

Construction of a Recombinant Wine Yeast Strain Expressing β -(1,4)-Endoglucanase and Its Use in Microvinification Processes

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A genetic transformation system for an industrial wine yeast strain is presented here. The system is based on the acquisition of cycloheximide resistance and is a direct adaptation of a previously published procedure for brewing yeasts (L. Del Pozo, D. Abarca, M. G. Claros, and A. Jiménez, *Curr. Genet.* 19:353-358, 1991). Transformants arose at an optimal frequency of 0.5 transformant per μg of DNA, are stable in the absence of selective pressure, and produce wine in the same way as the untransformed industrial strain. By using this transformation protocol, a filamentous fungal β -(1,4)-endoglucanase gene has been expressed in an industrial wine yeast under the control of the yeast actin gene promoter. Endoglucanolytic wine yeast secretes the fungal enzyme to the must, producing a wine with an increased fruity aroma.

The production of wine is a complex microbiological reaction involving the sequential development of various yeast strains and lactic acid bacteria. Traditionally, wines have been produced by natural fermentation caused by the development of yeasts originating from the grapes and winery. Results clearly show great variations in the quantity and distribution of the relevant yeast species which can be mainly related to differences in ecosystems (soil composition, climatological conditions, and variety of grape, etc.). These dissimilarities occur not only among different regions but also within the same region in different vintages (22). For this reason, during the last few years many wine makers have used pure yeast cultures isolated from their own wine region (25, 28). Such cultures, in the form of active dry yeasts, are supplied to the wineries, where they are inoculated into the fresh must in order to perform controlled fermentations. As a result of this practice, the final product has in successive vintages the typical sensory properties of the wine produced in that region (15).

The use of selected strains in the wine-making process requires the development of techniques that can clearly differentiate the inoculated yeast strain from the rest of the wild yeast strains present in the must. This would provide a convenient test in order to ensure that the fermentation process is really conducted by the inoculated yeast. As most of the strains belong to the same species, *Saccharomyces cerevisiae*, they cannot be identified by classical microbiological methods. To solve this problem, several techniques based on molecular polymorphisms have been used recently for wine yeast strain characterization (for a comparative study, see reference 24). Among all of them, mitochondrial DNA restriction endonuclease profiling has been revealed as the most efficient. Using a simplified mitochondrial DNA purification protocol, a study of the population dynamics of natural and inoculated wine fermentations has been carried

out (23). In the case of wine fermentations conducted by active dry yeast, the inoculated strain is mainly responsible for the fermentation but does not suppress significant development of natural strains during the early stages, thus permitting the influence during this period of naturally occurring strains which could have important effects on wine flavor.

From a biotechnological point of view, the use of active dry yeasts dramatically changes the microbiology of the wine process. Natural fermentation is a complex situation in which many different *S. cerevisiae* strains undergo sequential substitutions during fermentation. In the inoculated fermentation, however, a clear imposition and predominance of the active dry yeast is detected. This microbiological simplification opens the way to genetically modify the active dry yeast, constructing strains which express metabolic activities which affect positively the organoleptical characteristics of the wine produced. In contrast with this evident applied interest, only one preliminary report about the integration of the yeast K1 killer toxin gene into the genome of an industrial strain has been published (4).

One of the most important characteristics of a quality wine is its aromatic fragrance. The role of monoterpenes in muscat grapes and wine flavors is now well established (1). These compounds are present in the must partly as free volatile forms and partly as glycosidically bound nonvolatile precursors (8, 30). This last fraction is a potential source of flavors which generally remain odorless in the traditional wine-making process (9). Enzymatic hydrolysis of these grape glycosides has been suggested by some authors as a strategy to enhance wine flavor (9), and different cellulases and hemicellulases (10-12, 17, 27) have been added as exogenous enzymes with some positive results in microvinification experiments.

In this paper we describe a system for the genetic transformation of an industrial wine dry yeast. This system is based on the acquisition of resistance to the antibiotic cycloheximide which has been used previously by other authors in the transformation of brewing-yeast strains (5,

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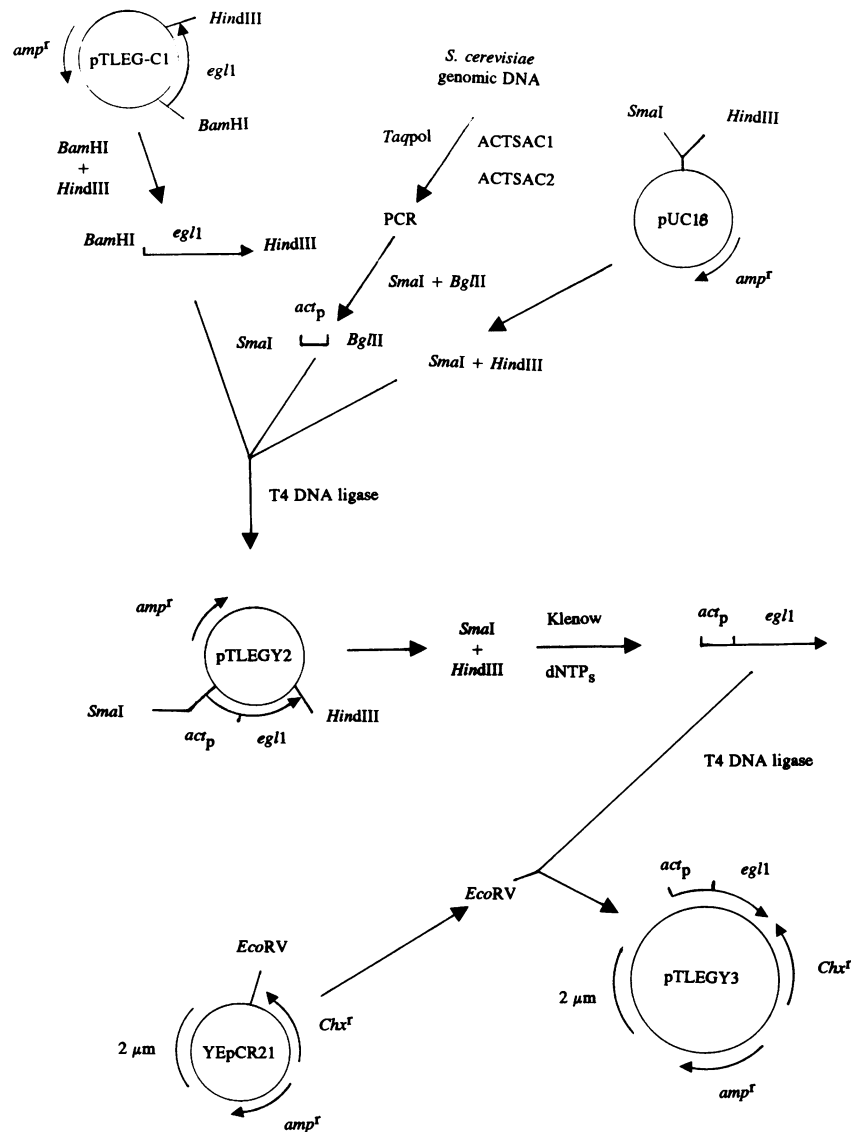


FIG. 1. Strategy used for the construction of the pTLEGY3 plasmid. Details are presented in Materials and Methods. dNTPs, deoxynucleoside triphosphates.

18). The transformed strain produces wine in the same way as the original strain. By this method, the *Trichoderma longibrachiatum egl1* gene (7), which encodes a β -(1,4)-endoglucanase, has been expressed in the industrial strain. In microvinification processes, the recombinant wine yeast strain secretes the fungal enzyme to the must, and it is possible to detect an increase in the flavor of the wine.

MATERIALS AND METHODS

Strains, plasmids, and media. The *S. cerevisiae* industrial wine yeast strain T₇₃ (CECT1894) is commercialized by Lallemand Inc. (Montreal, Quebec, Canada), and its selection (25) and molecular characterization (24) have been previously published. For cultures, the yeast strain was grown on YPD (1% yeast extract, 1.5% peptone, 2% glucose). All of the recombinant DNA experiments were per-

formed with *Escherichia coli* DH5 α (26) which was grown in LB medium (26).

The plasmid YEpcR21 was used as the transforming DNA (18). The construction of the plasmid pTLEGY3 appears in Fig. 1 and is summarized as follows. The *Bam*HI-*Hind*III fragment which contains the *T. longibrachiatum egl1* cDNA was recovered from plasmid pTLEG-C1 (7). An *S. cerevisiae* DNA fragment was synthesized by polymerase chain reaction using two oligonucleotides named ACTSAC1 (5'-GGACCCGGGTAAGCTGCC-3') and ACTSAC2 (5'-GCGT GAAAGATCTAAAAGCTGATGTAG-3') corresponding to the flanking sequences of the promoter of the actin gene (19) but with synthetic *Sma*I and *Bgl*II restriction sites, respectively. Amplification consisted of 30 cycles of 40 s at 95°C and 2 min at 55°C with a last polymerization step of 5 min at 72°C. The amplified fragment was digested with *Sma*I and *Bgl*II and ligated to pUC18 digested with *Sma*I and *Hind*III

in the presence of the *Bam*HI-*Hind*III fragment containing the *egl1* cDNA gene. The resulting plasmid, named pTL-EGY2, contains the *T. longibrachiatum egl1* cDNA under the control of the *S. cerevisiae* actin gene promoter. pTL-EGY2 was cut with *Sma*I and *Hind*III, and the fragment containing the expression cassette was treated with the Klenow fragment of *E. coli* DNA polymerase I in the presence of deoxynucleotides to create a filled fragment. Finally, this fragment was cloned into the *Eco*RV site of YEpCR21 to generate plasmid pTLEGY3.

Transformation protocols. *E. coli* was transformed by following the method described by Hanahan (13), and transformants were selected in LB medium plus ampicillin at 50 $\mu\text{g ml}^{-1}$. Transformation of the *S. cerevisiae* CECT1894 was achieved by using different protocols: electroporation of the cells (2), polyethylene glycol protoplast transformation (3), and lithium acetate treatment of intact cells (6). Selection and maintenance of the transformants were done on YPD plates (YPD with 2% agar) containing 1 μg of cycloheximide ml^{-1} .

General cloning procedures. The regular techniques described by Sambrook et al. (26) were followed throughout this work. Restriction enzymes and *Taq* polymerase were from Promega (Madison, Wis.), and T4 DNA ligase and Klenow DNA polymerase were from Boehringer (Mannheim, Germany). Yeast plasmid DNA was isolated by following the method of Pérez-Ortín and Estruch (21).

Endoglucanase determinations. Plate assays were performed as described previously (7) using carboxymethyl cellulose as the substrate. Endoglucanase activity in culture and must filtrates was assayed with azobarley β -glucan as the substrate by using a commercial kit from Biocon according to the instructions of the manufacturer. One unit of endoglucanase activity was defined as the amount of enzyme that gave 1 U of A_{590} . Western blot (immunoblot) detection of the *T. longibrachiatum Egl1* protein was achieved with a monoclonal antibody raised against the homologous *Trichoderma reesei* EG1 protein (16) by following published procedures (14).

Microvinification experiments and extraction of volatile compounds. Fermentation assays were carried out using 1.5 liters of sterilized must. The vinification process was as described previously (25). The organoleptical characteristics of the produced wines were tested by a panel of trained experts from the winery Vinival (Valencia, Spain). The wine was collected at the end of fermentation (sugar concentration, less than 2 g/liter) and centrifuged at 5,000 $\times g$ for 5 min at room temperature. A 500-ml centrifuged sample was taken for each assay. The sample was brought to 8% (wt/vol) NaCl, and 5.6 mg of the internal standard (thymol, 5-methyl-2-isopropylphenol) was added. The volatile compounds were extracted with 3:2 pentane-dichloromethane by continuous liquid extraction. The extracts were filtered after drying over anhydrous sodium sulfate and concentrated to a volume of 2 ml with a Vigreux column. The samples were stored at -20°C until the gas chromatographic analysis.

Gas-liquid chromatography. The gas chromatographic analysis of volatile compounds was carried out using a Hewlett-Packard capillary gas chromatograph, model 5890 series II, controlled with a 3365 Chemstation (flame ionization detector; column, Supelcowax 10 [Supelco, Inc., Bellefonte, Pa.] fused-silica capillary column [30 m by 0.25 mm, 0.25- μm film]; carrier gas, He at 25 cm s^{-1} ; injection detector temperature, 280°C; oven temperature, 60°C isothermal for 10 min and then programmed at 3°C min^{-1} until 230°C and held isothermal for 50 min).

Since the gas chromatographic analysis of the volatile compounds was focused mainly to determine whether there were relative differences between their amounts in wines produced by the different yeast strains, their chromatographic relative responses against the internal standard did not need to be determined. Following other authors (17), each volatile compound was quantified within each sample as a dimensionless number corresponding to the area of the chromatographic peak divided by the area of the internal standard.

Gas chromatography-mass spectrometry. The instrument used for gas chromatography-mass spectrometry was a Hewlett-Packard mass selective detector, model 5971 A. Chromatographic column and operating conditions were the same as given for gas-liquid chromatography. Operating conditions for the mass spectrometer were as follows: transfer line temperature, 280°C; ion source temperature, 180°C; scanned mass range, 2 to 400 atomic mass units; scan rate, 1 spectrum per s. Major volatile components of wine were identified by comparison of their mass spectra and retention times against those of the corresponding standards.

RESULTS

Genetic transformation of the industrial wine yeast strain T₇₃. Growth inhibition of the T₇₃ strain was tested by inoculation of YPD plates containing a range of concentrations of cycloheximide from 0.1 to 10 $\mu\text{g/ml}$. Growth was completely inhibited by 1 μg of cycloheximide per ml. At this dose, the frequency of spontaneous resistance to the antibiotic was less than 10^{-9} . Thus, a selection procedure based on the acquisition of cycloheximide resistance seemed feasible for this industrial wine yeast strain.

With plasmid YEpCR21 as the donor DNA, different yeast transformation methods were assayed (see Materials and Methods). Electroporation of intact cells or polyethylene glycol-mediated transformation of protoplasts did not give any positive results, despite the use of different amounts of plasmid DNA or competent cells or different recovery times before application of the selective pressure. Only by using the lithium acetate protocol was it possible to isolate transformants. Different recovery times between the plating of the transformation mixture and the addition of cycloheximide were studied, revealing that as in other fungal species, 18 h of recovery was the optimal time (data not shown). Under these conditions, transformation was achieved at a frequency of 0.5 transformant per μg of YEpCR21.

To study the fate of the transforming DNA, plasmid was isolated from some of the transformants. It was possible to detect the presence of autonomous replicating YEpCR21 plasmid in all transformants. Transformants were also examined for mitotic stability of the YEpCR21 plasmid. Purified single colonies of each transformant were grown on YPD plates without cycloheximide, and the resulting colonies were suspended in 1 ml of liquid YPD. Different dilutions of the suspension were inoculated onto YPD plates without cycloheximide or containing 1 μg of the antibiotic per ml, and the stability was defined as the percentage of colonies which appears in selective medium versus nonselective medium. A high level of mitotic stability (around 90%) was detected.

Microvinification experiments were carried out with one of the transformants, with the untransformed T₇₃ strain as a control. As can be seen in Table 1, the sugar degradation during both fermentations was very similar. Also, it was not possible to detect differences between the two wines by

TABLE 1. Residual sugars detected in the musts of wines produced by the inocula of T₇₃ and T₇₃ transformant strains

Time (days)	Residual sugars (g/liter ^a) in musts produced by:		
	T ₇₃	T ₇₃ (YEpCR21)	T ₇₃ (pTLEGY3)
1	200	200	200
2	184.5	ND	172.2
3	ND	ND	140.2
4	174.2	142.2	126.7
5	100.8	136.1	ND
6	74.3	81.7	56.7
7	38.2	62.7	37.8
8	19.9	37.8	19.1
9	ND	ND	8.6
11	6.9	ND	ND
12	5.2	3.2	ND
13	3.2	ND	ND
14	2.7	ND	3.2
15	2.4	ND	1.9

^a ND, not determined.

sensory analysis. We can conclude that the transformant strain was capable of producing wine in the same way that the industrial T₇₃ strain was.

Expression of the *T. longibrachiatum* *egl1* gene in the industrial wine yeast strain T₇₃. An expression cassette containing the cDNA of the *T. longibrachiatum* *egl1* gene under the control of the *S. cerevisiae* actin gene promoter was constructed as described above (see Materials and Methods). This cassette was cloned into the YEpCR21 plasmid to generate plasmid pTLEGY3 (Fig. 1). By the lithium acetate transformation system, this plasmid was used to transform the T₇₃ strain. Some of the transformants were characterized, and plasmid isolation revealed the presence of autonomous pTLEGY3 in these transformants. The mitotic stability of this plasmid in the absence of selective pressure was similar to that reported for the YEpCR21 transformants.

In addition to the acquired cycloheximide resistance, the successful transfer of the plasmid pTLEGY3 into the yeast could also be measured by Egl1 expression. The enzymatic activity of Egl1 in some of the presumptive transformants was detected on YPD plates with an overlay of carboxymethyl cellulose (Fig. 2). All the cycloheximide-resistant colonies tested were also capable of degrading carboxy-

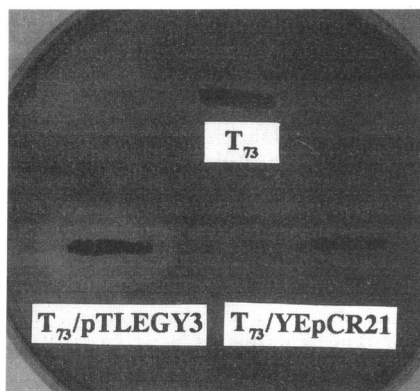


FIG. 2. Plate assay for the detection of endoglucanase activity (see Materials and Methods).

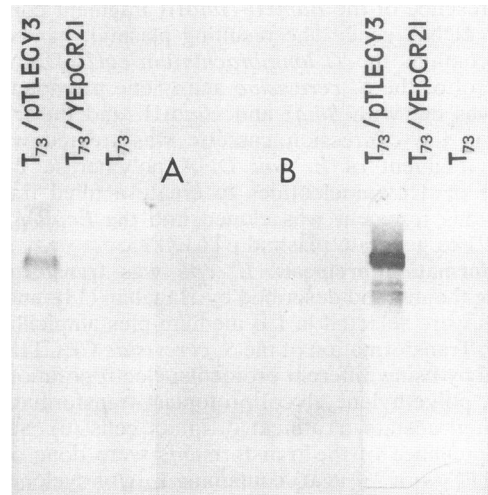


FIG. 3. Western blots of culture media (A) and musts (B) inoculated with T₇₃, a YEpCR21 transformant, and a pTLEGY3 transformant. The blots were incubated with the antibody raised against the *T. reesei* Egl1 protein.

methyl cellulose, indicating that the filamentous fungal gene was expressed in the industrial yeast strain. To confirm this, a Western analysis was carried out using a monoclonal antibody raised against the *T. reesei* Egl1 protein which, by virtue of the high level of similarity between both proteins (7), selectively recognizes the *T. longibrachiatum* Egl1 protein. Culture filtrates of YPD broth inoculated with the T₇₃ strain and one of each of the T₇₃ strains transformed with YEpCR21 and pTLEGY3 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (Fig. 3A). Only the pTLEGY3-transformed strain secreted the Egl1 protein into the media. The secreted protein is larger in apparent molecular weight than the native Egl1 produced by *T. longibrachiatum* (result not shown). The determination of endoglucanase activity in the supernatants of the YEpCR21- and pTLEGY3-transformed strains (Fig. 4A) clearly demonstrates that Egl1 is constitutively secreted into the media by the pTLEGY3-transformed strain.

Microvinification experiments using the recombinant wine yeast strain. The selected pTLEGY3 transformant was inoculated into sterile must to produce wine in a microvinification process. A parallel control fermentation using the T₇₃ strain was carried out. In the case of the must inoculated with the pTLEGY3 transformant it was possible to detect endoglucanase activity from the beginning of the process (Fig. 4B). Western blot analysis (Fig. 3B) revealed the presence of Egl1 protein in the must. In comparison with the protein secreted by the strain in YPD cultures, the Egl1 secreted to the must was heterogenous in size. At the end of the microvinification process, only a small percentage of the yeast population lost the endoglucanolytic phenotype (6.12, 7.32, and 8.16% in three different experiments).

Regarding the sugar degradation, as in the case of the wine produced by the YEpCR21 transformant, no significant differences were found between the wines produced by the pTLEGY3 transformant and the T₇₃ strain (Table 1). Also, the appearance of the wine was the same. However, the panel of experts detected an increased fruity aroma in the wine produced by the pTLEGY3 transformant strain. This result was confirmed in other microvinification processes

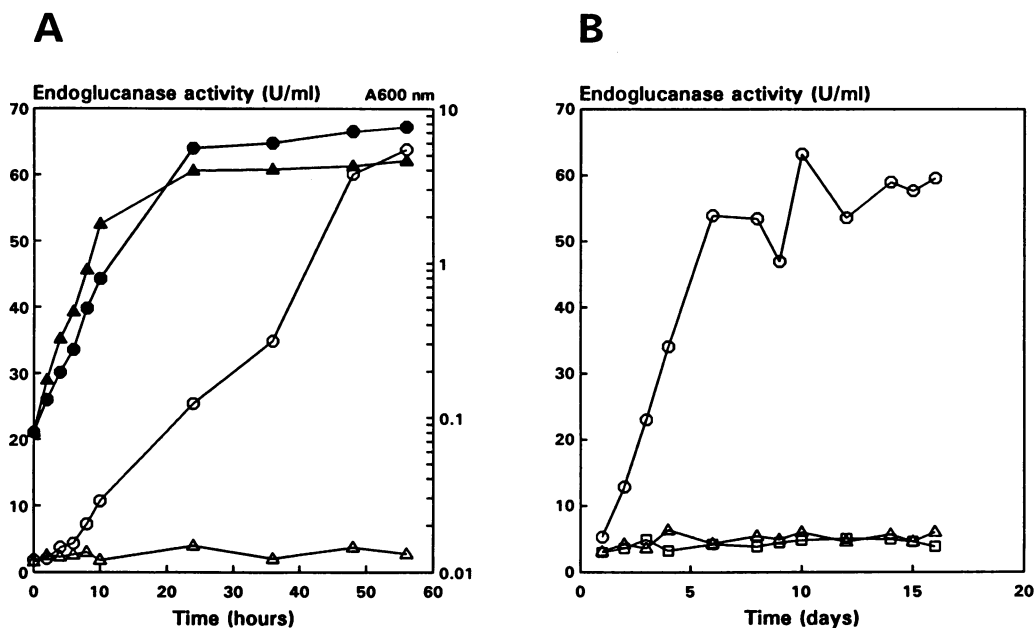


FIG. 4. (A) Growth (filled symbols) and endoglucanase activity (open symbols) in the supernatants of culture media inoculated with a YEpCR21 transformant (triangles) and a pTLEGY3 transformant (circles); (B) endoglucanase activity in musts inoculated with T_{73} (squares), a YEpCR21 transformant (triangles), and a pTLEGY3 transformant (circles).

using the same or other pTLEGY3 transformant strains (results not shown). The Egl1 protein hydrolyzes β -(1,4)-glycosidic bonds, the same bond present in the monoterpene glycosides of the must. For this reason we decided to compare the volatile compound profiles of the wines produced by the T_{73} strain and the pTLEGY3 transformant. The results clearly showed an increase of concentration for some major volatile compounds (Table 2). This increase was not detected in the case of microvinifications carried out by the YEpCR21 transformant strain (results not shown).

TABLE 2. Relationship between the concentrations of some volatile compounds in the musts produced by the inoculum of the T_{73} strain or the T_{73} transformant containing the pTLEGY3 plasmid

Volatile compound	Area of chromatographic peak/area of internal standard, ^a produced by:		
	T_{73}	T_{73} (pTLEGY3)	T_{73} (pTLEGY3)/ T_{73}
Ethyl propionate	—	0.031	NC
2-Butanol	0.918	2.211	2.408
Isoamyl acetate	0.032	0.079	2.469
Isoamyl alcohol	6.849	18.422	2.689
Ethyl caproate	—	0.041	NC
Acetoin	0.057	0.082	1.438
Ethyl lactate	0.077	0.137	1.779
1-Hexanol	0.019	0.039	2.053
Isobutyric acid	0.288	0.673	2.337
Isovaleric acid	0.187	0.466	2.492
Caproic acid	0.127	0.262	2.063
2-Phenyl ethanol	1.603	2.744	1.712
Thymol (internal standard)	1	1	1

^a Data are expressed as described in Materials and Methods. —, not detected; NC, not calculated.

DISCUSSION

Until recently, studies directed toward wine improvement have concentrated on the selection of new grape varieties or on fermentation and wine-making practices. Little attention has been paid to genetic improvement of the major microorganism involved in wine production, namely, the wine yeast. Transformation of laboratory *S. cerevisiae* strains is a trivial experiment. Contrariwise, some of the industrial *S. cerevisiae* strains are recalcitrant to the uptake of exogenous DNA. This is a well-documented problem in the case of brewing-yeast strains (18). A report concerning the genomic integration of the yeast K1 killer toxin gene in industrial wine yeasts has been recently published (4). This method provides stable transformants, but the selection scheme is time-consuming. The polyploid nature of some of the most common prototrophic markers (e.g., *leu2* and *ura3*) in industrial wine yeasts (unpublished results) makes their complementation impossible using the cloned wild-type alleles. Therefore, selection of transformants has to be based on dominant markers. The T_{73} industrial wine yeast strain is extremely sensitive to cycloheximide, so, as in other industrial yeasts (5), a selection system based in the acquisition of resistance to this antibiotic by complementation with the *CYH2* gene is possible. Transformants obtained in this way are quite stable, even in the absence of selective pressure, not only in laboratory cultures but also in microvinification experiments. In this latter case, the wines produced have the same organoleptical characteristics as those produced with the industrial strain T_{73} . From a commercial point of view, it will be desirable to modify this system in order to generate integrative transformants with no bacterial DNA. In this respect, directed integration at the resident *CYH2* locus is a reasonable alternative, and some *CYH2* disruption constructs are being prepared in our laboratory.

By using this system a previously cloned *T. longibrachiatum* *egl1* gene encoding a β -(1,4)-endoglucanase activity has been expressed in a wine yeast strain. The gene is expressed under the control of a strong constitutive yeast promoter, and the exogenous protein is secreted to the medium by using the fungal signal peptide (approximately 50% of the produced protein remains in the cell [unpublished results]). Other authors described previously the construction of endoglucanolytic brewing yeasts (20) which avoid the filtration problems associated with the presence of the barley β -glucans (29). To our knowledge this is the first report concerning the design of an endoglucanolytic wine yeast strain. This recombinant strain produces wine in the same way as the untransformed strain, but the aroma profile is different, being fruitier. From an industrial point of view this is a desirable change, probably due to residual β -glucosidase activity of the Egl1 protein. The expression of other cellulases and hemicellulases in the wine yeast strain could be of interest. With this sense, we are developing constructions for the expression of β -glucosidase or α -L-arabinofuranosidase activities.

Finally, it is important to notice that a new era in enology is emerging. The application of mitochondrial DNA restriction analysis to study the population dynamics of wine fermentations or to distinguish active dry yeasts (23, 24) and the construction of recombinant yeast strains are examples of the application of molecular biological techniques in enology. The possibility of constructing new industrial wine yeast strains is now a reality.

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