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Controlled Expression of the Dominant Flocculation Genes *FLO1*, *FLO5*, and *FLO11* in *Saccharomyces cerevisiae*▽

Patrick Govender,[1,2] Jody L. Domingo,[2] Michael C. Bester,[2] Isak S. Pretorius,[3] and Florian F. Bauer[2]*

Department of Biochemistry, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa[1]; Institute for Wine Biotechnology, Faculty of AgriSciences, University of Stellenbosch, Private Bag X1, Matieland (Stellenbosch) 7602, South Africa[2]; and The Australian Wine Research Institute, P.O. Box 197, Glen Osmond (Adelaide), South Australia 5064, Australia[3]

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In many industrial fermentation processes, the *Saccharomyces cerevisiae* yeast should ideally meet two partially conflicting demands. During fermentation, a high suspended yeast count is required to maintain a satisfactory rate of fermentation, while at completion, efficient settling is desired to enhance product clarification and recovery. In most fermentation industries, currently used starter cultures do not satisfy this ideal, probably because nonflocculent yeast strains were selected to avoid fermentation problems. In this paper, we assess molecular strategies to optimize the flocculation behavior of *S. cerevisiae*. For this purpose, the chromosomal copies of three dominant flocculation genes, *FLO1*, *FLO5*, and *FLO11*, of the haploid nonflocculent, noninvasive, and non-flor-forming *S. cerevisiae* FY23 strain were placed under the transcriptional control of the promoters of the *ADH2* and *HSP30* genes. All six promoter-gene combinations resulted in specific flocculation behaviors in terms of timing and intensity. The strategy resulted in stable expression patterns providing a platform for the direct comparison and assessment of the specific impact of the expression of individual dominant *FLO* genes with regard to cell wall characteristics, such as hydrophobicity, biofilm formation, and substrate adhesion properties. The data also clearly demonstrate that the flocculation behavior of yeast strains can be tightly controlled and fine-tuned to satisfy specific industrial requirements.

Industrial fermentations for the production of bioethanol, wine, beer, and other alcoholic beverages are performed in batch processes. At the end of fermentation, the suspended *Saccharomyces cerevisiae* yeast cells must be removed prior to further processing of the fermentation product. The separation of suspended yeast cells may have to be achieved by centrifugation or filtration, which are time-consuming and expensive procedures.

Alternatively, clarification can be achieved by natural settling of the yeast. While single yeast cells tend to settle over time, natural settling becomes a viable option in industrial processes only when cells aggregate, a process also referred to as flocculation. Flocculation is defined as the asexual, reversible, and calcium-dependent aggregation of yeast cells to form flocs containing large numbers of cells that rapidly sediment to the bottom of the liquid growth substrate (7, 53). Although flocculation could provide a seemingly ideal solution to the removal of yeast cells after primary fermentation, it should not occur before the fermentation is completed. As a matter of fact, early flocculation may result in sluggish or stuck fermentation and final products with high residual sugars and unsatisfactory aromatic characteristics (63).

Flocculation in *S. cerevisiae* is mediated by specific cell surface lectins (or flocculins) that are capable of binding directly to mannose residues of mannan molecules on adjacent cells (37, 53). This interaction leads to cellular aggregation and finally settling. In some specific cases, cellular aggregation does not lead to settling, but to yeast cells rising to the surface of the substrate and forming an air-liquid interfacial biofilm. This behavior is also referred to as flotation or flor formation (41, 70).

In *S. cerevisiae*, two distinct flocculation phenotypes have been characterized on the basis of their sensitivity to sugar inhibition, namely, Flo1 (mannose sensitive) and NewFlo (mannose and glucose sensitive) (55). Most brewer's yeast strains are of the NewFlo phenotype, and flocculation in these strains is initiated after the end of exponential respiro-fermentative phase of growth (47). The late onset of flocculation in yeast cells with the NewFlo phenotype makes them ideally suited to their task by aiding separation of biomass from the brew.

The genetic basis of flocculation has been the object of several investigations. These studies suggest that a family of subtelomeric genes, *FLO1*, *FLO5*, *FLO9*, and *FLO10*, encode specific lectins that are responsible for flocculation (56). A nonsubtelomeric gene, *FLO11*/*MUC1* (30, 35), on the other hand, encodes a protein that has been associated with flocculation, flor formation, invasive growth, and substrate adhesion (3, 18, 24, 35, 70). All Flo proteins are glycosylphosphatidylinositol-linked glycoproteins that share a common three-domain structure consisting of an N-terminal lectin domain, a central domain of highly repeated sequences rich in serine and threonine residues, and a carboxyl-terminal domain containing a glycosylphosphatidylinositol-anchoring sequence (reviewed in reference 62). In recent studies (33, 34), evidence was presented that the difference between the NewFlo and Flo1 flocculation phenotypes may be at least partially due to variations

* Corresponding author. Mailing address: Institute for Wine Biotechnology, Faculty of AgriSciences, University of Stellenbosch, Private Bag X1, Matieland (Stellenbosch) 7602, South Africa. Phone: 27 21 808 4346. Fax: 27 21 808 3771. E-mail: fb2@sun.ac.za.

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in the number of repeat sequences within the *FLO1* coding sequence.

The regulation of *FLO* gene expression is complex, and in particular, the promoter of *FLO11* has been intensively studied. *FLO11* expression is tightly controlled by environmental factors, and several signaling cascades, including the Ras-cyclic AMP-dependent kinase complex, the filamentous-growth-controlling mitogen-activated protein kinase, and the main glucose repression pathways have been directly linked to *FLO11* regulation (reviewed in reference 62). Two transcriptional regulators, Mss11p and Flo8p, have been shown to play a central role in the control of flocculation and flotation phenotypes (4, 32, 58). These investigations have shown that *FLO11* transcriptional regulation is particularly dependent on the nutritional status and specific composition of the growth environment. Less information is available regarding the regulation of other *FLO* genes, although it has been shown that *FLO1* expression is also controlled by nutritional status signals, such as carbon and/or nitrogen starvation (47), and other environmental indicators, such as pH (52) and ionic strength (26).

In addition to this transcriptional regulation, *FLO* gene activity has been shown to be modulated by other regulatory systems. In particular, data suggest that these genes are often under promoter-specific epigenetic control allowing *S. cerevisiae* cells in a homogenous population to reversibly switch between active *FLO* gene expression and silent modes (19). Furthermore, sequence analysis reveals that several DNA motifs in the central domain are conserved among different *FLO* genes, promoting diversity of adhesins by frequent intragenic recombination events (61).

Considering the complexity of *FLO* gene regulation, it is evident that manipulation of both physiological and environmental factors offers winemakers and brewers limited avenues to control or alter flocculation during fermentations. Therefore, it is not surprising that industrial yeast strains generally possess a less than optimal flocculation profile (8, 60). For this reason, replacement of the native promoters of these genes with less complex promoters conferring expression patterns that would be better adapted to industrial needs may result in yeast strains that display improved flocculation behavior for specific industrial purposes.

In previous attempts to modify flocculation behavior, the flocculation genes *FLO1* and *FLO5* were introduced into nonflocculent *S. cerevisiae* brewing yeast strains (2, 23, 65–67). However, the resultant modified yeast strains flocculated constitutively and displayed reduced fermentation performance or increased fermentation times. In an approach similar to the one described here, Verstrepen et al. (63) brought the chromosomal *FLO1* gene of the haploid nonflocculent *S. cerevisiae* FY23 laboratory strain under the transcriptional control of the *HSP30* stationary-phase promoter. The resulting strain showed strong flocculation toward the end of fermentation, resulting in a distinctly clearer beer than the beer obtained with wild-type cells (63).

In this study, we assess the suitability of six genome-integrated promoter-gene combinations to control stationary-phase-specific flocculation. For this purpose, the native promoters of the dominant flocculation genes *FLO1*, *FLO5*, and *FLO11* in the haploid *S. cerevisiae* FY23 strain were replaced with the inducible promoters *ADH2* and *HSP30*. The *ADH2* promoter is subjected to carbon catabolite repression and has been shown to be repressed several hundredfold during growth on glucose (16, 42). Derepression of the *ADH2* promoter generally coincides with transition to growth on ethanol (39). The *HSP30* promoter, on the other hand, has been shown to be induced during entry into the stationary phase of growth, which coincides with the depletion of glucose from the medium, which is found under low-stress nutrient-rich wort and wine fermentation conditions (15, 44, 45). In addition, the *HSP30* promoter is activated by several stress factors, including heat shock and sudden exposure to either ethanol or sorbate (40, 44, 49). Unlike *ADH2* regulation, which is reasonably well understood (14, 59, 69), the mechanism by which *HSP30* is induced in response to stress remains unclear.

TABLE 1. *S. cerevisiae* strains employed in this study

Strain	Genotype	Reference
FY23	*MAT***a** *leu2 trp1 ura3 flo8-1*	68
FY23-F1A	*MAT***a** *leu2 trp1 ura3 flo8-1 FLO1*::*SMR1-ADH2*	This study
FY23-F1H	*MAT***a** ***leu2 trp1 ura3 flo8-1*** *FLO1*::*SMR1-HSP30*	This study
FY23-F5A	*MAT***a** *leu2 trp1 ura3 flo8-1 FLO5*::*SMR1-ADH2*	This study
FY23-F5H	*MAT***a** *leu2 trp1 ura3 flo8-1 FLO5*::*SMR1-HSP30*	This study
FY23-F11A	*MAT***a** *leu2 trp1 ura3 flo8-1 FLO11*::*SMR1-ADH2*	This study
FY23-F11H	*MAT***a** ***leu2 trp1 ura3 flo8-1*** *FLO11*::*SMR1-HSP30*	This study

Since the dominant *FLO* genes are transcriptionally silent in the *S. cerevisiae* FY23 strain due to a nonsense mutation in the *FLO8* gene (32, 61, 68), expression regulated by either the *ADH2* or *HSP30* promoter constructs allows both assessments of the phenotypic consequences of the expression of a particular Flo protein and of the transcriptional character of a promoter in the same genetic background. Indeed, it is difficult to compare reports on flocculation in the literature due to the numerous techniques employed and the variations therein, coupled with the different yeast strain genetic backgrounds (26). Therefore, the inducible expression of three dominant flocculation genes, *FLO1*, *FLO5*, and *FLO11*, in the haploid laboratory strain *S. cerevisiae* FY23 strain reported in this study presents a unique opportunity to compare the adhesion characteristics (flocculation, invasive growth, and flor formation) of the aforementioned flocculation genes.

Our data show that each promoter-open reading frame (ORF) combination leads to specific flocculation and adhesion behaviors and results in additional important changes in cell surface properties, including hydrophobicity. The data indicate that highly specific flocculation behavior can be stably conferred to individual yeast strains.

MATERIALS AND METHODS

Strains. The yeast strains employed in this study are listed in Table 1. All strains were derived from *Saccharomyces cerevisiae* strain FY23 (32, 68). *Escherichia coli* DH5α (Gibco BRL/Life Technologies, Rockville, MD) was used as a host for all plasmid amplifications.

Media and cultivation conditions. Yeast strains were routinely cultivated at 30°C in rich YEPD medium, containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, and 2% (wt/vol) glucose. Single yeast colonies from 3-day-old YEPD

TABLE 2. Primers used in this study

Primer[a]	Sequence (5′→3′)[b]	Underlined sequence[c]
FLO1::SMR1-F	TGCGTCACTTTTCCTACGGTGCCTCGCACATGAATGTT ATCCGGCGCACGGGTACCGGCTTGGCTTCAGTT GCTG	Homologous *FLO1*p region matching nucleotides (nt) −813 to −764
FLO5::SMR1-F	GCAATAAACCACATGGCTACCGCACTTCTTGTCACTAT CCGGTACCGGCTTGGCTTCAGTTGCTG	Homologous *FLO5*p region corresponding to nt −1995 to −1956
FLO11::SMR1-F	TCACTGCACTTCAACTATGCCTTATAGCAACCAAGAA GCTAGAAAATGCCAACTATTAAAAAGATAACCTCTC GGTACCGGCTTGGCTTCAGTTGCTG	Homologous *FLO11*p region corresponding to nt −2710 to −2639
SMR1-R	CATGGGATCCAGCTTGCAATTTTTGACGGCCCC	BamHI restriction site
ADH2-F	TGACAGATCTAACTCGTTCCAGTCAGGATTG	BglII restriction site
ADH2-R	TGATAGTTGATTGTATGCTTTTTGTAGC	
ADH2::FLO1-R	CTGCCAAAAACATATAGCGATGAGGCATTGTCATTTT TGGATGTTCTGTTTACTGGTGACTGATAGTTGATTG TATGCTTTTTGTAGC	Homologous *FLO1*p region corresponding to nt −26 to +34
ADH2::FLO5-R	GCTAATCAATTTAAAGAAAATCAATTGCGGAATTTAC TGCAGAGCTGATAGTTGATTGTATGCTTTTTGTAGC	Homologous *FLO5*p region corresponding to nt −58 to −14
ADH2::FLO11-R	GGACCAAATAAGCGAGTAGAAATGGTCTTTGCATAGT GTGCGTATATGGATTTTTGAGGCTGATAGTTGATTG TATGCTTTTTGTAGC	Homologous *FLO11*p region corresponding to nt −26 to +34
HSP30-F	CATGAGATCTGATGGCATTGCACTCAAG	BglII restriction site
HSP30-R	TATTAAAGTCTCAAACTTGTTGTTTTG	
HSP30::FLO1-R	GCGATGAGGCATTGTCATTTTTGGATGTTCTGTTTACT GGTGACAAAAGATATTAAAGTCTCAAACTTG	Homologous *FLO1*p region corresponding to nt −26 to +18
HSP30::FLO5-R	GCTAATCAATTTAAAGAAAATCAATTGCGGAATTTAC TGCAGAGCTATTAAAGTCTCAAACTTG	Homologous *FLO5*p region corresponding to nt −58 to −14
HSP30::FLO11-R	GGACCAAATAAGCGAGTAGAAATGGTCTTTGCATAGT GTGCGTATATGGATTTTTGAGGCAAAAGATATTAAA GTCTCAAACTTGTTG	Homologous *FLO11*p region corresponding to nt −26 to +34
FLO1-F	AAGTGTGCGTCACTTTTCCTACGGT	
FLO1-F2	ATGGCACTAGTCGATCGAGG	
FLO1-R	AGCGATGAGGCATTGTCATTT	
FLO5-F	GCAATAAACCACATGGCTACC	
FLO5-F2	GGTTGTGTTCTA GGACTTTCTGACG	
FLO5-R	AGTGGTGCTAATCAATTTAAAGAA	
FLO11-F	CCTCTCACTGCACTTCAACTATGC	
FLO11-F2	TTACGGCCTAATGTCGAGAC	
FLO11-R	GGACCAAATAAGCGAGTAGA	

[a] The presence of a F or R at the end of the primer name indicates that the primer is a forward or reverse primer, respectively.
[b] Nonunderlined sequences correspond to *ADH2*, *HSP30*, and *SMR1-410* or *FLO* gene sequences as denoted by the primer name.
[c] Nucleotide numbering has been done by assigning the A in the ATG start codon of the open reading frame as base 1.

plates were used to inoculate starter cultures in 40 ml YEPD broth contained in 250-ml Erlenmeyer flasks, which were incubated at 30°C with shaking (160 rpm) for 18 h. These starter cultures were used to inoculate precultures at an initial cell density of 5×10^5 cells/ml which were incubated at 30°C with shaking (160 rpm) for 18 h. Thereafter, yeast cells for inoculation of experimental cultures were routinely prepared as follows using ice-cold reagents. Yeast cells from precultures were harvested by centrifugation (4,000 rpm, 5 min), washed once with 100 mM EDTA (pH 7) to ensure deflocculation, washed once with 30 mM EDTA (pH 7), and finally resuspended in 30 mM EDTA (pH 7). To determine the onset of flocculation, flocculent ability, glucose utilization, and growth rate of yeast in nutrient-rich medium, experimental cultures were seeded at an initial cell density of 5×10^5 cells/ml into 40 ml YEPD broth and incubated at 30°C with shaking (160 rpm). At 2-h intervals, for a period of 24 h and a 48 h time point, cell populations were harvested and deflocculated as described previously. The flocculation ability of FY23-F11A and FY23-F11H strains was also assessed in media with a composition identical to that of YEPD medium but with an alternative carbon source, namely, YEPE (3% [vol/vol] ethanol) and YEPGE (3% [vol/vol] ethanol together with 3% [vol/vol] glycerol) were used. In addition, flocculation and invasive growth tendencies were also assessed in chemically defined synthetic complete (SC) medium containing 0.67% (wt/vol) yeast nitrogen base (YNB) without amino acids (Difco, Detroit, MI) supplemented with all nutrients (50) and with the following as the sole carbon source: 2% (wt/vol) glucose (SCD medium), 0.2% (wt/vol) glucose (SCLD medium), 3% (vol/vol) ethanol (SCE medium), and 3% (vol/vol) ethanol with 3% (vol/vol) glycerol (SCGE medium). In addition to the above media, medium containing only the auxotrophic requirements (50) of strain FY23 (leucine, uracil, and tryptophan) was also used (SCD_{LUT} and $SCLD_{LUT}$ media). Flor medium containing 0.67% YNB and 3% (vol/vol) ethanol adjusted to pH 3.5 (24) containing all nutritional requirements was used to assess flor formation. For selection of sulfometuron methyl (SM)-resistant yeast transformants, SC medium containing 0.67% YNB and 2% (wt/vol) glucose was supplemented with amino acids for strain FY23 and 80 to 100 μg/ml SM (DuPont Agricultural Products, France). *E. coli* was grown at 37°C in Luria-Bertani (LB) medium (1% [wt/vol] Bacto tryptone, 0.5% [wt/vol] yeast extract, and 1% [wt/vol] NaCl), and bacterial transformants were selected using LB medium containing 100 mg/liter ampicillin. In this study, 2% agar (Difco) was used for all solid media. Bacterial and yeast strains were stored in LB medium containing 40% (vol/vol) glycerol and YPD supplemented with 15% (vol/vol) glycerol, respectively (1).

DNA manipulation, construction of promoter replacement cassettes, and yeast transformations. Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Expand High Fidelity PCR system (Roche Diagnostics GmbH) was employed for PCR amplifications. The amplification products were purified from agarose gels and cloned into pGEM-T Easy vector (Promega Corporation, Madison, WI) according to the specifications of the manufacturer. Standard procedures for bacterial transformations and plasmid isolation from *E. coli* were performed (46). Standard procedures for isolation and manipulation of DNA were employed in all other aspects of this study (1). The strategy for construction of promoter replacement cassettes was adapted from the strategy used in reference 64. The *ADH2* promoter region bearing a *FLO1* promoter (*FLO1*p) homologous sequence was amplified from pDLG5 plasmid (29) by PCR with ADH2-F and ADH2::FLO1-R primers (Table 2). The SM resistance yielding *SMR1-410* (*SMR1*) marker gene

TABLE 3. Real-time PCR primers and probes used in this study

Primer or probe[a]	Sequence (5′→3′)	Modification[b]
Primers		
FLO1-F (Taqman MGB)	ATGCCTCATCGCTATATGTTTTTG	None
FLO1-R (Taqman MGB)	GCTCCTGAGGCCACACTAGTTAG	None
FLO5-F (Taqman MGB)	AGCACCACTAAAAAAAATGACAATTG	None
FLO5-R (Taqman MGB)	GCCAGAAAGGCCAAGATTACC	None
Probes		
FLO1-probe	CAGTCTTTACACTTCTGGC	6-FAM 5′ label, 3′ minor groove binder/nonfluorescent quencher
FLO5-probe	ACCACTGCATATTTT	Vic dye 5′ label, 3′ minor groove binder/nonfluorescent quencher
FLO11-F-(QRT-PCR)	CCTCCGAAGGAACTAGCTGTAATT	None
FLO11-R-(QRT-PCR)	AGTCACATCCAAAGTATACTGCATGAT	None
PDA1-F-QRT-PCR	GGAATTTGCCCGTCGTGTT	None
PDA1-R-QRT-PCR	GCGGCGGTACCCATACC	None

[a] The presence of a F or R at the end of the primer or probe name indicates that the primer or probe is a forward or reverse primer or probe, respectively.
[b] 6-FAM, 6-carboxyfluorescein.

inclusive of promoter and terminator sequences was PCR amplified from plasmid pWX509 (9) with the SMR1-R and FLO1::SMR1-F primer pair. The *ADH2-FLO1*p 834-bp fragment in pGEM-T Easy was recovered by double restriction digestion with BglII and SpeI, while the *FLO1*p-*SMR1* insert (2,962 base pairs [bp]) was released by triple digestion with Alw441, BamHI, and SphI. Both fragments were subsequently ligated. The *FLO1*p-*SMR1-ADH2-FLO1*p vector was PCR amplified by using shorter primers FLO1-F and FLO1-R and ligation reaction mixture as the template. The integrating *FLO1* promoter replacement cassette (3,764 bp) was extracted from agarose gels and purified. A similar strategy was employed for the construction and synthesis of *FLO1*p-*SMR1-HSP30-FLO1*p, *FLO5*p-*SMR1-ADH2-FLO5*p, *FLO5*p-*SMR1-HSP30-FLO5*p, *FLO11*p-*SMR1-ADH2-FLO11*p, and *FLO11*p-*SMR1-HSP30-FLO11*p integrating promoter replacement cassettes. The primer pairs for different ORFs are as follows: primers FLO5-F and FLO5-R for *FLO5* and primers FLO11-F and FLO11-R for *FLO11*. Note that the *HSP30* promoter (*HSP30*p)-containing region was amplified using FY23 chromosomal DNA as the template. Yeast transformation with 5 μg of DNA was performed according to the lithium acetate method described by Gietz and Schiestl (17). Chromosomal integration was achieved by a double-crossover homologous recombination event in which the *FLO1*, *FLO5*, or *FLO11* gene was placed under transcriptional control of either the *ADH2* or *HSP30* promoter. The deletion of native promoters was confirmed by PCR using homologous primer sets. The primer pairs for transgenic strains were as follows: primers FLO1-F and FLO1-R for strains FY23-F1A and FY23-F1H, primers FLO5-F and FLO5-R for strains FY23-F5A and FY23-F5H, and primers FLO11-F and FLO11-R for strains FY23-F11A and FY23-F11H. In addition, the integration of promoter replacement cassettes in transformed yeast was further confirmed by PCR using heterologous primer sets that contained a forward primer from outside the region of integration and genomic DNA isolated from transformants. The primer pairs for different transformants were as follows: primers FLO1-F2 and ADH2-R for strain FY23-F1A, primers FLO1-F2 and HSP30-R for strain FY23-F1H, primers FLO5-F2 and ADH2-R for strain FY23-F5A, primers FLO5-F2 and HSP30-R for strain FY23-F5H, primers FLO11-F2 and ADH2-R for strain FY23-F11A, and primers FLO11-F2 and HSP30-R for strain FY23-F11H. The wild-type FY23 strain served as a control in these confirmation experiments.

Growth and enumeration of yeast populations. The cell density of suitably diluted yeast suspensions in 30 mM EDTA (pH 7) was determined either by direct cell counting with a hemocytometer or alternatively by measuring absorbance at 600 nm in a Cary 50 UV-visible spectrophotometer (Varian Inc., CA) using a standard curve as the reference.

Glucose determination. Cells were pelleted from 1-ml samples of experimental cultures by microcentrifugation (10,000 rpm, 1 min). The cell extracts were subsequently filtered through a 0.22-μm cellulose acetate filter and stored at −20°C until glucose analysis. The concentration of glucose in the culture medium was determined using a GAGO-20 glucose assay kit (Sigma, St. Louis, MO) according to the specifications of the manufacturer, using a Biotek 800ELX microplate reader (Biotek Instruments Inc., Winooski, VT).

Flocculation assays. The flocculent ability of yeast strains was established using the modified Helm's assay as described by D'Hautcourt and Smart (13). The percentage of flocculation reported in this paper represents the arithmetic mean of three independent determinations. To assess the influence of pH on flocculation, a composite suspension buffer with a very wide buffering range was adapted from the buffer used by Stratford (54) to replace the buffer employed in the above protocol. This buffer contained 10 mM calcium chloride, 50 mM Tris base, 50 mM succinic acid, 100 mM potassium hydroxide, and 4% (vol/vol) ethanol. The pH of the composite suspension buffer was adjusted with 5 M HCl, and flocculation was assessed as described above. To investigate sugar inhibition of *FLO1* and *FLO5* flocculation phenotypes, either mannose or glucose was added at various concentrations to both the washing and suspension buffers that are employed in the modified Helm's assay (13).

RNA extraction and cDNA synthesis. FY23, FY23-F11A, and FY23-F11A strains were precultured and treated as described above. Experimental batch cultures were inoculated in triplicate at an initial density of 5×10^5 cells/ml into 100 ml YEPD broth and incubated at 30°C with shaking (160 rpm) for 12, 16, and 48 h. To investigate the transcription of *FLO* genes, samples from batch cultures were washed with ice-cold H_2O, pelleted, and resuspended in ice-cold AE buffer (50 mM sodium acetate, 10 mM EDTA [pH 5.0]). Total RNA was isolated as previously described (48). DNA contamination was eliminated by DNase I (Roche Diagnostics) treatment. One microgram of total RNA was used as the template for cDNA synthesis using the ImProm-II reverse transcription system according to the manufacturer's instructions (Promega). cDNA samples were diluted 50 times with H_2O before real-time PCR analysis.

QRT-PCR analysis. Primers and probes used for quantitative real-time PCR (QRT-PCR) analysis are listed in Table 3 and were designed using Primer Express software version 3 (Applied Biosystems, CA). Reagents were purchased from Applied Biosystems and Kapa Biosystems (Cape Town, South Africa). QRT-PCR runs and collection of spectral data were performed with the 7500 cycler (Applied Biosystems). Sybr green was used for the detection of *PDA1* and *FLO11* amplicons with final primer concentrations of 100 nM. Specific probes and primers were designed to differentiate between the cDNA species corresponding to the extensively homologous *FLO1* and *FLO5* genes. Probes were modified by the addition of a 3′ minor groove binder and nonfluorescent quencher, as well as the 5′ attachment of fluorescent dyes as indicated in Table 3 (Applied Biosystems). Probe and primer concentrations were 250 nM and 900 nM, respectively, in QRT-PCRs. Cycling conditions during QRT-PCR were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s followed by 60°C for 1 min. When using Sybr green, a dissociation curve analysis was included to verify amplicon authenticity. Preliminary data analyses were performed with *S*ignal *D*etection *S*oftware (SDS) version 1.3.1. (Applied Biosystems). Individual QRT-PCR runs were performed at least in duplicate. The relative expression value for each sample was defined as $2^{-Ct(target)}$ where $Ct_{(target)}$ represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. Relative expression data were normalized to the relative expression value of the housekeeping gene *PDA1* in each respective sample, thus giving normalized relative expression for a target gene as $2^{-Ct(target)}/2^{-Ct(PDA1)}$.

Determination of hydrophobicity of yeast cell surfaces. The hydrophobicity of yeast cell surfaces was determined by measuring the distribution ratio of yeast

cells in a biphasic system consisting of a buffered solution and an organic solvent (22). Cultures in YEPD broth were incubated at 30°C for 48 h with shaking (160 rpm). The harvested cells from an experimental culture were deflocculated, washed, and diluted to a density of 5×10^6 cells ml^{-1} in 30 mM EDTA (pH 7). Yeast cells from a 20-ml aliquot of this suspension were washed twice and resuspended in 20 ml of phosphate-urea-magnesium (PUM) buffer (pH 7.1) (22). The absorbance of this suspension (*I*) was determined at 660 nm. Aliquots of 2.4 ml (three replicates) were dispensed into borosilicate glass tubes (15 by 75 mm), and 200 μl xylene was layered over the yeast suspension. The tubes were capped with rubber; samples were vortexed at maximum speed for 30 s and allowed to stand undisturbed for 15 min. The absorbance of the residual buffer layer (*F*) at 660 nm was determined. The average modified hydrophobic index (MHI) for a sample was calculated using the equation: MHI = 1 − (*F*/*I*).

Invasive growth plate assays. Yeast cultures processed as described above were adjusted to an optical density (measured at a wavelength of 600 nm) of 1.0, and 10-μl aliquots were dropped onto SCLD and $SCLD_{LUT}$ plates without piercing the agar surface and incubated for 5 days at 30°C. Using a gloved finger, superficial growth of yeast colonies was physically removed by washing the plates under a steady stream of water. Plates were allowed to air dry, and cells that invaded the agar were photographed.

Flor formation and buoyant cell density. Cells were precultured in YEPD medium, deflocculated, and washed as described above. Subsequently, 3×10^8 cells were recovered by microcentrifugation (10,000 rpm, 1 min), washed once, resuspended in 1 ml flor medium (pH 3.5), and added to test tubes (16 by 165 mm) containing 4 ml flor medium. Biofilm formation was photographed in natural light after 5 days of static incubation at 30°C. Alternatively, the cultures were incubated statically at 30°C for 60 h, after which 1-ml samples were withdrawn from just below the meniscus. The optical density of samples was determined spectrophotometrically at 600 nm.

Analysis of stress-induced expression of *FLO1*- and *FLO5*-encoded flocculins. FY23, FY23-F5H, and FY23-F1H strains were precultured and treated as described earlier. Experimental cultures were inoculated at an initial density of 5×10^5 cells/ml into 40 ml YEPD broth and incubated at 30°C with shaking (160 rpm) for 10 h. The incubation of untreated cells was extended for another 45 min at 30°C, whereas other cultures were exposed to the following stress treatments: heat shock for 30 min at 42°C, heat shock for 45 min at 42°C, 3% (vol/vol) ethanol for 30 min at 30°C, 6% (vol/vol) ethanol for 30 min at 30°C, and 6% (vol/vol) ethanol and heat shock for 30 min at 42°C. Ethanol (100%) was added directly to culture medium to yield a final concentration of 6% (vol/vol), and cultures were incubated at defined temperatures with shaking at 160 rpm. All cultures were placed on ice before flocculation was determined using the modified Helm's assay.

RESULTS

Yeast transformation. Following initial selection on SC plates containing SM, putative transformants were inoculated individually into YEPD broth and cultivated for 48 h at 30°C with shaking (160 rpm). A majority of strains transformed with the combinations of *FLO1*p-*SMR1-ADH2-FLO1*p, *FLO1*p-*SMR1-HSP30-FLO1*p, *FLO5*p-*SMR1-ADH2-FLO5*p, and *FLO5p-SMR1-HSP30-FLO5*p visually displayed strong flocculent phenotypes, suggesting that integration had occurred at the desired loci (Fig. 1A). Three independent transformants of each strain were selected for further analysis. No flocculent phenotype was detectable for putative transformants of strains FY23-F11A and FY23-F11H. A visual assessment of biofilm formation on flor medium was therefore used for the initial screen of putative *FLO11* transformants, and three independent flor-forming strains were retained for further analysis. For each of the selected strains, the deletion of native promoters was confirmed by PCR using homologous primer sets (Fig. 1B). In addition, integration at the correct gene locus was also confirmed by PCR (Fig. 1C) using primers in which the upstream primer was located outside the region of the inserted promoter replacement cassette.

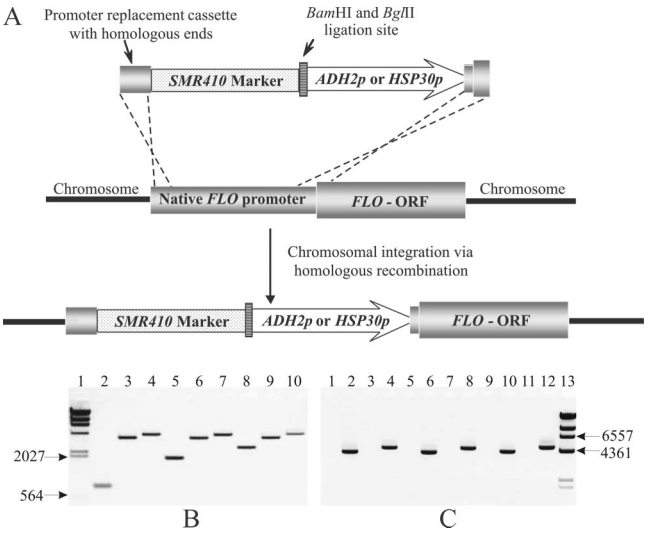

FIG. 1. Chromosomal integration of either the *ADH2* or *HSP30* promoter upstream of a dominant *FLO* gene in *S. cerevisiae* strain FY23. (A) Promoter replacement strategy. (B) The deletion of native promoters was confirmed by PCR using homologous primer pairs described in Materials and Methods. The amplification of the native promoter sequence was observed only in the wild-type FY23 strain (*FLO1*p [837 bp] [lane 2]), *FLO5*p [1,988 bp] [lane 5], and *FLO11*p [2,748 bp] [lane 8]), while only the integration cassette was amplified in strains FY23-F1A (*FLO1*p-*SMR1-ADH2-FLO1*p, 3,719 bp, lane 3), FY23-F1H (*FLO1*p-*SMR1-HSP30-FLO1*p, 4,198 bp, lane 4), FY23-F5A (*FLO5*p-*SMR1-ADH2-FLO5*p, 3,701 bp, lane 6), FY23-F5H (*FLO5*p-*SMR1-HSP30-FLO5*p, 4,180 bp, lane 7), FY23-F11A (*FLO11*p-*SMR1-ADH2-FLO11*p, 3,737 bp, lane 9), and FY23-F11H (*FLO11*p-*SMR1-HSP30-FLO11*p, 4,276 bp, lane 10). Lane 1 contained DNA molecular weight markers (phage lambda DNA restricted with HindIII). (C) The integration of promoter replacement cassettes were confirmed by PCR using heterologous primer sets that contained a forward primer from outside the region of integration and genomic DNA as the template as described in Materials and Methods. The amplification of *FLO1*p-*SMR1-ADH2*p (4,191 bp, strain FY23-F1A, lane 2), *FLO1*p-*SMR1-HSP30*p (4,670 bp, strain FY23-F1H, lane 4), *FLO5*p-*SMR1-ADH2*p (4,098 bp, strain FY23-F5A, lane 6), *FLO5*p-*SMR1-HSP30*p (4,577 bp, strain FY23-F5H, lane 8), *FLO11*p-*SMR1-ADH2*p (4,333 bp, strain FY23-F11A, lane 10), *FLO11*p-*SMR1-HSP30*p (4,812 bp, strain FY23-F11H, lane 12) is evident only in transformants, while lacking in the wild-type FY23 strain with corresponding primer pairs (lanes 1, 3, 5, 7, 9, and 11). Lane 13 contained DNA molecular weight markers (phage lambda DNA restricted with HindIII).

Stability, growth rates, glucose consumption, and flocculation. To assess the stability of the integrated promoter constructs, the selected transformed strains were cultivated in rich, nonselective medium in repeated batch cultures for more than 100 generations. For each strain, 20 individual colonies were then assessed for their flocculation behavior (*FLO1* and *FLO5* constructs) and flor-forming behavior (*FLO11* constructs). All tested colonies displayed the relevant phenotypes. The timing and intensity of the phenotypes were in all cases similar to those observed during the initial screen, indicating that the integration and resulting expression patterns are stable.

The growth rates and sugar utilization capabilities of the wild-type strain FY23 and its six transformants were assessed in YEPD medium containing 2% glucose at 2-hour intervals (Fig. 2 and 3). No significant differences between the wild-type FY23 strain and the transformants regarding biomass growth,

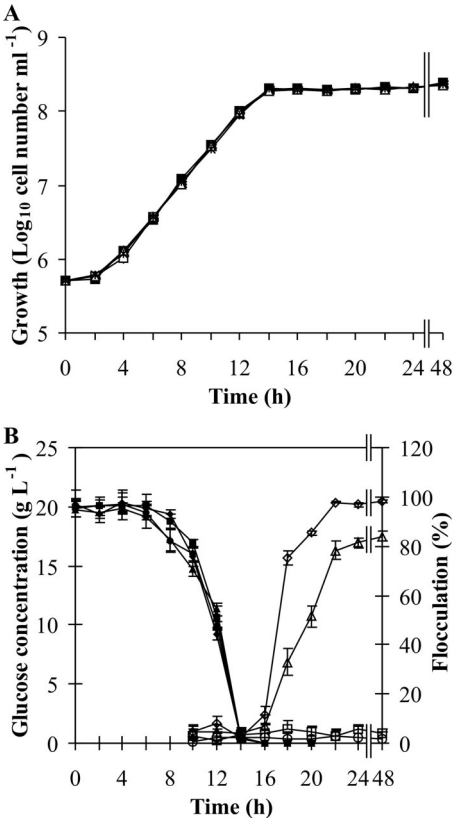

FIG. 2. (A) Growth of FY23 (wild type) (□), FY23-F1A (■), FY23-F5A (△), and FY23-F11A (218) strains. (B) Glucose utilization of FY23 (wild type) (■), FY23-F1A (◆), FY23-F5A (▲), and FY23-F11A (●) strains and flocculation profiles of FY23 (wild type) (□), FY23-F1A (◇), FY23-F5A (△), and FY23-F11A (○) strains. Yeast strains were cultivated in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm). Values represent the means of experiments performed in triplicate, and error bars represent standard deviations.

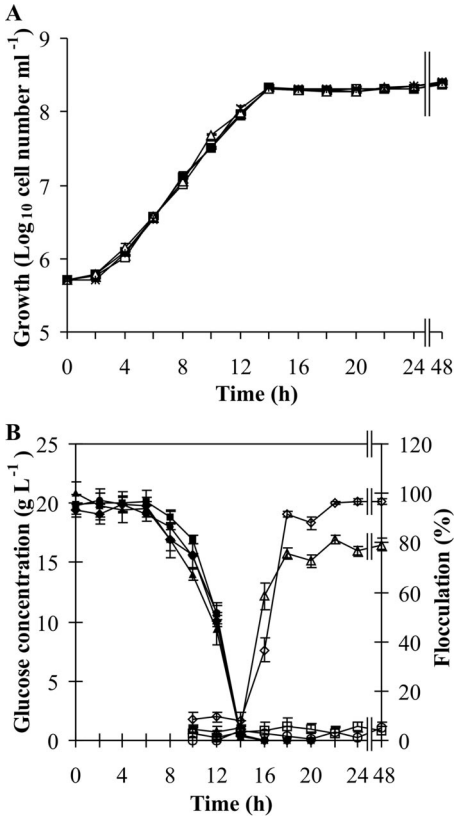

FIG. 3. (A) Growth of FY23 (wild type) (□), FY23-F1H (■), FY23-F5H (△), and FY23-F11H (218) strains. (B) Glucose utilization of FY23 (wild type) (■), FY23-F1H (◆), FY23-F5H (▲), and FY23-F11H (●) strains and flocculation profiles of FY23 (wild type) (□), FY23-F1H (◇), FY23-F5H (△), and FY23-F11H (○) strains. Yeast strains were cultivated in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm). Each point represents the mean of experiments performed in triplicate, and error bars represent standard deviations.

cell number, and sugar utilization capability were observed. As seen during the initial screen, strains transformed with combinations involving *FLO1* and *FLO5* ORFs showed flocculent behavior. Maximal flocculent ability of these strains was displayed 2 to 4 h after glucose depletion (Fig. 2B and 3B). In the *ADH2*p-*FLO1* and *ADH2*p-*FLO5* transformants, flocculation was observed approximately 2 h after glucose exhaustion, while maximum flocculation potential was evident after an additional 4 h (Fig. 2B).

After 48 h of growth in YEPD medium containing 2% (wt/vol) glucose, *FLO1* transformants (FY23-F1A [98% ± 1%] and FY23-F1H [97% ± 1%]) were more flocculent than the corresponding *FLO5* transgenic yeast strains (FY23-F5A [84% ± 2%] and FY23-F5H [79% ± 3%]) (Fig. 2B and 3B). This also suggests that the *ADH2* promoter (*ADH2*p)-controlled *FLO1* and *FLO5* phenotypes are slightly more flocculent than *HSP30*p-regulated phenotypes. This difference was obvious with macroscopic evaluation, where it was evident that *ADH2*p-induced *FLO1* and *FLO5* flocculent phenotypes are markedly stronger than *HSP30*p-mediated *FLO1* and *FLO5* flocculation phenotypes. *ADH2*p-*FLO1* flocs also formed larger clumps that remained at the bottom of the flasks even when agitated at 200 rpm (Fig. 4).

Interestingly, *FLO1* and *FLO5* transformants displayed decreased flocculation capacities in minimal media (data not shown). Under these conditions, the FY23-F1H and FY23-F5H strains, when cultivated in SCD medium containing all nutritional requirements or SCD_{LUT} medium that contained only the auxotrophic requirements of the strains displayed significantly higher flocculation abilities than the FY23-F1A

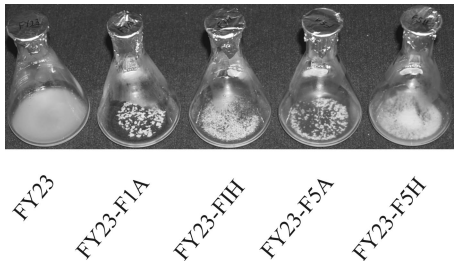

FIG. 4. Floc formation by FY23 (wild type), FY23-F1A, FY23-F1H, FY23-F5A, and FY23-F5H strains. Yeast strains were cultivated for 48 h in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm) and photographed in situ.

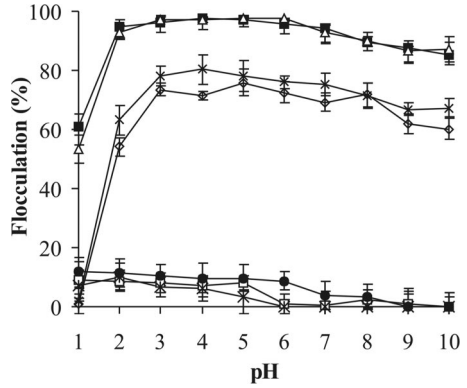

FIG. 5. Effect of pH on flocculation of FY23 (wild type) (□), FY23-F1A (■), FY23-F1H (△), FY23-F5A (×), FY23-F5H (◇), FY23-F11A (●), and FY23-F11H (218) strains. Yeast strains were grown for 48 h in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm). Flocculation was determined using a modified Helm's assay as described by D'Hautcourt and Smart (13) that incorporated a composite suspension buffer with a very wide buffering range from Stratford (54). Each point represents the mean of experiments performed in triplicate, and error bars represent standard deviations.

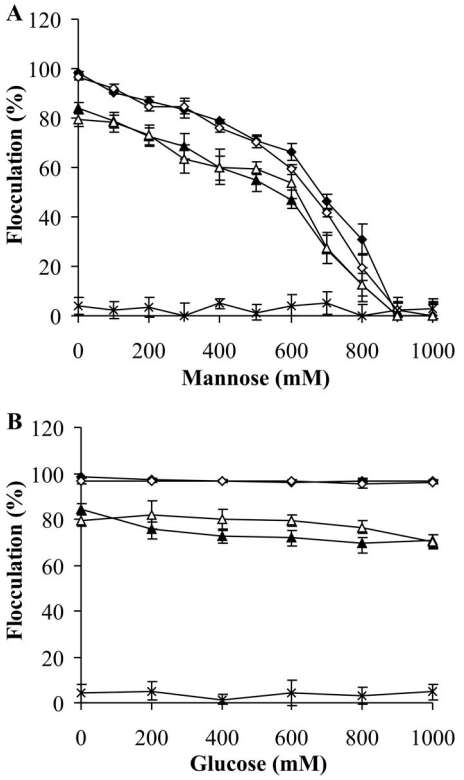

FIG. 6. Effects of mannose (A) and glucose (B) on flocculation of FY23 (wild type) (×), FY23-F1A (◆), FY23-F1H (◇), FY23-F5A (▲), and FY23-F5H (△) strains. Yeast strains were grown for 48 h in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm). Flocculation was determined using a modified Helm's assay as described in Materials and Methods. Values represent the means of experiments performed in triplicate, and error bars represent standard deviations.

and FY23-F5A strains did, with the latter strains not flocculating at all in SCD_{LUT} medium. *FLO11* expression mediated by either the *ADH2* or *HSP30* promoter in nutrient-rich YEPD medium (2% [wt/vol] glucose) (Fig. 2B and 3B), YEPE medium (3% [vol/vol] ethanol), or YEPGE medium (3% [vol/vol] ethanol and 3% [vol/vol] glycerol) and minimal media including SCD and SCD_{LUT} media did not yield a flocculent phenotype (results not shown).

The flocculent abilities of the wild-type FY23 strain and six transformed yeast strains were studied over a broad pH range (Fig. 5). The FY23-F11A and FY23-F11H strains and the wild-type FY23 strain displayed no significant flocculation ability over the entire pH range. The FY23-F1A and FY23-F1H strains displayed relatively stable flocculation between pH 2 and 10, whereas flocculation was reduced by nearly 40% at pH 1. In contrast, flocculation exhibited by the FY23-F5A and FY23-F5H strains was stable between pH 3 and 10, while flocculation was reduced by approximately 20% at pH 2 and completely abolished at pH 1. This supports previous findings which reported that Flo1-type flocculation displays a broad tolerance to pH (54), while a significantly reduced range (pH 4 to 5) was observed for NewFlo-type flocculation (51).

The relationship between sugar concentration and inhibition of flocculation in *FLO1* and *FLO5* transformants was also assessed (Fig. 6). Increasing concentrations of mannose were shown to have a progressively inhibitory effect on the flocculation of all these transformants, and flocculation was completely inhibited at 900 mM mannose (Fig. 6A). In contrast, no inhibitory effect was evident in the presence of glucose (Fig. 6B). Although Kobayashi et al. (28) reported residual flocculation of 22% at 10 mM mannose for a *FLO1*-expressing *S. cerevisiae* strain displaying Flo1-type flocculation, the overall mannose inhibitory profile reported is similar to this finding. It can be suggested that the concentration of mannose required for complete inhibition of Flo1-type flocculation is variable and strain dependent. This may simply be a consequence of Flo1p concentrations within the cell wall, with higher concentrations of Flo1p requiring a higher level of mannose to achieve inhibition. In addition, changes in *FLO1* sequences between different strains may be responsible for the difference. Since NewFlo-type flocculation is inhibited by both mannose and glucose, while Flo1-type flocculation is exclusively inhibited by mannose (55), this result clearly demonstrates that *FLO1*- and *FLO5*-encoded flocculins exhibit Flo1-type flocculation.

QRT-PCR analysis. In order to verify whether *ADH2*- or *HSP30*-mediated *FLO* gene expression is similar to the reported expression patterns of these two promoters, total RNA from FY23, FY23-F11A, and FY23-F11H cultures was processed from different growth phases after 12 h (exponential), 16 h (entry/early stationary), and 48 h (late stationary), and QRT-PCR was performed. It is clearly evident (Fig. 7) that both *ADH2* and *HSP30* are tightly repressed in the presence of glucose at 12 h. Entry into stationary phase shows strong induction. RNA levels, while slightly decreased in the late stationary phase, are maintained at high levels. Similar data were observed for the *FLO1* and *FLO5* constructs (data not shown). These transcription levels are strongly correlated with the onset of flocculation and adhesion phenotypes in all strains (Fig. 2 and 3). Moreover, the data clearly suggest that only the *FLO* gene carrying a modified promoter is activated and that the

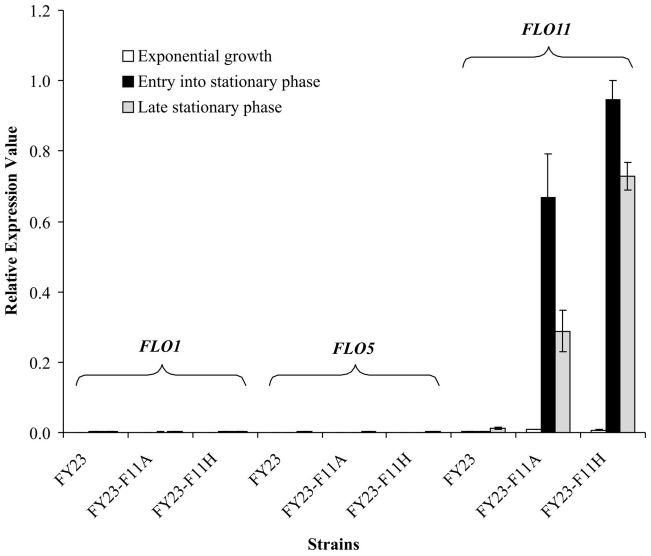

FIG. 7. Relative QRT-PCR expression of *FLO1*, *FLO5*, and *FLO11* transcripts in FY23 (wild type), FY23-F11A, and FY23-F11H strains. Samples were taken from sampling points corresponding to exponential growth phase, entry into stationary growth phase, and upon completion of fermentation. As indicated, a bracket denotes the expression of a particular *FLO* gene. The relative expression value for each sample was defined as $2^{-Ct(target)}$ where $Ct_{(target)}$ represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. Relative expression data were normalized to the relative expression value of the housekeeping gene *PDA1* in each respective sample, thus giving normalized relative expression for a target gene as $2^{-Ct(target)}/2^{-Ct(PDA1)}$. The highest mRNA expression level was arbitrarily set at 1.0. Values represent the means of experiments performed in triplicate, and error bars represent standard deviations.

two other genes that were monitored appear not to contribute to the observed phenotypes.

Heat shock and/or ethanol stress induction of flocculation in FY23-F1H and FY23-F5H strains. Both heat shock treatment and exposure to ethanol were reported as suitable induction conditions for the *HSP30* promoter (40, 49). Thus, it was probable that flocculent phenotypes conferred on transformed strains FY23-F1H and FY23-F5H under transcriptional regulation of *HSP30*p could be triggered when desired in response to these stress conditions. To assess these possibilities, only nonflocculent exponentially growing cell populations of these strains (determined from Fig. 2 and 3) were subjected to heat shock treatments and/or exposure to differing ethanol concentrations. The results clearly indicate that heat shock treatment for 45 min at 42°C elicited the strongest induction of flocculation in both FY23-F1H (94%) and FY23-F5H (65%) strains (Fig. 8). On the other hand, exposure to 3% (vol/vol) ethanol induced flocculation to a lesser extent in both FY23-F1H (70%) and FY23-F5H (28%) transformants. Both strains displayed similar flocculent abilities (approximately 10%) when exposed to 6% (vol/vol) ethanol, while no induction was evident for an ethanol/heat shock combination treatment.

Flor formation and invasive growth. As shown in Fig. 9A, only transgenic yeast FY23-F11A and FY23-F11H strains formed a biofilm after 5 days in flor medium at 30°C under static conditions. The FY23-F11A strain produced a distinctly

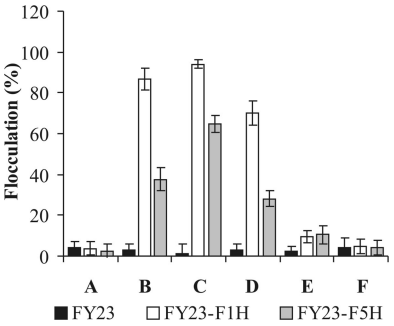

FIG. 8. Stress-induced expression of *FLO1*- and *FLO5*-encoded flocculins in *HSP30* transformants. Yeast strains cultivated for 10 h in YEPD were subjected to the following treatments: A, untreated (45 min at 30°C); B, heat shock for 30 min at 42°C; C, heat shock for 45 min at 42°C; D, 3% (vol/vol) ethanol for 30 min at 30°C; E, 6% (vol/vol) ethanol for 30 min at 30°C; F, 6% (vol/vol) ethanol and heat shock for 30 min at 42°C. The results are averages of three independent determinations, and error bars represent standard deviations.

thicker biofilm (Fig. 9A) and displayed threefold-higher suspended cell densities when evaluated 60 h postinoculation (Fig. 9B).

The ability of the wild-type FY23 strain and its six transformants to invade agar is shown in Fig. 10. Only *ADH2*-pro-

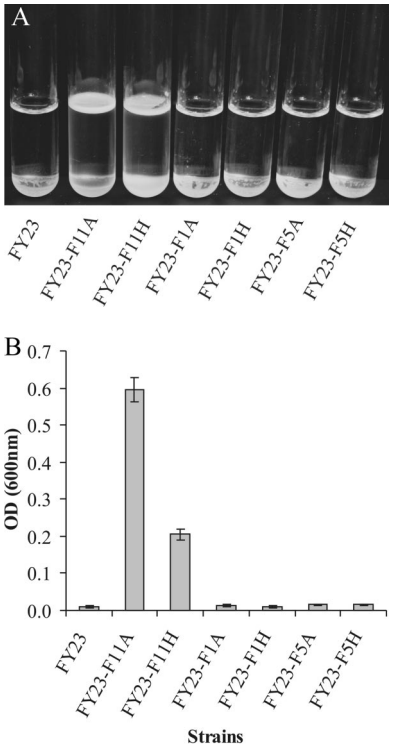

FIG. 9. (A) Biofilm formation. Cells were precultured in YEPD broth and recovered by centrifugation, washed once with flor medium, and resuspended at a density of 6×10^7 cells ml^{-1} in 5 ml flor medium contained in glass test tubes (16 by 165 mm). The tubes were photographed after 5 days of static incubation at 30°C. (B) Buoyant cell density determinations. The cultures were incubated statically at 30°C for 60 h, after which 1-ml samples were withdrawn from just below the meniscus. The optical density (OD) of samples was determined spectrophotometrically at 600 nm. The results are averages of three independent determinations, and error bars represent standard deviations.

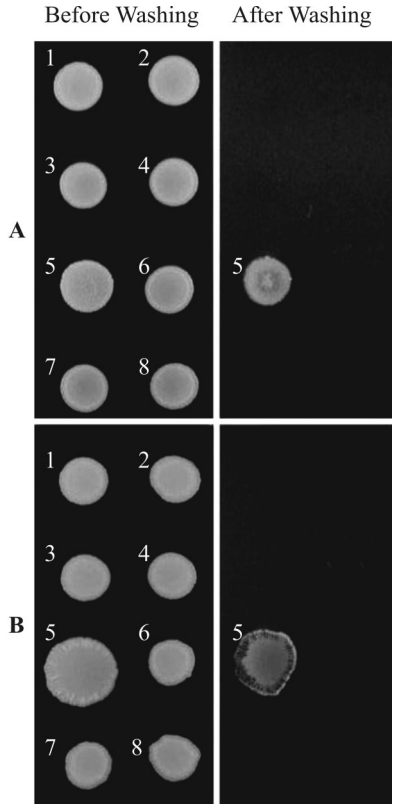

FIG. 10. Haploid invasive growth of FY23-F5A (colony 1), FY23-F5H (colony 2), FY23-F1A (colony 3), FY23-F1H (colony 4), FY23-F11A (colony 5), FY23-F11H (colony 6), and FY23 (wild type) (colonies 7 and 8) strains after 5 days growth at 30°C on $SCLD_{LUT}$ (A) and SCLD (B) media.

moted *FLO11* expression resulted in an invasive growth phenotype in SCLD and $SCLD_{LUT}$ agar media. Moreover, the FY23-F11A strain grew as a larger colony on SCLD agar, and it displayed more aggressive invasive growth behavior on SCLD agar plates than on $SCLD_{LUT}$ agar plates.

Effect of *FLO* gene expression on cell surface hydrophobicity. The hydrophobicity of yeast cell surfaces (Fig. 11) from yeast populations grown in YEPD medium for 48 h was determined by measuring the distribution ratio of yeast cells in a biphasic system consisting of a buffered solution and an organic solvent. The order of enhancement in terms of the MHI is FY23-F11A (0.83) > FY23-F11H (0.79) > FY23-F1A (0.64) > FY23-F1H (0.61) > FY23-F5A (0.29) > FY23-F5H (0.26) > FY23 (wild type) (0). Thus, it may be concluded that insertion of *FLO* gene-encoded glycoproteins Flo1p, Flo5p, and Flo11p into the yeast cell wall is responsible for increased cell surface hydrophobicity.

DISCUSSION

This is the first report that uses genome-integrated promoter-ORF combinations to compare the impact of various flocculation gene and promoter combinations on cell surface properties and cell surface-dependent phenotypes. The data show that integration confers stable (both in timing and intensity) expression properties to the targeted genes and demonstrate

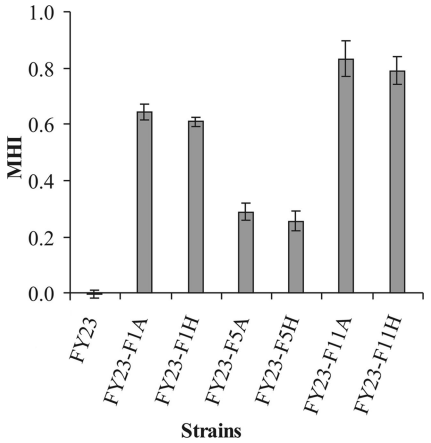

FIG. 11. Impact of *ADH2* and *HSP30* expression of *FLO* genes on cell surface hydrophobicity. The wild-type FY23 strain and transformants were cultivated for 48 h in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm). The MHI was determined according to the biphasic-solvent partition assay described by Hinchcliffe et al. (22). The results are averages of three independent determinations, and error bars represent standard deviations.

the possibility of adjusting flocculation and flor-forming behavior to specific industrial requirements. Importantly, all the engineered yeast strains displayed vegetative growth and fermentation properties that are comparable to those of the host strain, indicating that those industrially relevant characteristics were not compromised by modified *FLO* gene expression.

In this study, the genomic *FLO1*, *FLO5*, and *FLO11* ORFs were brought under the transcriptional control of promoters of the *ADH2* and *HSP30* genes by replacement of their native promoter sequences. The distinct advantage of the cloning strategy employed here over those used by other research groups (10, 12) is that no subcloning of the *FLO* genes is required. Furthermore, expression levels are independent of plasmid-related artifacts, such as variable copy numbers and the increased risk of intragenic recombinations. Indeed, *FLO* genes contain intragenic tandem repetitive sequences that have been previously reported as difficult to clone or even as "unclonable" sequences (57). Our data therefore provide reliable baseline information regarding the intrinsic ability of the three *FLO* genes to induce adhesion-related phenotypes.

The data show that *FLO1*-based constructs induce flocculation most efficiently, while *FLO5*-based constructs, while still leading to significant flocculation, are less efficient. *FLO11*-based constructs, on the other hand, induce flocculation only weakly. These constructs, however, strongly induced flor formation and cell adhesion, phenotypes that were not observed with *FLO1* or *FLO5*. Strains expressing *FLO11* also presented the highest cell surface hydrophobicity. Hydrophobicity was significantly lower in strains expressing *FLO5*, while strains expressing *FLO1* presented intermediate hydrophobicity levels. These data suggest that hydrophobicity per se is not a major determinant of adhesion-related phenotypes but that the specific sequences of the *FLO* genes are mainly responsible for phenotype specificity.

The observed flocculation patterns were in all cases consistent with the reported and measured expression patterns conferred by the two promoters. In the case of *HSP30*p-*FLO1* and

*HSP30*p-*FLO5* transformants, the onset of flocculation occurred toward the end of the respiro-fermentative exponential growth phase and was concomitant with the depletion of glucose from the medium. This is consistent with a previous study which showed in particular that the levels of *HSP30* mRNA increased before glucose exhaustion and climaxes with glucose exhaustion (44). The study also confirms the stress-inducible nature of *HSP30*p-controlled expression of *FLO1* and *FLO5* genes to yield flocculent phenotypes in response to specific stress conditions that include heat shock or exposure to ethanol. Although an ethanol concentration of 6% (vol/vol) is recommended for maximal induction of *HSP30*p, it is possible that this concentration brings about a toxic effect in the laboratory strains, which could be responsible for the absence of flocculation in these cells (11, 40).

Other groups have previously engineered the expression of individual *FLO* genes. The *FLO1* gene was constitutively expressed, thereby creating transgenic yeast strains that exhibited a constitutive flocculation property irrespective of the growth phase (2, 23, 66, 67). However, efficient fermentation requires a high suspended cell count, and constitutively flocculating yeast may lead to sluggish or stuck fermentations. These transgenic yeast strains are therefore not ideally suited for industrial batch-wise fermentation processes. Cunha and coworkers (12) reported controlled expression of the *FLO5* gene by employing a modified *ADH2* promoter. However, the native core promoter and ORF sequences of the *FLO5* gene used by Cunha et al. (12) were sourced from the YEp-*FLO5* plasmid. This plasmid was originally created by Bidard and coworkers (5) and was reported to contain the *FLO5* gene from the *S. cerevisiae* 17-13D strain. However, later studies by this research team retracted and confirmed that the *FLO5* gene used in the initial study was in fact identical to the *FLO1* gene sequence (6). We therefore assume that Cunha et al. (12) used the *FLO1* gene in their studies. This implies that our research study is the first to report inducible promoter-controlled *FLO5* and *FLO11* gene expression.

Cunha et al. (12) employed a multicopy plasmid-based strategy fusing the poly(T), *UAS1*, and *UAS2* regions of the *ADH2* promoter upstream of the native core promoter and ORF of the *FLO1* gene for expression in the laboratory yeast strain W303-1a. The same modified promoter was also employed to control *FLO1* gene expression by cloning an integrative cassette to disrupt the *CAN1* gene in a commercial baking yeast strain (Fleischmann). Similar to our study, the strains were reported to flocculate after glucose exhaustion in nutrient-rich medium (12). However, when using the native *ADH2* promoter, the onset of flocculation observed for *ADH2*p-*FLO1* and *ADH2*p-*FLO5* transformants in our study is in line with data published by Lee and DaSilva (31) who reported a similar native *ADH2* promoter-mediated expression pattern for β-galactosidase in *S. cerevisiae* transformed with a chromosomally integrated *ADH2*p-*lacZ* cassette. Moreover, the native *ADH2* promoter on multicopy plasmids was shown to drive β-xylanase production only after glucose exhaustion (27, 36), clearly suggesting that modification of the native *ADH2* promoter as suggested by Cunha et al. (12) is not necessary. Chambers et al. (10) employed the glucose-repressible *S. cerevisiae JEN1* promoter to regulate *FLO1* gene-mediated flocculation. However, the FY23-F1A and FY23-F5A strains reported here display a much later onset of flocculation in comparison to their *JEN1-FLO1* transgenic *S. cerevisiae* strain W303. These observations are clearly significant, as an early onset of flocculation might lead to a "stuck" or "hanging" fermentation because of insufficient contact between settled yeast cells and the medium. Some authors have reported nondetectable to significant decreases in ethanol production when converting nonflocculent yeast strains into flocculent strains (12, 23, 63, 65–67). Although decreased ethanol production will not meet the requirements of bioethanol production, it may be attractive to the alcoholic beverage industries that are currently faced with a growing consumer demand for lower-alcohol beers and wines (21, 38).

The decreased flocculation abilities observed for all strains in chemically defined minimal media may be attributed to starvation for auxotrophically required nutrients, as recent studies by Pronk (43) recommend increased supplementation of auxotrophic nutrients in comparison to those used in this study as prescribed by Sherman et al. (50). Lee and DaSilva (31) reported 10-fold-lower β-galactosidase activities for transgenic *S. cerevisiae* strains expressing *lacZ* under transcriptional control of the *ADH2* promoter when grown in minimal medium containing 2% glucose (wt/vol), which further supports these findings. Comparison of the relative promoter strengths of *ADH2*p and *HSP30*p for *FLO* gene expression in minimal media seems to suggest an increased nutritional demand for assimilable nitrogen by *ADH2*p. Although this study shows that *ADH2*p is responsible for later induction of flocculation and stronger flocculent phenotypes in nutrient-rich medium than *HSP30*p, it is most probable that *ADH2*p-controlled flocculation may not be suitable for certain industrial batch fermentation processes, such as winemaking because grape musts are sometimes deficient in assimilable nitrogen compounds (20).

Although no observable adhesion phenotype was evident for the *FLO11* transformants used in this study, Bayly et al. (3) presented evidence that *FLO11*-encoded flocculin yielded a strongly flocculent Flo1 phenotype in untransformed *S. cerevisiae* strain YIY345. However, it was also reported that *FLO11* overexpression in *S. cerevisiae* strain Σ1278b promotes very weak calcium-independent flocculation, while overexpression in *S. cerevisiae* strain S288C does not promote cell-to-cell adhesion (18, 62). It is possible that the flocculent ability of FY23-F11A and FY23-F11H strains may be too weak to be assessed by the modified Helm's assay employed in this study.

As mentioned previously, a nonsense mutation in the *FLO8* gene ensures that the dominant *FLO* genes are transcriptionally silent in the *S. cerevisiae* FY23 strain employed in this study (32, 61, 68). Thus, it is possible to eliminate contributions by other dominant *FLO* genes and exclusively assess the phenotypic consequences of *FLO11* expression. Therefore, it may be concluded that *ADH2*- and *HSP30*-facilitated *FLO11* expression is sufficiently responsible for flor formation. This finding is further supported by earlier reports that identified *FLO11* as a primary factor for flor formation in other *S. cerevisiae* strain types (24, 25, 70).

It has been proposed that flor wine yeast begins to form flor via a *FLO11*-mediated mechanism only when glucose repression of *FLO11* transcription is eliminated due to depletion of grape sugar after alcoholic fermentation (24). Based on the findings of this study, it can be suggested that the *ADH2* or

HSP30 promoter can be utilized to induce flor formation in nonflor wine yeast in a manner that will mimic natural flor wine yeast. The fact that the FY23-11A strain displayed decreased invasive growth in minimal agar that contained only auxotrophic nutritional requirements in comparison to complete nutrient supplementation further supports the previously mentioned notion that the *ADH2* promoter displays an increased demand for assimilable nitrogen. Surprisingly, no invasive growth phenotype was associated with the FY23-F11H strain. This suggests that growth on solid media is not an ideal induction condition for the *HSP30* promoter.

This study highlights that specific adhesion properties appear to be defined primarily by the properties of specific flocculins and not by general cell wall properties, such as hydrophobicity. Each *FLO* gene leads to specific phenotypes and phenotype intensities, with *FLO1* and *FLO5* resulting in cell aggregation and flocculation, whereas *FLO11* expression leads to invasive growth and flor formation. Clearly, the timing and intensities of the phenotypes are entirely dependent on the transcriptional regulation of each individual *FLO* gene.

The data clearly demonstrate that the flocculation behavior of industrial yeast can be fine-tuned to optimize specific production processes. The modified yeast strains used in this study contain only yeast-derived DNA sequences and can be regarded as self-cloned strains. Such modified strains are generally recognized as safe and may be approved more readily for industrial exploitation (60). The bioengineering of *S. cerevisiae* strains capable of controlled flocculation reported in this study may also benefit downstream processing in the pharmaceutical and nutraceutical industries which employ *S. cerevisiae* in batch-wise fermentations for the biosynthesis of high-value natural products, such as isoprenoids, flavanoids, and long-chain polyunsaturated fatty acids. We are currently investigating the impact of the same constructs in industrial wine yeast strains.

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