (Montreal, PQ, Canada), and its derivatives were used for transformation and microvinification experiments. Other industrial yeast strains are described in Table 1. *Escherichia coli* strain DH5α was used for the propagation of plasmids.

* Address correspondence to this author at the Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Apartado 73, E-46100 Burjassot, Spain (fax 346 3636301; e-mail jose.e.perez@uv.es).
[†] Instituto de Agroquímica y Tecnología de Alimentos.
[‡] Universitat de València.

S0021-8561(97)00653-5 CCC: $15.00
Published on Web 03/13/1998

Table 1. Source and Transformation Efficiency (Transformants per Microgram of DNA) of the Industrial Yeast Strains Used in This Study

strain	source	episomal	integration
T_{73}	Alicante (Spain), red wine	1000−12000	5−170
1476[a]	Montrachet (USA), white wine	270−850	65
1477[a]	Bordeaux (France), white wine	1800−2100	10
1485[a]	Jerez sherry (Spain), white wine	1500−6000	200
P29	Penedes (Spain), cava wine	100−5000	20
1170[a]	distillery	6000	350
1383[a]	distillery	30000−120000	1350
1331[a]	beer	10000	10
P22	bread	500−5500	25
T_{73}-4	recombinant wine yeast (this work)	15000−45000	1550

[a] CECT code (Spanish Type Culture Collection).

For laboratory cultures, yeast cells were grown at 30 °C in YPD-rich medium (1% yeast extract, 2% bacteriological peptone, 2% glucose) or in SD minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose). *E. coli* cells were grown at 37 °C in LBA medium (1% tryptone, 1% NaCl, 0.5% yeast extract, supplemented with 50 μg/mL of ampicillin). Solid media were supplemented with 2% agar.

Plasmids and DNA Manipulations. All DNA manipulations were carried out as described in Sambrook et al. (1989).

Plasmid pFA6-kanMX3 (Wach et al., 1994) was digested with the restriction enzymes *Eco*RI and *Sal*I. The fragment containing the kan^R gene of the *E. coli* transposon Tn903 was ligated to YEp352, a plasmid that contains the *URA3* gene (Hill et al., 1986), previously cut with the same enzymes, yielding plasmid YEp-KMX3.

The *Hin*dIII−*Hin*dIII fragment of pRB58 (Carlson and Botstein, 1982) containing the *S. cerevisiae URA3* gene was subcloned into pUC18 (Yanish-Perron et al., 1985) digested with the same enzyme, resulting in plasmid pURA3. *Sma*I−*Eco*RV fragments from either the pFA6-kanMX3 (excisable) or pFA6-kanMX4 (nonexcisable) cassette (Wach et al., 1994) containing the kan^R gene were ligated to the *Eco*RV cut plasmid pURA3, producing plasmids pURA::KMX3 or pURA::KMX4, respectively. Both plasmids were cut with *Nde*I and *Stu*I enzymes to obtain the *URA3* integrative cassettes.

Yeast Transformation Protocol. All industrial yeast strains were transformed according to the LiAc/SS-DNA/PEG protocol described by Gietz and Woods (1994), with the following modifications: after heat-shock, cells were centrifuged and resuspended in 1 mL of YPD and incubated with agitation for 2 h at 30 °C. They were then plated in YPD agar containing 100 μg/mL G418, and transformants were selected according to their resistance to the antibiotic (Wach et al., 1994). Transformant colonies usually appeared after 2 days at 30 °C.

Microvinification Experiments. Microvinifications were carried out at 22 °C with gentle orbital agitation (125 rpm) in 0.5 L glass bottles (Schott-Duran 86 ∅ × 181 mm) containing 0.5 L of red grape Bobal must. The initial yeast inoculum was of 3×10^5 cells/mL from YPD overnight cultures. Reducing sugar concentration was recorded as an indication of the fermentation progress. At the end of the fermentation, wines were centrifuged at 6000 rpm for 10 min to remove yeast cells and transferred to new bottles, which were kept at 4 °C prior to analysis. Enological parameters were determined as described in Querol et al. (1990).

Reagents. G418 (Geneticin) sulfate was from Gibco BRL and 5-fluoroorotic acid (5FOA) from Toronto Research Chemicals Inc.

RESULTS

Development of a High-Efficiency Transformation System for Wine Yeasts. The previously developed T_{73} transformation protocol (Pérez-González et al., 1993) yields recombinant cycloheximide-resistant strains (cyh^R) at very low frequency. In our search for other dominant markers, we discovered that the T_{73} strain is extremely sensitive to G418, its growth being prevented at concentrations of 100 μg/mL of this antibiotic. Prompted by this result, we decided to attempt a new strategy based on the protocol of Wach et al. (1994). A replicative plasmid named YEp-KMX3 containing the kan^R gene marker was constructed (see Materials and Methods for details). Using this plasmid and the lithium acetate protocol, a large number of G418-resistant transformants were obtained (a maximum of 1.2×10^4 transformants/μg of plasmid). No transformants or background growth was detected in control plates on which nontransformed cells were spread.

Other industrial yeast strains (wine, beer, distillery, and baker's yeasts) were then transformed using the same protocol and plasmid YEp-KMX3. As can be seen in Table 1, all of the strains tested were able to be transformed to G418 resistance. Transformation frequencies were between 10^3 and 1.2×10^5 transformants/μg of plasmid. This high efficiency is sufficient to attempt integrative transformations. For this purpose a vector (pURA::KMX4) containing the kan^R and *URA3* genes was constructed and an integrative cassette derived from it (for details see Materials and Methods) used to transform the yeast strains by the modified lithium acetate protocol. Transformants arose at a frequency of 5−1350/μg of DNA depending on the strain (see Table 1). A background of abortive transformants was seen, but this seems to be common for this procedure (A. Wach, A. Brachat, and P. Philippsen, University of Basel, Switzerland, private communication) and does not constitute a major problem because restreaking of the colonies onto G418-containing plates clearly selects for stable transformants.

Construction of a *ura3* T_{73} Strain. Efficient integrative transformations in the T_{73} strain allowed us to apply the method of Wach et al. (1994) to obtain food-grade recombinant strains. First, we studied the ploidy of the T_{73} genome. As for most wine yeast strains, this strain is homothallic and partially heterozygotic and sporulates well (data not shown). Using flow cytometry, we have determined that T_{73} has a DNA content which is compatible with its being diploid (not shown), although partial aneuploidy cannot be ruled out (J. Gimeno, E. Matallana, and J.E.P.-O., unpublished). Although this cannot guarantee that any particular gene is diploid, this is the most likely result.

As an example of the protocol to obtain a stable food-safe recombinant wine yeast, we have chosen to generate a *ura3* strain disrupted in the two predicted copies of the *URA3* locus. The *URA3* gene is the most frequently used gene marker in *S. cerevisiae*, and many molecular tools based on this gene have been constructed. We used 1 μg of the excisable cassette from plasmid pURA::KMX3 (see Materials and Methods) to transform T_{73} and obtained five transformants. By Southern analysis we demonstrated that all of them had the predicted insertion in the *URA3* locus. One of the kan^R transformants obtained, named T_{73}-1 (Figure 1), was used for further work. This transformant was grown for 25 generations in nonselective medium (YPD without G418), and 2×10^3 cells were plated onto YPD agar. Colonies were replica-plated onto G418-containing YPD plates. The frequency of kan^R excision was

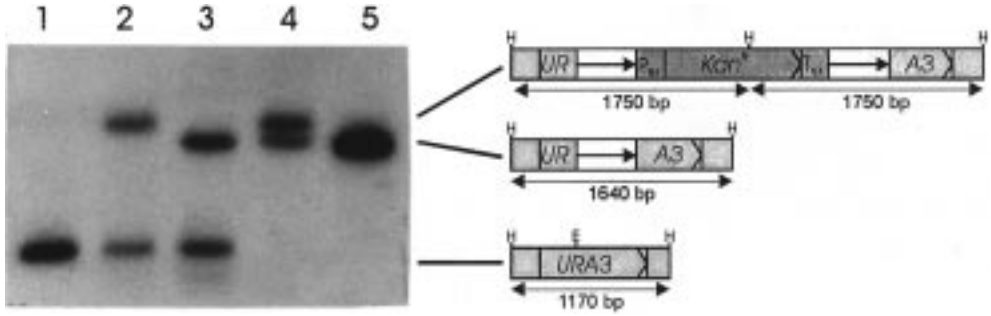

Figure 1. Southern analysis of *URA3* loci in several yeast strains. DNA from T_{73} (lane 1), T_{73}-1 (lane 2), T_{73}-2 (lane 3), T_{73}-3 (lane 4), and T_{73}-4 (lane 5) were digested with *Hin*dIII and electrophoresed in a 2% agarose gel. The gel was capillary transferred to nylon membrane and hybridized with a 1.1 kb *Hin*dIII *URA3* probe. An explanation of the three possible bands obtained is shown on the right: the wild-type locus produces a 1170 bp band (lanes 1−3), kan^R insertion produces two almost identical 1750 bp bands (lanes 2, 4), and after excision, the locus yields a 1640 bp band (lanes 3−5). *Hin*dIII (H) and *Eco*RV (E) restriction sites are indicated. *URA3* and kan^R genes are indicated as boxes and direct repeats as arrows.

10^{-3}, within the range described by Wach et al. (1994). One colony that did not grow on these plates was analyzed by Southern blotting (Figure 1) and shown to have excised the kan^R gene, leaving behind a 515 bp fragment. This strain (T_{73}-2) was used for a second round of transformation. In this case we used 1.5 μg of the excisable cassette and obtained 15 transformants. One transformant (T_{73}-3) was shown by Southern analysis to have the predicted insertion of the cassette into the remaining intact *URA3* locus (integration into the already disrupted locus is also possible and, in fact, 50% of the transformants isolated were of this type). The Southern analysis shows that no other copies of the wild type *URA3* locus are present, confirming that the original T_{73} strain is diploid for this locus (Figure 1). Finally, using the procedure of excision described above, we obtained some colonies that had lost their resistance to G418. Two were analyzed by Southern blotting and proved to have excised the second copy of the kan^R gene (Figure 1). One of them (T_{73}-4) lacks any copy of the *URA3* gene and is consequently unable to grow in synthetic media without uracil and can resist up to 1 mg/mL 5FOA, a substance toxic for $ura3^+$ cells (data not shown). This strain can be transformed to uracil prototrophy using either episomal plasmid YEp352, which contains the *URA3* marker, or the isolated gene itself (as a 1.1 kb *Hin*dIII fragment). As can be seen in Table 1, transformation frequencies were improved up to 4- or 9-fold, respectively, with regard to kanamycin selection. These results are similar to the frequencies obtained for most yeast strains and indicate that the recalcitrance to transformation shown by industrial yeasts mostly relates to the selection step. In laboratory strains selection for G418 resistance instead of a prototrophy results in a decrease of, at least, an order of magnitude in the number of transformants obtained (S.P. and S. Rodríguez, unpublished observations).

Microvinification Experiments. To show that the natural vinification ability of the T_{73} strain is conserved in recombinant strains, we carried out three independent microvinification experiments with T_{73} and the recombinant T_{73}-4/YEp352, which carries the *URA3* gene. The latter is necessary because *ura3* strains grow very slowly, and this can affect microvinification indirectly. The enological parameters of the wines obtained are shown in Table 2. Results from three experiments clearly show that no significant differences exist between T_{73} and its recombinant derivative T_{73}-4/YEp352. These two strains also fermented at similar rates (not shown).

Table 2. Enological Parameters of the Wines Obtained with T_{73} and T_{73}-4/YEp352

	T_{73}[a]	T_{73}-4/YEp352
reducing sugar (g/L)	2.1 ± 0.4	3.2 ± 0.6
ethanol (%)	11.4 ± 0.1	11.6 ± 0.1
pH	3.22 ± 0.10	3.12 ± 0.05
total acidity (g/L)	7.78 ± 0.10	6.95 ± 0.48
volatil acidity (g/L)	0.32 ± 0.09	0.21 ± 0.03
total SO_2 (mg/L)	31 ± 2	38 ± 7
free SO_2 (mg/L)	9 ± 2	5 ± 2
glycerol (g/L)	4.3 ± 0.1	4.4 ± 0.1

[a] Standard deviation of three independent experiments.

DISCUSSION

As the yeast *S. cerevisiae* is one of the model systems used for studying the molecular biology of eukaryotes, many genetic engineering techniques and protocols are available for it. However, natural wine yeasts have biological properties and commercial standards that make them more difficult to modify. The main problems concern their very low transformation efficiencies and the fact that the procedures available cannot guarantee the stability and marketable use of those strains. We have focused on the problem of genetic instability because of the block it imposes on the improvement of industrial yeast strains through current genetic engineering methods. Commercial yeasts are produced without screening through any selection system. Hence, transformation of industrial yeasts with autonomously replicating recombinant plasmids is not a suitable strategy because of their instability. However, the strategy of integrating exogenous DNA into specific genetic loci can in principle solve this problem. For commercial applications it is also preferable that any inessential DNA be removed. In the work reported, only a 470 bp repeat and a 45 bp polylinker tract remain after autoexcision of the kan^R cassette. There is no open reading frame longer than 200 bp inside this 515 bp fragment, thus making the consequent occurrence of an undesirable phenotype highly unlikely.

An essential requirement for any protocol aimed at genetic manipulation is a highly efficient transformation method. Methods for the genetic transformation of laboratory strains of *S. cerevisiae* were developed many years ago. They include the protoplast method (Hinnen et al., 1978), which is generally efficient but strain dependent and time-consuming (Johnston et al., 1981), and the lithium acetate method originally developed by Ito et al. (1983) and recently improved (Gietz and Woods, 1994; Gietz et al., 1995), which is much simpler

and has broader applications; other methods, such as electroporation (Simon, 1993) and biolistic transformation (Armaleo et al., 1990), have also been used. However, successful application of such methods to industrial strains of this genus has not been described.

We have investigated the transformation conditions for the T_{73} wine yeast and found that the efficiency obtained previously (Pérez-González et al., 1993) can be more than a >1000-fold. Integrative transformation, which is hundreds of times less efficient than transformation with autonomous plasmids, is now feasible. We have succeeded in obtaining many integrative transformants, not only for this particular strain but also for many other wine and food yeast strains (Table 1). We have chosen the *URA3* locus as the integration site because it is a very well characterized gene that is present as an auxotrophic marker in many useful yeast plasmid vectors. Moreover, it encodes an essential enzyme in de novo nucleotide biosynthesis and hence can be expected to be present in most (if not all) yeast strains. Our results show that the *URA3* locus in all strains tested is similar to the laboratory S288c strain, given that extensive sequence homology is needed for integrative recombination (Rothstein, 1991).

Industrial *ura3* strains may be useful for many purposes, for example, for routine laboratory assays in which the auxotrophic strain may be transformed with specific members of the large variety of recombinant replicative plasmids available in order to carry out basic preliminary studies. As *URA3* is itself a yeast gene, it can be used as an integration marker in the same cassette with the exogenous DNA of interest and not require subsequent elimination. Due to the slow growth of *ura3* strains in musts and other "complete" media (S.P., unpublished observations), this provides a fortuitous system for autoselection of the recombinant yeast.

The presence of a heterozygotic locus leads, through gene conversion events, to heterogeneity of the yeast population due to the absence of a selective pressure either for or against a particular phenotype. As both copies of the modified *URA3* locus are identical in strain T_{73}-4, no gene conversion is possible so the genetic stability is very high. This is very important for a naturally diploid homothallic yeast strain. We have preliminary evidence (Puig et al., unpublished results) that the heterozygotic strain, T_{73}-1, can reorganize (with a frequency of 10^{-5}) its *URA3* locus under various culture conditions, producing both types of homozygotes: *URA3*/*URA3* and *ura3*Δ/*ura3*Δ. This potential for reorganization could be a serious drawback and would imply that an ideal recombinant industrial yeast strain should be homozygotic for the desired recombinant loci to be sure that it is genetically stable, as we have done in the case of T_{73}-4. Alternatively, this phenomenon could be used to eliminate the second copy of the *URA3* gene by selecting for the *ura3*Δ/ura3Δ mutant, which appears spontaneously in 5FOA medium. Alternative methods, such as sporulation, which will also produce *ura3*Δ/*ura3*Δ strains after mating type switching and conjugation of the **a** and α cells, are less suitable because they would produce a homozygotic strain different from the parent.

The method used here for complete disruption of the *URA3* locus may also be used for the integration of exogenous genes in the yeast genome. We have constructed recombinant yeast strains containing various enologically interesting enzymes which show similarly stable phenotypes to that shown by T_{73}-4 (S.P., D.R., and J.E.P.-O., unpublished results). The protocol we have described constitutes a powerful tool with which to manipulate industrial yeasts, either to replace natural alleles or to insert new genes in such a way that the subsequent use of the recombinant strains for food production may be feasible without any potential risk of strain impairment or consumer rejection.

ACKNOWLEDGMENT

We thank Dr. P. Philippsen for the generous gift of the pFA series plasmids. We thank Dr. J. E. O'Connor for help with flow cytometry and A. Cortell (Vinival S.A.) for doing enological analyses. We also thank Drs. José A. Pérez-González and A. Querol for helpful discussions and Dr. A. MacCabe for critical reading of the manuscript.

LITERATURE CITED

Armaleo, D.; Ye, W.; Klein, T. M.; Shark, K. B.; Sanford, J. C.; Johnson, S. A. Biolistic nuclear transformation of *Saccharomyces cerevisiae* and other fungi. *Curr. Genet.* **1990**, *17*, 97−103.

Carlson, M.; Botstein, D. Two differentially regulated mRNAs with different 5′ ends encode secreted and intracellular forms of yeast invertase. *Cell* **1982**, *28*, 145−154.

Gietz, R. D.; Woods, R. A. High efficiency transformation with lithium acetate. In *Molecular Genetics of Yeast*; Johnson, J. R., Ed.; IRL Press: Oxford, U.K., 1994; pp 121−134.

Gietz, R. D.; Schiestl, R. H.; Willems, A. R.; Woods, R. A. Studies on the transformation of intact yeast cells by the LiAc/ss-DNA/PEG procedure. *Yeast* **1995**, *11*, 355−360.

Goffeau, A., et al. The yeast genome directory. *Nature* **1997**, *387 (Suppl.)*, 1−105.

Hill, J. E.; Myers, A. M.; Koerner, T. J.; Tzagoloff, A. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* **1986**, *2*, 163−167.

Hinnen, A.; Hicks, J. B.; Fink G. R. Transformation of yeast. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 1929−1933.

Ito, H.; Fukuda, Y.; Murata, K.; Kimura, A. Transformation of intact cells treated with alkali ions. *J. Bacteriol.* **1983**, *153*, 163−168.

Johnston, J.; Hilger, F.; Mortimer, R. Variation in frequency of transformation by plasmid YRp7 in *Saccharomyces cerevisiae*. *Gene* **1981**, *16*, 325−329.

Linko, Y.-Y.; Javanainen, P.; Linko, S. Biotechnology of bread baking. *Trends Food Sci. Technol.* **1997**, *8*, 339−344.

Pérez-González, J. A.; González, R.; Querol, A.; Sendra, J.; Ramón, D. Construction of a recombinant wine yeast strain expressing β-(1,4)-endoglucanase and its use in microvinification processes. *Appl. Environ. Microbiol.* **1993**, *59*, 2801−2806.

Querol, A.; Ramón, D. The application of molecular techniques in wine microbiology. *Trends Food Sci. Technol.* **1996**, *7*, 73−78.

Querol, A.; Jiménez, M.; Huerta, T. Microbiological and enological parameters during fermentation of musts from poor and normal grape-harvest in the region of Alicante (Spain). *J. Food Sci.* **1990**, *55*, 114−122.

Querol, A.; Barrio, E.; Huerta, T.; Ramón, D. Strain for use as dry yeast in fermentation of Alicante wines: Selection and DNA patterns. *J. Food Sci.* **1992a**, *57*, 183−185.

Querol, A.; Barrio, E.; Huerta, T.; Ramón, D. Molecular monitoring of wine fermentations conducted by active dry yeast strains. *Appl. Environ. Microbiol.* **1992b**, *58*, 2848−2953.

Rothstein, R. Targeting, disruption, replacement, and allele

rescue: Integrative DNA transformation in yeast. *Methods Enzymol.* **1991**, *194*, 281−301.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

Simon, J. R. Transformation of intact yeast cells by electroporation. *Methods Enzymol.* **1993**, *217*, 478−483.

Snow, R. Genetic improvement of wine yeasts. In *Yeast Genetics Fundamental and Applied Aspects*; Spencer, J. F. T., Spencer, D. M., Smith, A. R. W., Eds.; Springer-Verlag: Berlin, 1983; p 439.

Wach, A.; Brachat, A.; Pöhlmann, R.; Philippsen, P. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae. Yeast* **1994**, *10*, 1793−1808.

Yanisch-Perron, C.; Vieira, J.; Messing, J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **1985**, *33*, 103−110.

Received for review July 28, 1997. Revised manuscript received January 30, 1998. Accepted February 3, 1998. This work was supported by projects ALI93-0809 (to D.R.) and ALI95-0566 (to J.E.P.-O.), both from Comisión Interministerial de Ciencia y Tecnología of the Spanish government, and project GV-3124/95 (to J.E.P.-O.) from Generalitat Valenciana. S.P. is a recipient of a Spanish F.P.I. fellowship.

JF9706538