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Three Different Targets for the Genetic Modification of Wine Yeast Strains Resulting in Improved Effectiveness of Bentonite Fining

Daniel Gonzalez-Ramos,[‡] Manuel Quirós,[†] and Ramon Gonzalez*[,†,‡]

[†]Instituto de Ciencias de la Vid y del Vino (CSIC-UR-CAR) Logroño (La Rioja), Spain, and
[‡]Instituto de Fermentaciones Industriales (CSIC), Madrid, Spain

Bentonite fining is used in the clarification of white wines to prevent protein haze. This treatment results in the loss of a significant portion of the wine itself, as well as aroma compounds important for the quality of white wines. Among other interesting effects on wine quality, yeast cell wall mannoproteins have been shown to stabilize wine against protein haze. A previous work showed that wine yeast strains engineered by deletion of *KNR4* release increased amounts of mannoproteins and produce wines showing attenuated responses in protein haze tests. This paper describes the technological properties of several new recombinant wine yeast strains, deleted for genes involved in cell-wall biogenesis, as well as the regulatory gene *KNR4*. Stabilization of wines produced by three of the six recombinant strains analyzed required 20−40% less bentonite than those made with their nonrecombinant counterparts. The availability of multiple targets for genetically improving yeast mannoprotein release, as shown in this work, is relevant not only for genetic engineering of wine yeast but especially for the feasibility of genetically improving this character by classical methods of strain development such as random mutagenesis or sexual hybridization.

KEYWORDS: Wine yeast; mannoproteins; protein haze; bentonite fining; genetic engineering

INTRODUCTION

The influence of yeast cell wall macromolecules, and particularly mannoproteins, on wine quality has been one of the hot topics of wine biotechnology during the past 15 years (*1*). Stabilization against protein haze was one of the first positive properties described for mannoproteins (*2−6*). Wine spoilage by protein haze formation can cause considerable economic loss in product returns. It results from the aggregation of grape, heat unstable, proteins during storage and handling. The most common way to circumvent this problem is bentonite fining; this, however, generates large amounts of sediment and associated wine loss, as well as loss of aroma compounds (*7−9*). Wines aged "sur lies" have lower haze potential than wines matured without lees due to the protective effect of the mannoproteins released from yeast cell walls (*10*). The addition of mannoproteins to wine results in higher protein stability, and the specific contributions of particular mannoproteins to wine quality have been analyzed (*2−5*, *10*). In particular, two mannoproteins, Hpf1p and Hpf2p (from haze protecting factor), overexpressed in *Saccharomyces cerevisiae* laboratory strains, were shown to contribute to reduced turbidity of wine (*11*).

There is an ever-growing interest in the selection and development of wine yeast strains able to release mannoproteins more efficiently than currently available strains. In a previous work we constructed *KNR4* deleted industrial wine yeast strains in two different backgrounds and showed increased heat stability of the wines obtained (*12*) estimated by reduced haziness in the heat test, depending on the genetic background. The potential of *KNR4* as a genetic engineering target for increased mannoprotein release, together with three other cell wall biogenesis related genes, had been identified in a screen using laboratory strains (*13*). Among them, *KNR4* was the only gene coding for a regulatory protein, Knr4p, required for the correct targeting of the Stl2p MAP kinase (*14*). It is part of the main cell integrity pathway and participates in the coordination of cell wall synthesis with bud emergence (*15*). The other potential targets identified in that screen encoded proteins with enzymatic activities involved in the synthesis of cell wall constituents. *GPI7* encodes an enzyme required for the synthesis of the GPI anchor (*16*, *17*), a structure mediating the linkage of some proteins to the plasma membrane or to the cell wall. Most *S. cerevisiae* cell wall mannoproteins are synthesized as GPI-anchored precursors and covalently linked to the cell wall through a GPI anchor remnant (*18*). In *GPI7*-deficient strains, GPI-anchored proteins are not efficiently linked to the cell wall and are released to the extracellular medium (*17*). *FKS1* encodes a subunit of the β-1,3-glucan synthase responsible for β-1,3-glucan synthesis during growth in glucose and in the growing bud (*19*, *20*). Deletion of *FKS1* results in cell walls with a lower β-1,3-glucan content, slow-growth phenotype, and release of glycosylated proteins to the medium (*19*, *21*). *GAS1* encodes a glycoprotein of the plasma membrane. Gas1p has β-1,3-glucanosyltransferase activity and is

*Address correspondence to this author at ICVV, Instituto de Ciencias de la Vid y del Vino (CSIC-UR-CAR), C. Madre de Dios 51, 26006 Logroño, Spain (telephone +34 941299684; e-mail rgonzalez@icvv.es).

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Table 1. Strains Employed in This Study

strain	relevant genotype	reference or origin
EKD-13	EC1118; *KNR4::ARO4-OFP/KNR4::KanMX4*	*12*
EGD-13	EC1118; *GPI7::ARO4-OFP/GPI7::KanMX4*	this study
EFD-13	EC1118; *FKS1::ARO4-OFP/FKS1::KanMX4*	this study
TKD-123	T73-4; *KNR4::ARO4-OFP/KNR4::KanMX4/KNR4::URA3*	*12*
TGD-13	T73-4; *GPI7::ARO4-OFP/GPI7::KanMX4*	this study
TGASD-31	T73-4; *GAS1::ARO4-OFP/GAS1::KanMX4*	this study
Y05251	BY4741, *FKS1:: KanMX4*	Euroscarf
Y00897	BY4741, *GAS1::KanMX4*	Euroscarf
Y01361	BY4741, *GPI7::KanMX4*	Euroscarf

Table 2. Primers Used in This Work

primer	sequence 5′−3′
CDFKS-f	GAAATAGTCTCACTTACTGGGCGAC
CDFKS-r	CTGAAGAGCCATGAGACAATTGC
CDGAS-f	CAACAACGATACTGGTCCAAATG
CDGAS-r	CTGACAAAGAAGCTGCCTCATTC
CDGPI-f	CTTTTTCAAGGCAATATGCTCG
CDGPI-r	TTCAAAACGATAGGCTTTTCTTGC
PFKS-f	CCAGTCACGACGTTGTAAAACGACGGCGTTTTGATGAAGCACAGGAAG
PFKS-r	GGTACCGAGCTCGAATTCACTGGGACCGTTGTATGAAAGACTTGATTTC
PGARO-f	GGATCCTCTAGAGTCGACCTGCGGTGAAGTGTGCGTGGTAGATG
PGARO-r	GATTACGCCAAGCTTGCATGCCTGGTGTCACGGGCTCTGTTTAC
PGAS-f	CCAGTCACGACGTTGTAAAACGACGGCAACACCAACTTTACCTACCTTTAGGAC
PGAS-r	GGTACCGAGCTCGAATTCACTGGCTGTGTTTGTTGTTTTTGTTTTATCAGAC
TFKS-f	GGATCCTCTAGAGTCGACCTGCCAATACTTGCTTGAACGCTTGATTT
TFKS-r	GATTACGCCAAGCTTGCATGCCTCAATAATGGCTGCGTAAAAATTTTG
TGARO-f	CCAGTCACGACGTTGTAAAACGACGGCATCGTGATAGTGTCATCCTC
TGARO-r	GGTACCGAGCTCGAATTCACTGGGGACAGCGATAATTGAGTGGTGG
TGAS-f	GGATCCTCTAGAGTCGACCTGCGCTTCGACACATACATAATAACTCGATAAG
TGAS-r	GATTACGCCAAGCTTGCATGCCTGAGTCAATTGATTGAAAATAATTCGC

involved in the elongation of β-1,3-glucan branches (*21*, *22*). Strains deleted for *GAS1* show a phenotype similar to *FKS1* defective strains (*21*).

We have now constructed several recombinant wine yeast strains, partially or totally defective for *GAS1*, *GPI7*, or *FKS1* in two different industrial genetic backgrounds. Some of the newly constructed strains, as well as the previously described EKD-13 (defective for *KNR4*), allowed a 20−40% reduction in bentonite requirement for complete protein haze stabilization of the wines. Interestingly, a correlation between the responses to heat treatment of the untreated wines and the amount of bentonite required for stabilization was not always apparent.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions. EC1118 is a wine yeast strain commercialized by Lallemand Inc. (Montreal, Canada). T73-4 is a uridine auxotroph derived from the winemaking strain T73 (*23*). All other yeast strains used in this work are listed in **Table 1**. *Escherichia coli* strain DH5α (*supE44*, Δ*lacU*169 [Φ80 *lacZ*Δ*M*15], *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*) was used for the construction and amplification of the plasmids employed in this study. Several laboratory media were used in this work: YPD broth (2% glucose, 2% peptone, 1% yeast extract); YPD plates (YPD + 2% agar); GCY (2% glucose, 2% Bacto Casaminoacids (BD, Sparks, MD), 0.67% Difco yeast nitrogen base (BD)); SD + PFP plates (0.67% yeast nitrogen base without amino acids (Difco Laboratories Inc., Detroit, MI), 2% dextrose, 1.67% purified agar, 0.9 g/L L-tyrosine, 2 g/L *p*-fluoro-DL-phenylalanine (PFP)); YPD + G418 plates (YPD plates + G418 40 μg/mL). For the fermentation of natural Sauvignon Blanc must, Sauvignon Blanc grape berries were pressed and potassium metabisulfite was added to the juice to a final concentration of 60 mg/L.

For the quantification of the release of mannoproteins, yeast cells were grown in GCY medium. Each strain was inoculated from a fresh preculture in the same medium to a starting OD_{600} = 0.1 unit and incubated at 30 °C and 150 rpm to stationary phase. Media were then recovered by centrifugation for the analysis of polysaccharide content.

For the fermentation experiments, precultures were grown in YPD broth and must was inoculated to a final concentration of 10^6 cells/mL. Fermentation kinetics assays were carried out in small volumes (50 mL), and Sauvignon Blanc must clarified by centrifugation was used. For bentonite fining assays 500 mL of unclarified must was used, and in both cases fermentations were carried out at 20 °C in Erlenmeyer flasks closed with Müller valves. The fermentation time course was monitored by recording CO_2 production as weight loss until constant weight. Wines were then recovered, and yeast cells were removed by centrifugation. Dominance of the recombinant strains in these experiments was assessed by isolating yeast colonies in YPD from samples taken at the end of the fermentation process and replica plating in YPD + G418 plates.

Molecular Biology Techniques. Unless otherwise specified, general molecular biology techniques were used to construct the deletion cassettes (*24*). Construction of EKD-13 and TKD-123 was previously described (*12*). Two different markers, *ARO4-OFP* and *KanMX4*, were used for the construction of new recombinant strains deleted for two copies of the target genes. Deletion cassettes consisted of the marker gene flanked by 500 base pairs corresponding to the promoter and terminator region of the cognate target gene. First, *ARO4-OFP* was isolated from the plasmid pEA2 (*25*) by digestion with *Sac*I and *Bam*HI and inserted by ligation into pUC19 (*24*) digested with the same restriction enzymes. The resulting plasmid was named pUCARO. Promoter regions were amplified from *S. cerevisiae* genomic DNA with primer pairs PGARO-f/PGARO-r, PGAS-f/PGAS-r, and PFKS-f/PFKS-r for *GPI7*, *GAS1*, and *FKS1*, respectively (**Table 2**). Terminator regions were amplified with primer pairs TGARO-f/TGARO-r, TGAS-f/TGAS-r, and TFKS-f/TFKS-r for *GPI7*, *GAS1*, and *FKS1*, respectively (**Table 2**). These inserts were cloned in pairs, sequentially, in pUCARO by the primer extension technique (*26*), and the resulting plasmids were named pDGPI7-1, pDGAS1-1, and pDFKS1-1, carrying *ARO4-OFP* flanked, respectively, by promoter and terminator regions of *GPI7*, *GAS1*, and *FKS1*.

For yeast transformation experiments with PFP selection, deletion cassettes were PCR amplified from the cognate plasmid using primer pairs PGARO-r/TGARO-f, PGAS-f/TGAS-r, and PFKS-f/TFKS-r (**Table 2**). For transformations with G-418 selection, deletion cassettes were amplified from genomic DNA from haploid laboratory strains carrying the cognate gene replaced by *KanMX4*: Y01361 for *GPI7*, Y00897 for *GAS1*, and Y05205 for *FKS1*.

Yeast Transformation and Analysis of Transformants. Transformation of *S. cerevisiae* was carried out by the lithium acetate method (*27*). Briefly, yeast strains were transformed with 20 μL of the appropriate PCR amplification reaction (see above). For the *ARO4-OFP* marker, after the transformation experiment, cells were diluted 10 times in YPD and incubated during 17 h at 30 °C and 200 rpm to allow the expression of the resistance allele before selective pressure was applied. Transformants were selected on SD + PFP plates after 5 days of incubation at 30 °C (*25*). For the *KanMX4* marker, cells were diluted to 1:2 in YPD medium and incubated for 1 h at 30 °C and 200 rpm to allow the expression of the resistance allele. Transformants were selected on YPD + G418 (40 μg/mL) after 2 days of incubation at 30 °C.

The resistance phenotype of transformants was confirmed by replica plating in selection media, and positive strains were grown in YPD at 30 °C and 200 rpm. Genomic DNA was extracted as described by Querol et al. (*28*). Correct insertion was confirmed by PCR amplification of the whole locus, using primer pairs CDGPI-f/CDGPI-r, CDGAS-f/CDGAS-r, and CDFKS-r /CDFKS-r (**Table 2**) for *GPI7*, *GAS1*, and *FKS1* deletion, respectively, and verification of the amplicon size by agarose gel electrophoresis.

Analysis of Mannoproteins and Polysaccharides. For the quantification of total polysaccharides, monosaccharides were removed from the supernatans of cultures in GCY medium by gel filtration in Econo-Pac 10DG columns (Bio-Rad, Alcobendas, Spain), following the recommendations of the manufacturer. The concentration of total mannoproteins and polysaccharides in the eluted fraction was determined against a standard curve of commercial mannan (Sigma, Tres Cantos, Spain) by the phenol−sulfuric acid method as described by Segarra et al. (*29*). Five replicates were performed for each determination, and data were analyzed by one-way ANOVA and Dunett test for comparison of means using SPSS 15.0 software.

For the specific detection of mannoproteins, Sauvignon Blanc wines were resolved by SDS-PAGE (*30*). Ten microliters of wine fermented by different strains was loaded in each well. The proteins were transferred to a nitrocellulose membrane using the Mini Protean transfer system (Bio-Rad) following the directions of the manufacturer. The mannoproteins present in the membrane were detected by the use of peroxidase-conjugated concanavalin A (Sigma) as described by Klis et al. (*31*).

Protein Haze Analysis (Heat Test). For bentonite fining assays, bentonite was previously suspended and hydrated in distilled water at 50 g/L. Different amounts of the homogenized suspension were added to 40 mL of wine to reach 0, 12, 24 36, 48, or 60 g/hL. Closed tubes were incubated at room temperature in a rocking table for 30 min. Wines were then clarified by centrifugation, 5 min at 3000*g*, and filtered through a 0.45 μm PVDF filter.

The stability of bentonite-treated wines was assayed by incubating 5 mL aliquots at 85 °C for 30 min and cooling on ice. The turbidity of the wines was determined in a nephelometer (Hach, Loveland, CO). Three fermentation experiments were performed for most strains and two in the case of EGD-13 and EFD-31. Three to six replicates of the stability test were performed for each fermentation experiment. Results were analyzed by multifactor analysis and Dunett test by using SPSS 15.0 software. The strain factor appeared as significant at the $p < 0.05$ level for all of the comparisons.

HPLC Analysis of Wines and Grape Juice. Samples from fermentation experiments were analyzed by HPLC to quantify the amount of sugars, glycerol, and ethanol. Chromatography was performed on a Thermo (Thermo Electron Corp., Waltham, MA) chromatograph equipped with a P400 SpectraSystem pump, an AS3000 autosampler, and a Thermo SpectraSystem RI150 refraction index detector was used. The column was an HPX-87H (BIO-RAD). The conditions used in the analysis were as follows: eluent, H_2SO_4 1.5 mM; flux, 0.6 mL/min; column temperature, 50 °C. Samples were filtered through a 0.45 μm PVDF filter (Teknokroma, Spain), diluted 2 or 10 times depending on the sugar content expected, and injected in duplicate.

RESULTS

Construction of New Recombinant Wine Yeast Strains. EC1118 or T73-4 was independently transformed with each of the deletion cassettes amplified from pDGPI7-1, Y00897, or Y05205 genomic DNA for *GPI7*, *GAS1*, and *FKS1* deletion, respectively. Transformants were selected for PFP (*GPI7* deletion) or G418 resistance (*GAS1* or *FKS1* deletion). Three to 24 transformants were obtained, depending on the specific gene deletion, and were PCR analyzed as described under Materials and Methods. One transformant from each type, carrying one intact copy of the cognate gene and one replaced by the selection cassette, was used for a second round of transformation (data not shown). In this second round, deletion cassettes amplified from Y01361 genome, pDGAS1-1 or pDFKS1-1, were used to get both alleles of *GPI7*, *GAS1*, or *FKS1* deleted. In the EC1118 background, six double-deleted strains were obtained for *GPI7* and three for *FKS1*. However, none of the 22 transformants from four transformation experiments obtained for the transformation of the *GAS1* deletion cassette showed the expected pattern of amplification bands in the PCR analysis. All of them contained at least two alleles: the original locus and that replaced by *ARO4-OFP*. Considering that there was probably a deleterious effect for total loss of function of *GAS1* in EC1118, this combination was discarded for further analysis. Double-deleted strains were obtained in all cases in the T73-4 background. However, a third copy of *FKS1* was found in this background. Taking advantage of the uridine auxotrophy of T73-4, the URA3 marker was used to delete this additional copy of *FKS1*, but after unsuccessfully analyzing 22 positive transformants, this combination was also discarded (data not shown). The growth pattern of the strain carrying two deleted and one wild type copy of *FKS1* was similar to that of the wild type (data not shown). Further analyses were carried out with the strains completely lacking each of the other two genes: EGD-13 and TGD-13 (for *GPI7* deletion); EFD-31 (for *FKS1* deletion); TGASD-31 (for *GAS1* deletion) as well as the previously published strains EKD-13 and TKD-123 (both deleted for *KNR4*); and the original strains EC1118 and T73-4.

Release of Mannoproteins in Laboratory Media. GCY liquid medium was inoculated with each strain to an OD_{600} of 0.1, growth was monitored to stationary phase, and the amount of polysaccharides released during growth in GCY medium was measured in the supernatants (**Figure 1**). As expected from previous studies on laboratory strains (*13*) and from results recently published for EKD-13 (*12*), the strains deleted for all copies of *KNR4*, *GPI7*, *FKS1*, or *GAS1* released significantly higher amounts of polysaccharides than their unmodified counterparts in both genetic backgrounds. These results encouraged us to proceed to the technological characterization of the deleted strains.

Fermentation Kinetics. A Sauvignon Blanc grape juice with a sugar content of 25% (w/v) was used for fermentation assays with EC1118, T73-4, and the six recombinant strains. Fermentation profiles are shown in **Figure 2**. Strains deleted in *KNR4* showed negligible impairment of fermentation kinetics, whereas strains deleted in *GPI7*, *FKS1*, or *GAS1* fermented wine more slowly than their unmodified counterparts. Notwithstanding, residual sugar, as well as ethanol and glycerol, productions were similar in most fermentation assays (data not shown), perhaps with a slightly lower ethanol production in favor of higher glycerol content, but these subtle differences would require specific confirmation.

Bentonite Fining Assays. Wines fermented as described under Materials and Methods were clarified by centrifugation at the end of the fermentation process and subjected to protein stability

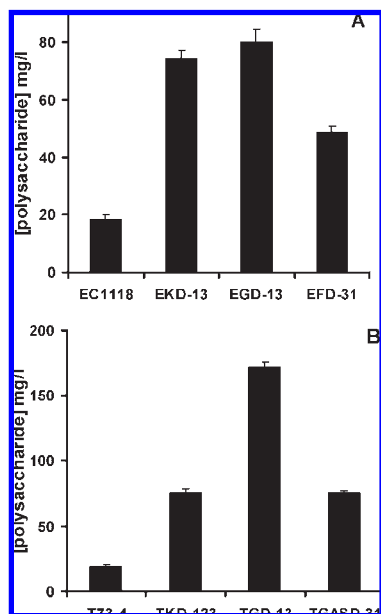

Figure 1. Concentration of the polysaccharides released in GCY medium: (**A**) strains in the EC1118 background; (**B**) strains in the T73-4 background.

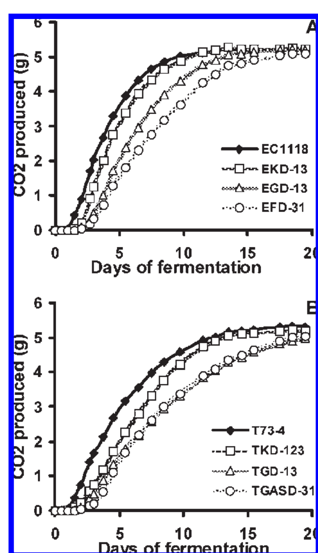

Figure 2. Time course of the fermentation of Sauvignon Blanc must: (**A**) strains in the EC1118 background; (**B**) strains in the T73-4 background.

assays after fining with different amounts of bentonite. Dominance of the recombinant strains was 60−100% at the end of the fermentation process for recombinant strains in the EC1118 background, as well as TKD-123, but it was below 10% for strains TGD-13 and TGASD-31. This indicates that the properties of these two wines would depend on the indigenous microbiota rather than on the inoculated strains. Therefore, they were not included in the stability assays. For strains in the EC1118 background only EKD-13 showed improved stability before treatment (about 30% reduction in turbidity, **Figure 3**). The other two strains showed similar (EFD-31) or higher turbidity (EGD-13) than the control strain. Curiously, wines fermented by EFD-31 or EGD-13 were much more responsive to bentonite fining than wines fermented by EC1118 or EKD-13. Therefore, whereas EC1118 required the use of 48−60 g/hL of bentonite for total

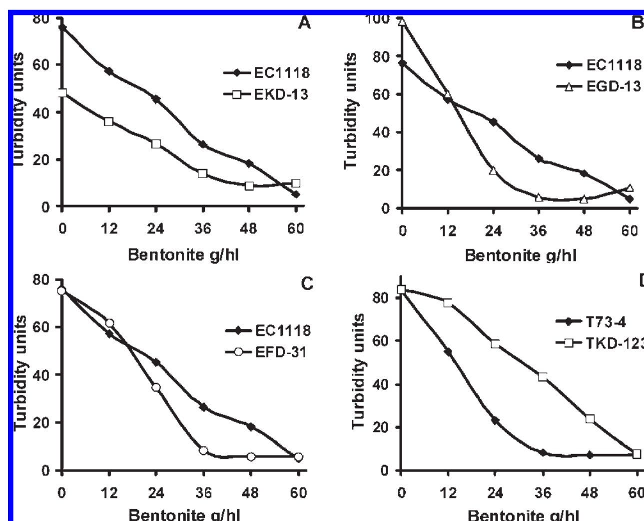

Figure 3. Effect of bentonite fining on the heat-test results of Sauvignon Blanc wines fermented with the recombinant strains compared to their unmodified counterparts. The identities of the strains are indicated in each panel. Results are the mean of two or three replicates except for panel **D**, where the results of a representative experiment are shown.

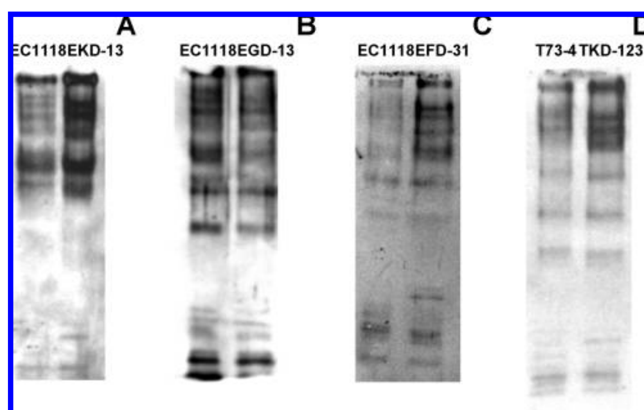

Figure 4. Mannoproteins released during fermentation of a Sauvignon Blanc wine by the recombinant strains compared to their unmodified counterparts. The identities of the strains are indicated in each panel.

stabilization, similar turbidity values were obtained for the wines fermented by any of the recombinant strains with a treatment of 36−48 g/hL (**Figure 3**), as a consequence of either the initial improved stability (EKD-13) or improved response to bentonite treatment (EGD-13 and EFD-31). These wines were completely stable because no additional stabilization was obtained with increasing doses of bentonite. Mannoproteins released during the fermentation of this natural must are shown in **Figure 4**. There is not a clear correlation between the increase in mannoprotein release and the increment in bentonite responsiveness. For example, EGD-13 produces wines with improved response to bentonite fining (**Figure 3**) but does not show a parallel increase in mannoprotein content of the wines (**Figure 4**).

In the T73-4 background, levels of haziness of untreated wines fermented by either TKD-123 or the wild type strain were similar in the heat test, or higher for TKD-123, depending on the experiment. However, the response of the recombinant strain to bentonite treatment was lower, requiring almost twice the amount of bentonite for complete stabilization, as compared to the unmodified strain (**Figure 3**). The lower protein stability of wines fermented by TKD-123 is not explained by its failure to

overproduce mannoproteins; actually, it releases increased amounts of mannoproteins (**Figure 4**).

DISCUSSION

The interest in EKD-13 (an EC1118 derivative deleted for *KNR4*) for improving protein stability of white wines was already suggested in a previous work (*12*). In the present work we have tried to answer two questions arising from these previous results. The first question was whether the interesting properties shown by strains deleted for *KNR4*, at least on the EC1118 genetic background, were exclusive of this gene, or if deletion of genes involved in cell wall biosynthesis, instead of signal transduction pathways, would also result in technologically interesting phenotypes concerning mannoprotein release. The second question was whether these changes in wine composition resulted in any technologically relevant advantage, given that the increased stability of wines fermented by EKD-13 was not enough to avoid the need for bentonite fining (*13*).

We have now shown that deletion of *GPI7* or *FKS1* in the EC1118 background also results in increased release of mannoproteins in laboratory conditions. However, in contrast to *KNR4* deletion, the initial protein haze susceptibility of wines fermented with these strains is similar to or clearly higher than for the unmodified strain. The finding that these wines respond quite better to bentonite fining treatments clearly compensates for this apparently discouraging result. It seems now clear that bentonite fining requirement is a better parameter to compare strain suitability for improved protein stability than simply performing the heat test on the untreated wines. Indeed, all of the EC1118 derivatives tested in this work required 20−40% less bentonite to reach complete stabilization.

On the other hand, the strong dependence on the genetic background previously reported for *KNR4* deletion (*12*) is in agreement with the results obtained in the present work. None of the recombinant strains derived from T73-4 produced wines improved for bentonite fining, rather the contrary. In addition, for *GPI7* and *KNR4*, for which a comparison was possible, strains in the T73-4 background were much more impaired in fermentation behavior than their counterparts in the EC1118 background. We consider three nonexclusive explanations for the failure to improve bentonite fining in TKD-123: it could be related to the growth and fermentation kinetics impairment observed for T73-4 derivatives, the fact that T73-4 wines already require less bentonite fining than EC1118, or the mechanism of stabilization discussed below. Notwithstanding, the former would not completely explain the results because TKD-123 not only fails to improve the response of wines to bentonite fining, but this treatment is clearly less effective for these wines. Also indicative of the influence of the genetic background are the difficulties encountered in obtaining specific gene deletions in one or the other background (*GAS1* in EC1118 or *FKS1* in T73-4).

Concerning the mechanism of stabilization of white wines fermented by these strains, there is no obvious explanation for the fact that wines showing similar or reduced stability as compared to the control before bentonite fining respond significantly better to bentonite. One hypothesis would be that some of the substances released by yeast cells, including mannoproteins and other molecules, would negatively contribute to the protein stability of wines, the final susceptibility to heat treatment being the result of interactions between all of these molecules. The second part of the hypothesis would be that increased effectiveness of bentonite fining in these wines would result from high reactivity of yeast-derived unstable molecules to bentonite. Indeed, some of these recombinant strains gave rise to wines more heat-unstable than the wild type, by using a bentonite clarified white grape must (data not show). The specific mannoproteins released by each strain might also influence bentonite fining effectiveness, according to results from several authors that have shown the specific influence on protein haze of particular mannoproteins or fractions (*2*−*6*, *11*). This is illustrated by strain EGD-13, apparently releasing fewer mannoproteins than EC1118, but producing wines clearly more responsive to bentonite fining, or TKD-123 for the opposite (**Figures 3** and **4**).

Finally, considering their better fermentation performance and mannoprotein release, EKD-13 and EFD-31 seem to be the more suitable strains, and consequently *KNR4* and *FKS1* better target genes, for improving mannoprotein release of industrial wine yeast strains through genetic engineering. There is, however, an interesting conclusion to be drawn from the results with EGD-13 and EFD-31: inactivation of genes other than *KNR4* also results in increased mannoprotein release in industrial strains and, especially, improved response to bentonite fining. Because the four genes targeted in this work constitute a limited sample of the wine yeast genome, there is a chance that loss of function of some other genes would also lead to improved strains in terms of heat stability and bentonite responsiveness of the wines produced. It is also possible that mutation/deletion of genes not directly related in cell-wall biogenesis would result in phenotypes similar to those described here. The fact that several genes are appropriate targets to improve mannoprotein release opens the way to genetically improving wine yeast strains through classical genetic improvement methodologies that, given their randomness, would not be feasible in the case of a single target gene. These methodologies are becoming popular in the enological field and would result in strains commercially suitable in most countries, in contrast to the generalized restriction on the use of genetically engineered strains. In this work we chose protein haze stabilization as a model application due to its amenability to laboratory-scale experimentation and the possibility of getting quantitative results. Nevertheless, the applications of strains improved for mannoprotein release would be eventually wider, especially considering the remarkable effect of mannoproteins on several other quality traits of red, white, and sparkling wines (*1*).

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