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Comparison of Two Alternative Dominant Selectable Markers for Wine Yeast Transformation

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Genetic improvement of industrial yeast strains is restricted by the availability of selectable transformation markers. Antibiotic resistance markers have to be avoided for public health reasons, while auxotrophy markers are generally not useful for wine yeast strain transformation because most industrial *Saccharomyces cerevisiae* strains are prototrophic. For this work, we performed a comparative study of the usefulness of two alternative dominant selectable markers in both episomic and centromeric plasmids. Even though the selection for sulfite resistance conferred by *FZF1-4* resulted in a larger number of transformants for a laboratory strain, the *p*-fluoro-DL-phenylalanine resistance conferred by *ARO4-OFP* resulted in a more suitable selection marker for all industrial strains tested. Both episomic and centromeric constructions carrying this marker resulted in transformation frequencies close to or above 10^3 transformants per μg of DNA for the three wine yeast strains tested.

During the past century, the selection of microbial strains has been one of the main sources of improvement for traditional fermentation processes, including the production of fermented foods such as wine or dairy products. For enology, the natural variability of *Saccharomyces cerevisiae* strains has been exploited by means of the isolation of wild yeast strains from grapes, musts, or successful fermentations; characterization of the isolates; and selection of the most suitable strains for each particular wine-making process or production area. Classical genetic methods such as the isolation of spontaneous or induced mutants or sexual crosses have been seldom used for the improvement of *S. cerevisiae* strains used for wine making (2, 19, 23, 26, 37), probably because of the difficulties in the design of selection procedures and because of the fact that, very often, the progeny of an excellent wine-making strain performs poorer than the parent strain (18). During the last 15 years, several attempts to construct genetically engineered yeast strains have been published, and very interesting improvements in the wine-making process or the quality of the wine obtained have been reported, including improved primary and secondary flavors, malic acid decarboxylation by yeast, increased resveratrol, lactic acid, or glycerol contents, and improved survival properties under technological conditions (6, 11, 16, 17, 20, 27, 32, 34, 38, 43, 46, 47). However, because wild yeast strains are usually prototrophic, most of the strains obtained for these works contained antibiotic resistance genes which were used for selection of the recombinant strains and would be unacceptable for marketing. Puig et al. (35) published an interesting method for obtaining recombinant wine yeast strains that are free of antibiotic selection markers. In this method, a *ura3*Δ strain is first constructed by sequential gene replacement of the wild-type *URA3* gene, and the kanamycin resistance cassette used to this end is removed by homologous recombination. The resulting auxotrophic strain can then be transformed by using the *URA3* marker, and the recombinant strains obtained are free of any sequence coding for antibiotic resistance. This strategy, which is appropriate for working with a single or very limited number of yeast strains, would be difficult to use as a general strategy to obtain recombinant strains from several wild isolates that would typically be diploid or aneuploid and heterozygous (5, 7). For this reason, we have turned our sight back to dominant selectable markers other than antibiotic resistance. Of the few sequences that have been described as potentially useful as dominant selectable markers for *S. cerevisiae*, we chose *ARO4-OFP* (41) and *FZF1-4* (31) for the construction of centromeric and episomic transformation vectors and a comparison of their relative efficiencies in transformation experiments with several industrial strains and a laboratory yeast strain. We chose these two markers because they can confer additional interesting phenotypes a priori.

The *ARO4-OFP* allele was first identified in a screen for mutants with a resistance phenotype against OFP (*o*-fluoro-DL-phenylalanine), a phenylalaline structural analogue, and against *p*-fluoro-DL-phenylalanine (PFP) plus L-tyrosine (14). Interest in these mutants came from a previous observation that industrial OFP-resistant mutants overproduce β-phenylethyl alcohol, an interesting aromatic compound found in fermented beverages (15). *ARO4-OFP* codes for a DAHP (3-deoxy-D-*arabino*-heptolusonate-7-phosphate) synthase that is insensitive to feedback inhibition by tyrosine (13, 29, 44). The release of feedback inhibition in the *ARO4-OFP* gene product results from a C-to-A transversion at base 496 of the open reading frame, which results in a Q166K amino acid substitution (12).

Sulfite is currently used as an antioxidant and as a powerful antimicrobial agent in the manufacture of a wide variety of food products, including wine (45). *FZF1* codes for a zinc finger transcription factor (8) that activates the expression of

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SSU1 (4), which in turn codes for a plasma membrane protein that is required for sulfite efflux, which plays a central role in sulfite tolerance (3, 30). The isolation of sulfite-resistant mutants (9, 48) has permitted the cloning of *FZF1* dominant alleles that are responsible for this phenotype (10, 31), such as *FZF1-4*, which promotes increased transcription rates of *SSU1* (30). *FZF1-4* has an A-to-G transition at position 170 of the open reading frame, resulting in a C57Y substitution in the protein (31).

MATERIALS AND METHODS

Strains. The wine yeast strains used for this work were as follows: EC1118 (Lallemand Inc., Montreal, Canada), a commercial *S. cerevisiae* second-fermentation strain that was isolated from the Champagne region (France); IFI473, an *S. cerevisiae* strain from the collection of the Instituto de Fermentaciones Industriales that was isolated in Sant Sadurni d'Anoia (Spain) and previously shown to be suitable as a starter for second fermentation (28); and T_{73-4} (35), a *ura3*Δ derivative of *S. cerevisiae* T_{73} (Lallemand), which in turn is a commercial strain that was isolated in Alicante (Spain). Other industrial strains used for this work were IFI228, an *S. cerevisiae* baker's yeast strain from the collection of the Instituto de Fermentaciones Industriales, and IFI234, IFI237, and IFI239, all of which are brewer's yeast strains from the same collection. The laboratory strain *S. cerevisiae* BY4741 (*MAT***a** *his3 leu2 met15 ura3*) was also used for transformation experiments. *Escherichia coli* strain DH5α (*supE44* Δ*lacU169* [ϕ*80 lacZ*Δ*M15*] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) (22) was used for the construction and production of the plasmids used in this study.

DNA manipulations and plasmid construction. The *ARO4* and *FZF1* genes, including promoter and terminator sequences, were amplified from yeast genomic DNA (BY4741) with the primer pairs ARO4U (5′-TGAATCACGTG ATCAACAGC-3′)-ARO4D (5′-CATGATCACCTAATTAGCCGT-3′) (14) and FZF1U (5′-CGGTGCTGGTATCATGGCTTTGATC-3′)-FZF1D (5′-ACT CTTGCCTTTTCTTTGTTTC-3′) (31), respectively. *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) was used for PCR amplifications, and the amplification products were cloned into pGEM-T (Promega Corporation, Southampton, United Kingdom) according to the instructions of the respective suppliers. In both cases, the insert was released by digestion with NcoI and NotI, the extremes were filled in by treatment with Klenow, and then the genes were cloned into a pRS316 (42) or YEp352 (24) vector that had previously been digested with SmaI. The resulting plasmids were named pCA1, for *ARO4* cloned in pRS316; pCF1, for *FZF1* cloned in pRS316; pEA1, for *ARO4* cloned in YEp352; and pEF1, for *FZF1* cloned in YEp352. *ARO4-OFP*- and *FZF1-4*-containing centromeric or episomic plasmids were constructed by site-directed mutagenesis of pCA1, pCF1, pEA1, and pEF1 by use of a QuickChange in vitro mutagenesis kit (Stratagene). *ARO4* mutagenesis was performed with the primer 5′-GCTTGATACCATTTCTCCTAAATACTTGGCTGATTTGG-3′ and its complementary primer 5′-CCAAATCAGCCAAGTATTTAGGAGAAATGGT ATCAAGC-3′, and *FZF1* mutagenesis was performed with the primer 5′-GCA AAAAATTTATAAGACCGTACCATCTACGAGTTCACAAATGG-3′ and its complementary primer 5′-CCATTTGTGAACTCGTAGATGGTACGGTCT TATAAATTTTTTGC-3′. The resulting plasmids were named pCA2 (*ARO4-OFP* in pRS316), pCF2 (*FZF1-4* in pRS316), pEA2 (*ARO4-OFP* in YEp352), and pEF2 (*FZF1-4* in YEp352). Maps of these four plasmids are shown in Fig. 1. The plasmids were purified from *E. coli* cells by use of a High Pure plasmid isolation kit (Roche Diagnostics SL, Barcelona, Spain) and from yeast cells by the method described by Robzyk and Kassir (39). Yeast genomic DNAs were extracted by the method of Querol et al. (36). DNA concentrations were calculated by measuring the optical densities at 260 nm. DNA fragments resolved in agarose gels were purified by use of a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). *E. coli* was transformed by electroporation (40). All other nucleic acid manipulations were performed as described by Sambrook et al. (40).

Transformation experiments. Yeast cells were grown overnight in YPAD medium (1% yeast extract, 2% peptone, 2% dextrose, and 0.01% adenine hemisulfate) at 30°C and 200 rpm. The total number of cells in each transformation tube was adjusted to 10^8 by a direct microscopic count, and transformation was performed according to the lithium acetate method described by Ito et al. (25), as modified by Agatep et al. (1). Industrial yeast strains were transformed with 20 μg of DNA, while laboratory strains were transformed with 2 μg of DNA. Before the selection of transformants, cells were diluted 10 times in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) and incubated for 17 h at 30°C and 200 rpm to allow the expression of resistance alleles (31). The cells that were transformed with pCF2 or pEF2 plasmids were selected on YPD + TA plates (2× YPD containing 150 mM L-tartaric acid for buffering at pH 3.5 and 2% agar and were separately autoclaved in equal amounts, mixed and sulfite was added, in the necessary quantity for each strain, from a sterile 100× solution). The cells that were transformed with pCA2 or pEA2 plasmids were selected on SD + Tyr plates (0.67% yeast nitrogen base without amino acids [Difco Laboratories Inc., Detroit, Mich.], 2% dextrose, 1.67% agar for minimal medium, 0.9 g of L-tyrosine and PFP/liter, in an estimated quantity for each strain, and the required supplements, the last two of which were added from sterile 100× solutions). All plates were incubated at 30°C, and colonies were counted after 2 to 5 days. Additionally, for comparisons of the transformation frequencies obtained by using different selection criteria, the transformants of BY4741 and T_{73-4} strains were selected after 3 days of incubation at 30°C on SD plates without uridine (0.67% yeast nitrogen base [Difco Laboratories], 2% dextrose, 1.67% agar for minimal medium, and the required supplements). True and false-positive transformants were distinguished by enzyme restriction analyses of plasmid DNAs extracted from a limited number of putative transformants. The actual percentage of false-positive results was deduced from the transformation frequencies discussed below.

Determination of MICs. The MICs of sulfite and PFP were calculated for each strain in the selection media described above. First, the cultures were adjusted to 10^7 cells/ml, and 2-μl aliquots of these suspensions or serial dilutions of them (1/10, 1/100, and 1/1,000) were spotted onto plates of the cognate selection media with various amounts of sulfite (0, 1, 2, 3, 4, 5, 6, or 7 mM) or PFP (0, 1, 2, 3, 4, or 5 g/liter) and then incubated for 3 to 5 days at 30°C. In a second round of culture, plates containing amounts of inhibitor differing by 0.1 mM (sulfite) or 0.1 g/liter (PFP) from the previous value were used. This test was repeated twice, and identical results were obtained. Finally, these results were confirmed by plating 4×10^7 cells from a YPD overnight culture on selection medium containing the previously calculated MIC, as well as slightly higher and lower concentrations of sulfite (±0.2 mM) or PFP (±0.2 g/liter), to test for the spontaneous appearance of resistant colonies.

RESULTS

MICs. For each of the wine yeast strains, as well as strain BY4741, the MIC of sulfite or PFP was determined, as described in Materials and Methods, in order to decide the amount of each inhibitor to be used for transformation experiments. Sulfite resistance was clearly higher for wine yeast strains than for the laboratory strain, while resistance to PFP was similar for all of the strains, with the exception of T_{73-4}, which had a resistance about twice that of the other strains (Table 1).

The high MIC of sulfite in the case of wine yeast strains seems to be related to their niche of origin, because baker's (IFI228) and brewer's (IFI234, IF237, and IFI239) yeast strains showed resistance levels that were similar to that of the laboratory strain (0.8 to 1.5 mM) (data not shown).

Transformation of industrial and laboratory yeast strains. In order to obtain comparative results, we cloned both selective markers into the same vector backgrounds, i.e., YEp352 as a multicopy plasmid (24) and pRS316 as a centromeric plasmid (42). Three wine yeast strains and one laboratory strain were transformed, as described in Materials and Methods, with pCA2, pCF2, pEA2, or pEF2. Several transformation experiments were performed for each combination of strain and transforming plasmid. Control experiments included pRS316, YEp352, and no-DNA transformations. The amount of PFP or sulfite used for each strain was the MIC shown in Table 1. For comparison, *ura3*Δ strains (BY4741 and T_{73-4}) were selected in parallel for the *URA3* marker that was present in all of the constructions. The results of these transformation experiments are summarized in Table 2.

As expected, the highest transformation frequencies were

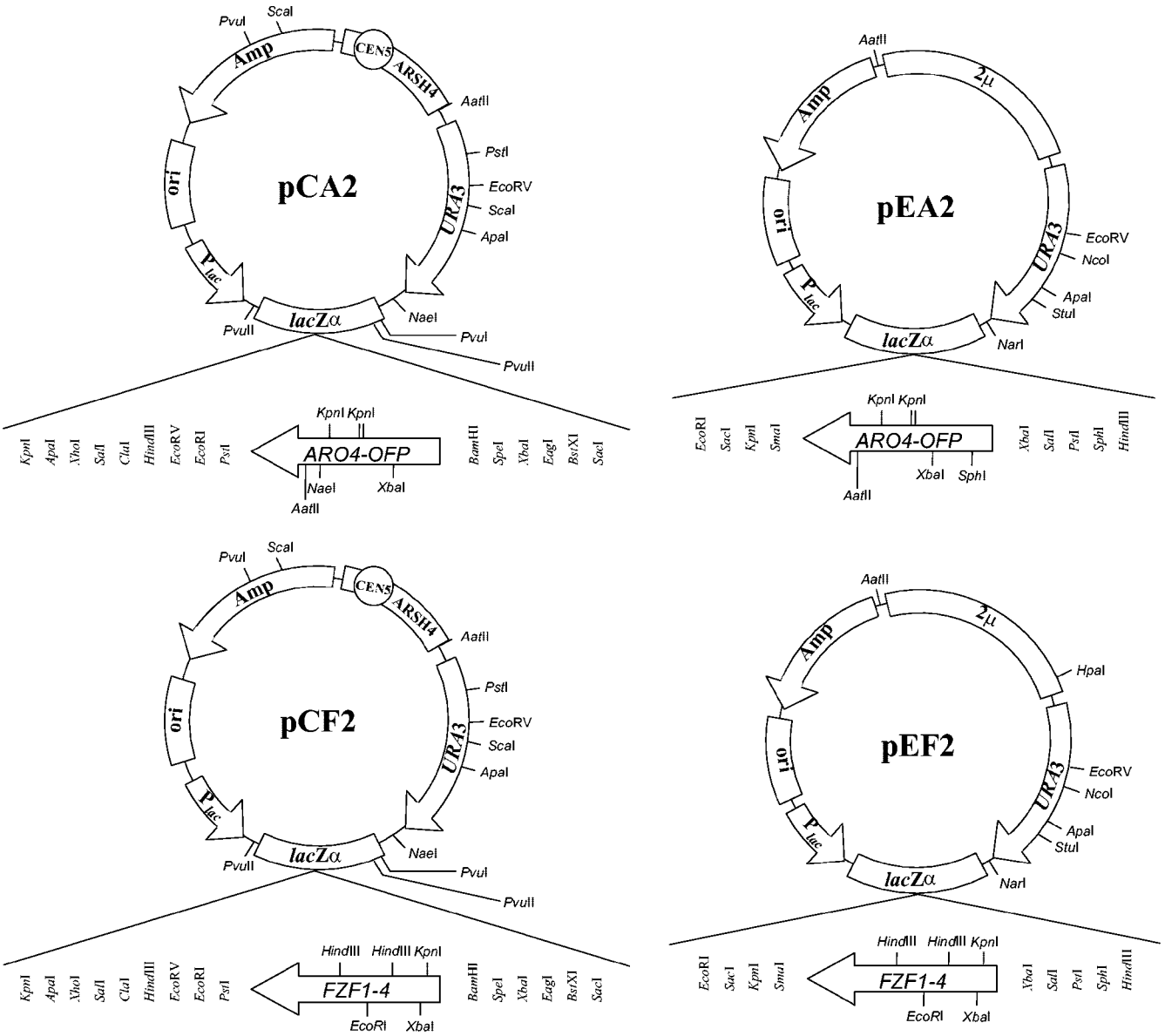

FIG. 1. Restriction maps of centromeric and episomic plasmids carrying the resistance genes tested in the yeast transformation experiments. *E. coli*-derived sequences: ori, replication origin of ColE1; *amp*, beta-lactamase-encoding gene; *lacZ*α, α fragment of the *lacZ* gene; P_{lac}, promoter of *lacZ*. *S. cerevisiae*-derived sequences: 2μ, replication origin of the natural 2μm plasmid; ARSH4, autonomously replicating sequence; CEN5, centromeric sequence; *URA3*, *URA3* gene; *ARO4-OFP*, mutant allele of *ARO4* conferring resistance to PFP; *FZF1-4*, mutant allele of *FZF1* conferring sulfite resistance.

obtained with the laboratory strain BY4741 and the *URA3* marker. Transformation frequencies were also high for BY4741 when *ARO4-OFP* or *FZF1-4* was used as a selection marker, with no clear trend towards the episomic or centromeric constructions (frequencies above 10^3 were attained for all four constructions tested [Table 2]).

TABLE 1. MICs of PFP and sulfite for *S. cerevisiae* strains[a]

Reagent	MIC (g/liter [PFP] or mM [sulfite]) of reagent for strain			
	BY4741	EC1118	T_{73-4}	IFI 473
PFP	1.3	2	3.7	2
$Na_2S_2O_5$	1.5	5	5	4.5

[a] The results were identical for two independent repetitions.

Among wine yeast strains, the highest transformation frequencies were obtained with strain T_{73-4}, with very similar values for vectors carrying the *URA3* or *ARO4-OFP* marker. Transformation of the prototrophic industrial strains EC1118 and IFI473 with the *URA3* marker could not be tested, but the frequencies were high for pCA2 and pEA2 transformation.

The relative transformation frequencies for the two dominant selection markers for T_{73-4} were opposite those for the laboratory strain BY4741. IFI473 could not be transformed to sulfite resistance with either pCF2 or pEF2, while EC1118 could only be transformed with the episomic construction pEF2.

TABLE 2. Transformation frequencies obtained for several constructions and yeast strains[a]

Plasmid	Transformation frequency (no. of transformants/μg of DNA)											
	BY4741			EC1118			T_{73-4}			IFI 473		
	Mean	Max	n^b	Mean	Max	n^b	Mean	Max	n^b	Mean	Max	n^b
pCA2	1.6×10^4	7×10^4	6	390	10^3	5	25×10^3	5×10^3	4	100	375	4
pEA2	300	10^3	6	750	2×10^3	5	5×10^3	10^4	4	400	10^3	4
pCF2	1.3×10^5	2×10^5	10	0	0	8	2.5	17	8	0	0	10
pEF2	6×10^4	9×10^4	10	1.3	5	8	6	47	8	0	0	10
pRS316[c]	5×10^5	2×10^6	16	Not tested			5×10^3	10^4	4	Not tested		
YEp352[d]	3×10^5	10^6	16	Not tested			4×10^3	10^4	4	Not tested		

[a] The industrial strains EC1118 and IFI473 are prototrophic, and therefore transformation with the *URA3* marker was not tested.
[b] *n*, number of transformation experiments performed.
[c] Results from transformation experiments using the *URA3* marker for pRS316 and its derivatives pCA2 and pCF2.
[d] Results from transformation experiments using the *URA3* marker for YEp352 and its derivatives pEA2 and pEF2.

Occasionally, some colonies appeared in the control plates (pRS316, YEp352, or no-DNA transformations) in PFP resistance experiments. Because their numbers were sometimes equivalent to about 1/10 the number of transformants in the pCA2 or pEA2 plates, our transformation frequencies may have been overestimated because of the presence of false-positive transformants. We took advantage of the double markers of our constructs (*ARO4-OFP* and *URA3*) and the fact that two of the yeast strains were auxotrophic for uridine (BY4741 and T_{73-4}) to easily test this possibility. PFP-resistant strains from each transformation experiment were replica plated on SD without uridine and YPD. The results were unequivocal, as 159 of the 160 tested colonies from pCA2 or pEA2 transformations were prototrophic for uridine, indicating that they had actually incorporated the transforming plasmid, while the strains from pRS316, YEp352, or no-DNA transformations were uridine auxotrophs. For the first transformation experiments with strains EC1118 and IFI473, a limited number of colonies was also analyzed in order to verify "true" transformation. This was done by plasmid DNA extraction and restriction analysis of the plasmids obtained. As expected, 14 of the 14 strains analyzed from pCA2 or pEA2 transformations contained the plasmid used for transformation.

Sulfite resistance was a more uncertain selection marker for yeast transformation. Several transformation experiments had to be discarded because of an unacceptable background level resulting in a lawn of colonies for the negative controls. In spite of this random factor, we were able to perform several successful transformation experiments and to test the authenticity of the transformants (as described above for PFP-resistant strains). Two hundred nineteen of the 243 tested colonies from pCF2 or pEF2 transformations were confirmed to be true transformants, either by uridine prototrophy (BY4741 and T_{73-4} derivatives) or because they contained a plasmid with the expected restriction map. However, the rate of false transformants in some particular experiments with the *FZF1-4* marker was as high as 80%. Only confirmed transformants were taken into account to compute the transformation frequencies shown in Table 2. Transformation of a non-wine industrial yeast strain, brewer's yeast strain IFI234, which had a sulfite tolerance level equal to that of the laboratory strain, was unsuccessful.

DISCUSSION

There are two main limitations to the development of genetic transformation systems for industrial yeast strains. First, there are almost no naturally auxotrophic strains, and the isolation of spontaneous or induced auxotrophic mutants is hindered by the ploidy of the strains (5). Second, the use of antibiotic resistance markers may limit the commercial application of strains due to restrictions on the release of genetically modified organisms carrying antibiotic resistance genes. Some years ago, Puig et al. (35) developed a method to overcome these limitations based on the genetic engineering of a target strain in order to make it auxotrophic for uridine and the subsequent use of the *URA3* marker in order to introduce traits of interest. Ingenious as it is, we found this strategy too laborious for routine work with several wine yeast strains. In a quest for non-antibiotic resistance dominant selectable markers, we decided to compare the relative merits of *ARO4-OFP* and *FZF1-4* as transformation markers for wine yeast strains.

The range of transformation frequencies obtained for each combination of strain and plasmid was quite large (Table 2). This may have been due partly to the long incubation period before plating, allowing for variable numbers of generations. However, this would not account for all of the variability found, and other parameters affecting transformation should be blamed. In spite of this variability, it is still possible to extract some conclusions from the transformation frequencies observed, thanks in part to the large number of experiments performed (150 experiments) (Table 2). Our results clearly favor the use of *ARO4-OFP* over *FZF1-4* as a marker for the transformation of wine yeast strains, for two reasons. First, in the case of wine yeast strains, the transformation frequencies obtained were clearly higher for the *ARO4-OFP* marker than for *FZF1-4*. Indeed, IFI473 could not be transformed to sulfite resistance at all, while EC1118 could only be transformed with the episomic construction. This lack of success with the centromeric constructions makes us pessimistic about the performance of this marker for integrative transformation, a requisite for obtaining stable recombinant yeast strains for industrial applications. Second, the problems derived from the random appearance of high background levels in *FZF1-4* transformations were never encountered with PFP resistance transformations. The spontaneously resistant colonies that appeared in

the negative controls were not found in real transformation experiments.

These problems with sulfite transformation were probably due to a loss of effectiveness of the sulfite added to the selection medium, but changes in the method of addition (melted medium or plate surface) and standardization of the temperature of addition did not solve the problem (data not shown). Goto-Yamamoto et al. (21) identified the allele *SSU1-R*, which confers increased sulfite resistance to some wine yeast strains. The *SSU1-R* allele is the consequence of a translocation between chromosomes VIII and XVI involving the *SSU1* and *ECM34* promoter regions. Several variants of this translocation are found in wine yeast strains from different geographical origins (33), suggesting the unaware selection of this event through the generalized use of sulfite in wine making. One consequence of this translocation event is the presence of a variable number of copies (depending on the particular strain) of 76-bp repeats that contain putative binding sites for the transcriptional activator Fzf1p. The larger number of Fzf1p binding sites in the promoter of the *SSU1-R* allele than in the wild-type allele would explain the higher *SSU1* transcription rates and the higher resistance level observed for strains carrying the translocation (21). At first sight, the poor results that we obtained for the transformation of wine yeast strains to sulfite resistance seemed to be related to the high tolerance levels already shown by the host strains. This may be because Ssu1p levels are no longer limiting for sulfite tolerance in these strains, because further increases in the mRNA levels have no effect on Ssu1p levels, or finally, because the product of *FZF1-4* is not more effective than Fzf1p as a transcriptional activator from the recombinant promoter. However, the lack of success in the transformation of IFI234 does not support this view.

In conclusion, we propose the use of *ARO4-OFP* as a marker for the transformation of wine yeast strains. This marker did not have the problem of false-positive transformants and allowed us to obtain yeast transformants with both multicopy and centromeric plasmids. With pCA2 or pEA2 used as a scaffold, new constructions that place together the sequences of interest and *ARO4-OFP* can be made. These constructions would be directly utilizable for the overexpression of a gene of interest or to create gene disruptions. However, in order to make recombinant wine yeast strains that are devoid of bacterial antibiotic resistance genes, it would be more appropriate to use linear DNA fragments derived from these constructions for yeast transformation. In this way, safer recombinant wine yeasts can be constructed, allowing for improvements in wine quality and technological properties (flavor, taste, astringency, body, color, filterability, etc.). In the particular case of second-fermentation strains used for the manufacture of sparkling wines, we are interested in accelerating the aging process. To do that, we intend to use *ARO4-OFP* as a transformation marker to construct wine yeast strains overexpressing genes involved in autophagy.

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