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Genetic Determinants of the Release of Mannoproteins of Enological Interest by *Saccharomyces cerevisiae*

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Cell wall mannoproteins released by *Saccharomyces cerevisiae* during wine fermentation and aging have recently attracted the attention of enologists and researchers in enology due to their positive effect over a number of technological and quality properties of the wines, including protein and tartaric stability, aroma and color stability, astringency, mouthfeel, malolactic fermentation, or foam properties of sparkling wines. This work has investigated the effect of deletions involving genes related to cell wall biogenesis on the release of mannoproteins, as well as the effect of the released mannoproteins on wine protein stability. When available, the phenotypes have been studied in different genetic backgrounds, in haploid or diploid strains, and in homo- or heterozygosis. Strains deleted for *GAS1*, *GPI7*, or *KNR4* release higher amounts of mannoproteins and polysaccharides to the medium. These increased amounts of mannoproteins and polysaccharides lead to a stronger stability of Sauvignon Blanc wines against protein haze.

KEYWORDS: Wine yeast; mannoproteins; protein haze; mutant; genetic improvement

INTRODUCTION

The cell wall of *Saccharomyces cerevisiae* is an elastic structure that provides osmotic and physical protection to the cell. It consist of two layers: an inner layer made of β-1,3-glucan and chitin, which represents about 50−60% of the cell wall dry weight, and an outer layer, which consist of β-1,6-glucan and heavily glycosylated mannoproteins. The mannoproteins are glycoproteins having carbohydrate fractions made of around 98% mannose and 2% glucose. Most of them are covalently linked to the inner cell wall layer, either directly to the β-1,3-glucan matrix or indirectly via a β-1,6-glucan branch (*1*).

During the alcoholic fermentation of grape must, *S. cerevisiae* ferments the sugars to ethanol and other metabolites such as glycerol, acetate, succinate, pyruvate, and several esters, all of them contributing to the sensorial properties of the wine. In addition, yeast cells release cell constituents such as proteins or polysaccharides, also contributing to the quality of the wine (*2*). Among them, cell wall mannoproteins deserve special attention due to the high number of positive enological properties described during the past 15 years (*3*).

Chemical stabilization of white wines was one of the first enological properties described for mannoproteins. In some white wines, grape proteins aggregate and precipitate due to the high temperatures or the long storage time. If precipitation takes place in the bottle, this haziness is perceived as spoilage by the consumer (*4*). Wines aged on yeast lees have lower haze potential than wines aged without lees, and this is due to the protective effect of the mannoproteins released from yeast cell walls. In fact, the addition of some mannoproteins to wine results in higher protein stability (*5*). In addition, wines aged on lees are more stable against potassium bitartrate precipitation, which could affect unstabilized wines at low temperatures (*6*).

Even more interesting, yeast mannoproteins have been associated with several wine properties related to sensorial quality. This includes retention of aroma compounds (*7*, *8*), reduction of astringency (*9*), and increased body and mouthfeel (*8*, *10*, *11*), especially appreciated in red wines. Finally, mannoproteins seem to stimulate the growth of lactic acid bacteria (*12*, *13*) and, consequently, malolactic fermentation, and improve the foam quality of sparkling wines (*14*, *15*).

Industrial wine yeast strains differ in the amount of polysaccharides released during winemaking (*9*). Because, depending on their quality and type, strains releasing higher amounts of mannoproteins would produce higher quality wines, release of mannoproteins is a potential target for strain selection and/or genetic improvement of wine yeast.

To improve our understanding of the genetic determinants of mannoprotein release in *S. cerevisiae* and to help the design of strategies for the genetic improvement of industrial wine yeasts for mannoprotein production, we have studied mannoprotein release by laboratory strains of *S. cerevisiae* deleted for specific genes. These genes (listed below) have been chosen on the basis of published cell wall related phenotypes, such as release of cell wall constituents or altered cell wall composition (*16*−*20*).

GPI7 (YJL062w) encodes for an enzyme involved in the synthesis of the GPI anchor (*17*, *21*). The GPI anchor is a

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Table 1. Strains Used in the Study and Their Genotypes

strain	genotype
BY4741 derivatives	
BY4741	MATa; his3D1; leu2D0; met15D0; ura3D0
FKS1-4741	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YLR342w::kanMX4
GAS1-4741	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YMR307w::kanMX4
GPI7-4741	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YJL062w::kanMX4
KNR4-4741	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YGR229c::kanMX4
YBR183w-4741	BY4741; MAT a; his3zΔ1; leu2Δ0; met15Δ0; ura3Δ0; YBR183w::kanMX4
YDL231c-4741	BY4741; MAT a; his3zΔ1; leu2Δ0; met15Δ0; ura3Δ0; YDL231c::kanMX4
YNL080c-4741	BY4741; MAT a; his3zΔ1; leu2Δ0; met15Δ0; ura3Δ0; YNL080c::kanMX4
YOL092w-4741	BY4741; MAT a; his3zΔ1; leu2Δ0; met15Δ0; ura3Δ0; YOL092w::kanMX4
10000M derivatives	
10000M	MATa; ura3-52; leu2D 1; trp1D63; his3D200; GAL2
GPI7-10000	10000M; MATa; ura3-52; LEU2; his3Δ200; trp1Δ63; GAL2; YJL062w::kanMX4
KNR4-10000	10000M; MATa; ura3-52; LEU2; HIS3; TRP1; GAL2; YGR229c::kanMX4
YBR183w-10000	10000M; MATa; ura3-52; LEU2; his3Δ200; TRP1; GAL2; YBR183w::kanMX4
YDL231c-10000	10000M; MATa; ura3-52; LEU2; HIS3; trp1Δ63; GAL2; YDL231c::kanMX4
YNL080c-10000	10000M; MATa; ura3-52; leu2Δ1; HIS3; TRP1; GAL2; YNL080c::kanMX4
YNL294c-10000	10000M; MATa; ura3-52; leu2Δ1; his3Δ200; trp1Δ63; GAL2; YNL294c::kanMX4
W303 derivatives	
GAS1-303	MAT α; ade2-1; can1-100; his3-11; leu2-3; 112trp1-1; ura3-1; gas1::Leu2
W303 1B	MAT α; ade2-1; can1-100; his3-11; leu2-3; 112trp1-1; ura3-1
BY4743 derivatives	
BY4743	MATa/MATα; his3D1/his3D1; leu2D0/leu2D0; met15D0/MET15; LYS2/lys2D0; ura3D0/ura3D0
FKS1-4743A	BY4743; Mat a/α; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YLR342w::kanMX4/YLR342w
FKS1-4743B	BY4743; Mat a/α; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YLR342w::kanMX4/YLR342w::kanMX4
GAS1-4743A	BY4743; Mat a/a; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YMR307w::kanMX4/YMR307w
GAS1-4743B	BY4743; Mat a/α; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YMR307w::kanMX4/YMR307w::kanMX4
GPI7-4743A	BY4743; Mat a/α; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YJL062w::kanMX4/YJL062w
GPI7-4743B	BY4743; Mat a/α; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YJL062w::kanMX4/YJL062w::kanMX4
KNR4-4743A	BY4743; Mat a/α; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YGR229c::kanMX4/YGR229c
KNR4-4743B	BY4743; Mat a/α; his3D1/his3D1; leu2D0/leu2D0; lys2D0/LYS2; MET15/met15D0; ura3D0/ura3D0; YGR229c::kanMX4/YGR229c::kanMX4

structure mediating the linkage of some proteins to the plasma membrane or the cell wall. In *GPI7*-deficient strains, GPI-anchored proteins are not efficiently linked to the cell wall and are released to the medium (*17*).

GAS1 (YMR307w) encodes for a glycoprotein of the plasma membrane. Gas1p has β-1,3-glucanosyltransferase activity (*16*) and is involved in the elongation of β-1,3-glucan branches (*22*). Deletion of *GAS1* results in cell walls with a lower β-1,3-glucan content and in the release of glycosylated proteins to the medium (*16*).

FKS1 (YLR342w) encodes for a subunit of the β-1,3-glucan synthase. Strains deleted for *FKS1* show a phenotype similar to the gas1Δ mutant (*16*).

The gene product of *KNR4* (YGR229c) is also involved in the biosynthesis of the β-1,3-glucan (*23*). *KNR4* deletion results in a higher chitin content of the cell wall and a reduction of the β-1,3-glucan synthase activity (*23*, *24*).

YBR183w, YDL231c, YNL080c, YOL092w, and YNL294c were identified in a systematic screening for deletions inducing cell wall related phenotypes (*20*). Compared to the control strain, strains deleted in any of them release increased amounts of one or more cell wall components to the medium (*20*).

MATERIALS AND METHODS

Strains, Media, and Growth Conditions. The *S. cerevisiae* strains used in this study are listed in **Table 1**. BY4741, BY4743, and their derivatives were obtained from EUROSCARF. W303-1B deleted for *GAS1* was a gift from Laura Popolo (Università degli studi di Milano, Italy). Strain 10000M was obtained from EUROSCARF. 10000M derivatives were a gift from José Manuel Rodríguez-Peña (Universidad Complutense de Madrid, Spain). Cultures were performed in GCY medium [2% glucose, 2% Bacto Casaminoacids (BD, Sparks, MD), 0.67% Difco yeast nitrogen base (BD)] supplemented with 0.34 mM uridine for uridine auxotrophs. Each strain was inoculated from a fresh preculture in the same medium, for a starting $OD_{600} = 0.1$ unit, and incubated at 30 °C and 150 rpm up to stationary phase. Media were then recovered by centrifugation for the analysis of polysaccharide content. YPD (2% glucose, 2% peptone, 1% yeast extract) or minimal medium [2% glucose, 0.67% Difco yeast nitrogen base (BD) supplemented with 0.34 mM uridine for uridine auxotrophs] was used in some experiments as described below.

Quantification of Total Mannoproteins and Polysaccharides. Macromolecules in the culture media were separated from monosaccharides by gel filtration in Econo-Pac 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) according to the phenol−sulfuric method as described by Segarra et al. (*25*). Five replicates were performed for each determination, and data were analyzed using SPSS 13.0 by one-way ANOVA and Student−Newman−Keuls or Dunett tests for comparison of means. To compensate for the different biomass yields, data on polysaccharide content were also compared after standardization for 1 OD_{600} unit.

Concanavalin A Detection. The proteins from the supernatants were resolved by SDS-PAGE (*26*). Different amounts of supernatant were

Table 2. Concentration of Total Mannoproteins and Polysaccharides Released by the Haploid Strains

strain	[polysaccharide] (mg/L)	[polysaccharide]/DO_{600} (mg/L)/DO_{600}	strain	[polysaccharide] (mg/L)	[polysaccharide]/DO_{600} (mg/L)/DO_{600}
BY4741	13	1.5	10000M	19	3.8
FKS1-4741	37[a]	4.5[a]	GPI7-10000	16	5.1
GAS1-4741	85[a]	13.7[a]	KNR4-10000	81[b]	9.4[a]
GPI7-4741	111[a]	14.9[a]	YBR183w-10000	19	2.6
KNR4-4741	28[a]	3.8[a]	YDL231c-10000	18	3.9
YBR183w-4741	13	1.6	YNL080c-10000	26	3.7
YDL231c-4741	15	1.9	YNL294c-10000	25	4.9
YNL080c-4741	26[a]	2.9[a]	GAS1-303	73[c]	21.0[a]
YOL092w-4741	14	1.8	W303 1B	26	4.5

[a] Concentration of polysaccharides significantly different ($n = 5$) from the control strain BY4741 [b] Concentration of polysaccharides significantly different ($n = 5$) from the control strain 10000M. [c] Concentration of polysaccharides significantly different ($n = 5$) from the control strain W303 1B.

used to have the same OD_{600} equivalent 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. (*27*).

Haze Protection Studies. A Sauvignon Blanc wine was prepared in the laboratory to perform stabilization tests, because commercial white wines are already stabilized by bentonite treatment and are not suitable for haze protection studies. Twenty kilograms of Sauvignon Blanc grapes was pressed in laboratory baskets, and the must was inoculated with the winemaking strain of *S. cerevisiae* EC1118 (Lallemand, Ontario, Canada) to a concentration of 10^6 cells/mL. Fermentation was performed at 20 °C in Erlenmeyer flasks containing 500 mL of must, closed with Müller valves, and monitored by loss of weight until it was constant. Immediately after the end of fermentation, yeast cells were removed by centrifugation and the wine was stored at 4 °C.

Haze protection potential was studied using freeze-dried macromolecules from 30 mL of culture medium prepared by gel filtration as described above. They were dissolved in 45 mL of Sauvignon Blanc wine and divided in 4.5 mL aliquots for stability testing. In some experiments, invertase (Sigma) was used as a control. Stability was assayed by incubating at 85 °C for 30 min and cooling on ice. The turbidity of the wines was determined in a nephelometer (Hach, Loveland, CO), and data from nine replicates were analyzed by ANOVA as described above.

RESULTS

Release of Mannoproteins. Preliminary assays to estimate the release of mannoproteins and polysaccharides by the different strains were performed with the standard yeast complete medium YPD, but the high background signal values obtained precluded any acceptable quantification (data not shown). Because, on the other hand, minimal medium resulted in far too low biomass yields, we finally developed GCY medium (2% glucose, 2% casaminoacids, and 0.67% YNB), which results in biomass yields similar to YPD and lower background for polysaccharide quantification. A second methodological adjustment, involving the method used for the separation of mannoproteins and polysaccharides from monomeric or oligomeric sugar, was necessary to further reduce the background. This was due to the observation that glucose present in the noninoculated medium precipitated by using previously published methods for polysaccharide precipitation. This problem was overcome by replacing the precipitation step by size exclusion chromatography, as described under Materials and Methods. The method finally chosen consisted in using GCY as the growth medium and size exclusion chromatography for the purification of mannoproteins and polysaccharides, followed by quantification according to the phenol−sulfuric method.

In a first step we used this optimized method to quantify the mannoproteins and polysaccharides released by all of the haploid strains listed in **Table 1**. The results are shown in **Table 2**. For each deletion mutant the average amount of mannoproteins and polysaccharides was compared with the cognate wild-type strain. In the BY4741 genetic background five strains released more mannoproteins and polysaccharides than the control. Three of them, strains deleted in *FKS1* (FKS1-4741), *YNL080c* (YNL080-4741), and *KNR4* (KNR4-4741), showed moderate, albeit statistically significant, increments in released polysaccharides, whereas the amount released by strains deleted in *GAS1* (GAS1-4741) or *GPI7* (GPI7-4741) was 7 or 9 times higher, respectively, than the control (**Table 2**). The results were similar after standardization based on the final OD_{600} of the cultures (**Table 2**). This standardized value was calculated also for samples taken all through the growth curve, and it showed to be stable from midexponential to the stationary phase (data not shown). In the 10000M genetic background, only *KNR4* deletion resulted in an increased polysaccharide content in the supernatant (**Table 2**). The amount of polysaccharides was 4 times higher than the control strain (2.4 after standardization, **Table 2**). Finally, *GAS1* deletion was assayed in a W303 background, showing a 3-fold increase in the amount of polysaccharides, which is lower than observed in the BY4741 background.

To assess the relationship between release of polysaccharides and actual mannoprotein content in the culture media, supernatants of all cultures were analyzed by SDS-PAGE and concanavalin A detection as described under Materials and Methods. The results are shown in **Figure 1** and are in accordance with the previous data on total polysaccharide content. Mannoprotein bands corresponding to *FKS1*, *GAS1*, and *GPI7* deletions in a BY4741 background were more intense than the control, even though the method is not quantitative. Also in accordance with previous data, *KNR4*-deleted strains in the 10000M background showed mannoprotein bands more intense than its cognate control (**Figure 1**). In this background *GPI7* deletion shows mannoprotein bands similar to *KNR4* deletion, even though the measured total mannoprotein and polysaccharide content for GPI7-10000 is low.

To help the design of genetic engineering strategies to improve mannoprotein production by wine yeasts, it was interesting to know the behavior of diploid strains either heterozygous or homozygous for the deletions of interest. On the basis of the previous results *FKS1*, *GAS1*, *GPI7*, and *KNR4* were chosen for this study. All four mutations were typically recessive; none of the heterozygous strains released higher amounts of mannoproteins than the control (data not shown). Conversely and as expected, homozygous strains showed a phenotype very similar to their haploid counterparts, with

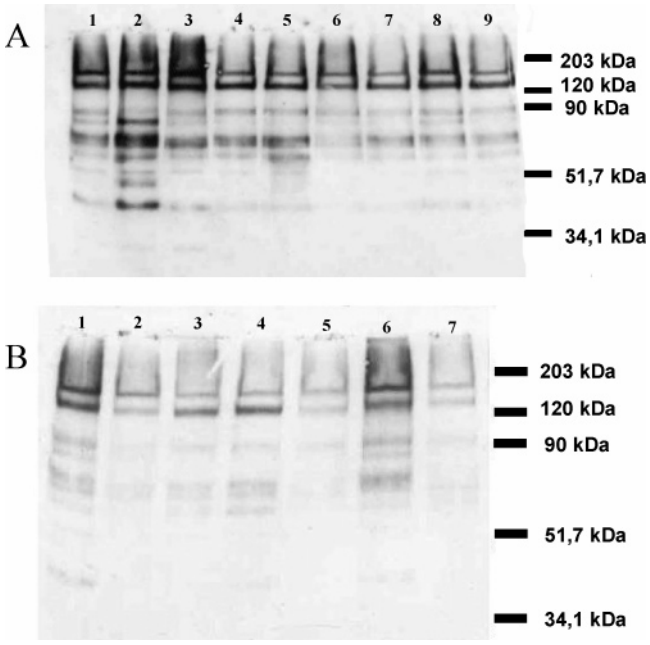

Figure 1. Mannoproteins detected by hybridization with peroxidase-conjugated concanavalin A: (**A**) (lane 1) FKS1-4741, (lane 2) GAS1-4741, (lane 3) GPI7-4741, (lane 4) YBR183-4741, (lane 5) YDL231-4741, (lane 6) YNL080-4741, (lane 7) YOL092-4741, (lane 8) KNR4-4741, (lane 9) BY4741; (**B**) (lane 1) GPI7-10000, (lane 2) YDL231c-10000, (lane 3) YNL080c-10000, (lane 4) YBR183w-10000, (lane 5) YNL294c-10000, (lane 6) KNR4-10000, (lane 7) 10000M.

Table 3. Concentration of Total Mannoproteins and Polysaccharides Released by the Diploid Homozygous Strains

strain	[polysaccharide] (mg/L)	[polysaccharide]/DO_{600} (mg/L)/DO_{600}
FKS1-4743B	37[a]	6.6[a]
GAS1-4743B	78[a]	11.3[a]
GPI7-4743B	96[a]	17.2[a]
KNR4-4743B	28[a]	4.2[a]
BY4743	15	2.0

[a] Concentration of polysaccharides significantly different ($n = 5$) from the control strain BY4743.

significantly higher amounts of polysaccharides for all of the deletion strains as compared to BY4743 (**Table 3**). The strains deleted for *GAS1* and *GPI7*, showing the highest increases in released polysaccharides, also showed more intense mannoprotein bands when analyzed by SDS-PAGE and concanavalin A detection (data not shown).

Wine Stabilization. Macromolecules recovered from the cultures of the haploid strains that showed the best results in release of polysaccharides and the highest intensity mannoprotein bands by concanavalin A detection were used for haze protection studies as described under Materials and Methods. All of the preparations resulted in enhanced stability of the Sauvignon Blanc wine against protein haze, with turbidity values ranging from 65 to 90% of those of the untreated wine. In the BY4741 background, macromolecules of the supernatants of strains deleted for *GAS1* and *GPI7* showed a protein haze protection effect superior to that of the control strains and similar to that obtained with 0.25 mg/mL invertase (**Table 4**). In addition, macromolecules from the strains deleted for *KNR4* in the 10000M background induced higher stability than the control (**Table 4**). Macromolecules from the strains that released only moderately higher amounts of mannoproteins (as compared to the control) resulted in stabilization levels similar to the control strains.

Table 4. Percentage of Turbidity of Wines Treated with Mannoproteins Released by the Strains

sample	% turbidity	sample	% turbidity
control wine	100.0	KNR4-4741	77.3
Invertase 0.25 mg/L	62.6[a]	BY4741	76.6
FKS1-4741	80.5	KNR4-10000	77.5[b]
GAS1-4741	68.8[a]	10000M	90.1
GPI7-4741	65.7[a]		

[a] Turbidity significantly different ($n = 9$) from the control strain BY4741. [b] Turbidity significantly different ($n = 9$) from the control strain 10000M.

DISCUSSION

The experimental approach used in this work, involving the use of GCY medium, gel filtration, total polysaccharide quantification, and specific detection of mannoproteins, as a confirmatory step, allows the fast identification of genes the deletion of which resulted in increased levels of mannoproteins in the culture medium. Mannoprotein detection with concanavalin A is not quantitative and is less sensitive than total polysaccharide quantification; this could partly explain why strains showing only moderate increases in total polysaccharides in the culture medium did not result in more intense mannoprotein bands. Alternatively, because not all of the mutations assayed are specific for mannoproteins, but result in general dysfunctions of the cell wall, it is possible that some mutants release increased amounts of other cell wall polysaccharides (namely glucans) or the mannoproteins released by each strain are different.

There is a clear effect of the genetic background on the release of mannoproteins and polysaccharides. This is clearly exemplified by strains deleted for *GPI7*, showing a huge release of mannoproteins and polysaccharides in BY4741 background, but much more moderate in the 10000M background; similarly, *GAS1* deletion resulted in a higher increase in the released polysaccharides in the BY4741 background than in W303 background (**Table 2**). The results obtained for homozygous and heterozygous diploid strains indicate a recessive phenotype for the deletions tested. This is in accordance with the fact that they are loss-of-function mutations and, additionally, indicate that the proteins coded by these genes are not in limiting amounts in the growth conditions tested.

The secreted polysaccharides are active in the stabilization of Sauvignon Blanc wine against protein haze. Our results do not indicate a linear correlation between the amount of polysaccharides released and the level of stabilization obtained, because moderate increases in the amount of polysaccharides did not result in any appreciable protein haze stabilization. This could be related to the potentially different natures of the polysaccharides released by each strain, as discussed above, or to the fact that the macromolecular extracts were added to a wine already containing mannoproteins from the strain used for fermentation. Polysaccharides from *GAS1*- and *GPI7*-deleted strains resulted in stabilization levels similar to those obtained by adding 0.25 mg/mL invertase, used as a control (**Table 4**), the stabilizing effect of which was recognized earlier (*28*, *29*).

Among the genes studied, deletion of *GPI7*, *GAS1*, or *KNR4* resulted in the highest release of polysaccharides, including mannoproteins. The first one is involved in the synthesis of the GPI anchor, mediating among other functions the binding of

many mannoproteins to the yeast cell wall glucan. Its effect on the release of mannoproteins seems to be direct. Products of the two other genes are involved in the synthesis of β-1,3-glucan. The effect of the cognate deletions is probably indirect, due to the failure of GPI-anchored cell wall proteins to bind a defective glucan layer.

The results presented in this work suggest ways to genetically engineer wine yeast to improve mannoprotein release and protein stability (or other quality parameters affected by mannoprotein content) of the wines obtained. This would consist in the deletion of *GPI7*, *GAS1*, or *KNR4*. Unfortunately, deletion of *GPI7* or *GAS1* results in strains with greatly impaired growth (*30*−*32* and data not shown). Deletion of these genes could result in wine yeast strains unable to perform alcoholic fermentation in industrial conditions. In our hands (data not shown) the growth of *KNR4*-deleted strains is much more similar to the wild type. In addition, albeit results in **Table 2** indicate an effect of the genetic background on the release of polysaccharides by *KNR4* or *GPI7* defective strains, it is also shown that *KNR4* deletion leads to an increase in the release of polysaccharides in the two genetic backgrounds tested. Consequently, and considering the uncertainty about the effect of industrial genetic backgrounds over mannoprotein release, deletion of *KNR4* in industrial strains would be preferred. In principle, because we have shown that the deletion of any of these genes is recessive and wine yeasts are expected to carry two or more copies of any gene (*33*), two or more rounds of deletion would probably be necessary to obtain the desired phenotype. A possible shortcut would come if, in the wine yeast genetic background, and in wine fermenting conditions, the product of the chosen gene becomes limiting in heterozygosis, so that the expected phenotype could be obtained after the first round of deletion.

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