RESEARCH ARTICLE

Phenotypic diversity of Flo protein family-mediated adhesion in *Saccharomyces cerevisiae*

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Abstract

The *Saccharomyces cerevisiae* genome encodes a Flo (flocculin) adhesin family responsible for cell–cell and cell–surface adherence. In commonly used laboratory strains, these *FLO* genes are transcriptionally silent, because of a nonsense mutation in the transcriptional activator *FLO8*, concealing the potential phenotypic diversity of fungal adhesion. Here, we analyse the distinct adhesion characteristics conferred by each of the five *FLO* genes in the S288C strain and compare these phenotypes with a strain containing a functional copy of *FLO8*. Our results show that four *FLO* genes confer flocculation, but with divergent characteristics such as binding strength, carbohydrate recognition and floc size. Adhesion to agar surfaces, on the other hand, largely depended on two adhesins, Flo10 and Flo11. Expression of any *FLO* gene caused a significant increase in cell wall hydrophobicity. Nevertheless, the capacity to adhere to plastic surfaces, which is believed to depend on hydrophobic interactions, differed strongly between the adhesins. Restoring Flo8 yielded both flocculation and cell–surface adherence, such as invasive growth, a phenotype not observed when any of the single *FLO* genes was overexpressed. Taken together, this study reveals how *S. cerevisiae* carries a small reservoir of *FLO* genes that allows cells to display a wide variety of adhesive properties.

Introduction

For decades, the budding yeast *Saccharomyces cerevisiae* has been commonly studied as a single-cell organism living freely in suspension. However, in its natural environments, *S. cerevisiae* often forms complex multicellular structures that differ significantly from the planktonic cultures often used in laboratories (Palkova, 2004; Dickinson, 2005; Vopálenská *et al.*, 2005). Recent research shows that at least some of these morphological transitions are physiological responses to starvation and stressful conditions, giving yeast cells an advantageous opportunity to forage for nutrients or to escape harmful environments (Zaragoza & Gancedo, 2000; Prusty *et al.*, 2004; Gognies *et al.*, 2006; da Silva *et al.*, 2007; Smukalla *et al.*, 2008). Cell–cell and cell–surface adherence are requisites for the initiation and development of an impressive spectrum of aggregation structures, such as yeast flocs, filaments and biofilm structures (Verstrepen & Klis, 2006).

Many adhesion phenotypes depend on a specific family of cell surface proteins, the so-called adhesins or flocculins, which are encoded by the *FLO* ('flocculation') genes (for a review, see Verstrepen & Klis, 2006). At the carboxy-terminus, adhesins contain a glycosylphosphatidylinositol anchor, which is trimmed off at the plasma membrane before incorporation in the cell wall (for a review, see Lipke & Ovalle, 1998; Pittet & Conzelmann, 2007). The central part of the adhesins consists of highly repeated amino-acid sequences that are heavily N- and O-glycosylated (Dranginis *et al.*, 2007). The N-terminal part of the protein is required for ligand binding and is thus thought to confer the specificity of adhesion phenotypes (Kobayashi *et al.*, 1998; Zupancic *et al.*, 2008). Each yeast cell contains a small reservoir of different adhesin-encoding genes. The *FLO* gene family evolves and diverges very quickly (Hahn *et al.*, 2005), and each yeast strain contains a different set of *FLO* alleles. The commonly used laboratory strain S288C, for

example, contains five *FLO* genes: *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11* (Caro *et al.*, 1997). Additionally, several *FLO*-structural-related pseudogenes are located near the telomeres (Harrison *et al.*, 2002). The fast evolution and divergence of the *FLO* genes are driven by the presence of the large tandem repeat regions, located within each *FLO* gene. These unstable repeats drive slippage and recombination reactions within and between *FLO* genes, leading to the constant generation of novel *FLO* alleles and pseudogenes (Verstrepen *et al.*, 2004, 2005).

The Flo proteins confer adhesion through two distinct mechanisms. First, cell–surface adhesion is believed to depend on the hydrophobic interactions between certain domains in the adhesins and the surfaces they are binding. Cell–cell adhesion (flocculation), on the other hand, depends on reversible, lectin-like interactions between a sugar-binding domain in the Flo proteins, and oligosaccharides that decorate the outer cell wall (Bony *et al.*, 1997). Flocculation is divided into three subphenotypes, depending on which sugars competitively inhibit adhesion: mannose sensitive (flo1-type), mannose–glucose sensitive (newflo type) and mannose insensitive (Masy *et al.*, 1992). The flo1 type is believed to be caused by a specific N-terminal mannose-binding domain found in Flo1 and highly similar proteins such as Flo5 and Flo9 (Teunissen & Steensma, 1995). A homologous protein, Lg-Flo1, has been isolated from *Saccharomyces pastorianus* (Kobayashi *et al.*, 1998). This mannose–glucose-sensitive adhesin is of considerable importance in the brewing and biotechnological industries because its relaxed sugar specificity ensures that flocculation will only occur at the end of fermentation, when all sugars that competitively bind the adhesin and inhibit flocculation are consumed. Consequently, this results in a cost-effective and environmental-friendly technique to separate cells from the beer (Verstrepen *et al.*, 2003; Sampermans *et al.*, 2005). Another protein with relaxed sugar specificity is Flo10, which only shares 58% homology with Flo1, and is involved in a mannose-, glucose-, maltose- and sucrose-sensitive flocculation phenotype (newflo) when overexpressed in the Σ1278b strain (Guo *et al.*, 2000). The most studied member of the adhesin family, Flo11, is not involved in flocculation in *S. cerevisiae* strains. On the other hand, Flo11 is required for flo1-type flocculation in *Saccharomyces diastaticus* strains, which can possibly be attributed to the strain-specific mannosylation of this adhesin (Bayly *et al.*, 2005; Douglas *et al.*, 2007). Because flocculation is believed to depend on the lectin-like N-terminal domain of the Flo proteins, it seems likely that the sugar specificity largely depends on the N-terminal Flo protein sequence. Kobayashi *et al.* (1998) have identified the amino acids in the N-terminal responsible for the carbohydrate recognition of Flo1 and Lg-Flo1. Surprisingly, however, Liu *et al.* (2007) observed that the altered number of tandem repeats in the central part of a *FLO* gene caused the conversion of the flocculation phenotype from flo1 to newflo.

In some *S. cerevisiae* haploid yeast strains, such as Σ1278b, glucose depletion or amino-acid starvation causes a developmental switch that control cell shape and budding pattern and allows cells to invade agar medium. This process is called invasive growth (Roberts & Fink, 1994; Cullen & Sprague, 2000; Braus *et al.*, 2003; Ceccato-Antonini & Sudbery, 2004). The adhesin Flo11, which is the most diverged Flo family member, plays an important role in the invasiveness, filamentation and plastic adhesion of yeast. A deletion of the *FLO11* gene in the Σ1278b background inhibits these adhesion phenotypes (Lo & Dranginis, 1998; Reynolds & Fink, 2001). The large promoter sequence of *FLO11* is the main downstream target of the two most important transduction pathways controlling invasive growth: the MAP kinase cascade and the Ras2/cAMP-dependent protein kinase pathway (Lo & Dranginis, 1998; Rupp *et al.*, 1999; Gancedo, 2001; Gagiano *et al.*, 2002). In most genetic backgrounds, different types of stresses such as glucose depletion are insufficient to trigger differentiation, and additional activation of the cAMP pathway is required. In a Σ1278b background, on the other hand, the Ras2/cAMP pathway appears to be overactive and a stressful situation, such as depletion of fermentable carbon source, is sufficient to induce these morphological changes (Stanhill *et al.*, 1999).

In the S288C yeast strain, a nonsense mutation in the transcriptional regulator *FLO8* inhibits adhesive phenotypes such as flocculation, pseudohyphal growth and invasive growth by the repression of *FLO1* and *FLO11*. When the defective *FLO8* gene is replaced by an intact version or when the intact *FLO8* gene is expressed from a high copy number plasmid, *FLO1* and *FLO11* are activated and the adhesive properties are restored (Liu *et al.*, 1996; Kobayashi *et al.*, 1999; Bester *et al.*, 2006; Fichtner *et al.*, 2007). The silent *FLO5*, *FLO9* and *FLO10* cannot be induced, even when different suppressors of adhesive growth, such as *SFL1*, *TUP1*, *SSN8*, *SIN4* and *SRB8*, were deleted (Fichtner *et al.*, 2007). In a recent study, Govender *et al.* (2008) showed that the flocculation and adhesion behaviour in an S288C strain could be tightly regulated with the controlled expression of *FLO1*, *FLO5* and *FLO11*. Because of the lack of adhesion in wild-type (WT) (*flo8*$^{-}$) S288C cells, much of the research has focused on a different yeast strain, in most cases Σ1278b. This strain only expresses one *FLO* gene, *FLO11* (Guo *et al.*, 2000; Halme *et al.*, 2004; Reynolds *et al.*, 2008). Recently, it has been found that the Flo1-mediated adhesion in the Σ1278b strain is abolished because of the absence of a Flo8-binding site in the *FLO1* promoter sequence and two major deletions in its ORF (Fichtner *et al.*, 2007). Remarkably, when the regulatory gene *SFL1* was deleted or when loss-of-function mutations in either *IRA1* or *IRA2* occurred, *FLO10* is derepressed in Σ1278b and is able to compensate for an inactivated *FLO11* in both invasive and pseudohyphal

growth, even under the control of its own promoter (Guo *et al.*, 2000; Halme *et al.*, 2004). In neither condition could *FLO5* or *FLO9* mRNA be observed in the Σ1278b strain. By means of a very elegant technique, Halme *et al.* (2004) proved that both *FLO10* and *FLO11* were also regulated epigenetically. Under inducing nitrogen-starvation conditions, yeast-form cells are observed when *FLO11* is silenced by the histone deacetylase Hda1p and pseudohyphal filaments are observed when *FLO11* is expressed. In Ira$^-$ mutants, the expression of *FLO10* is also variegated by means of a distinct set of histone deacetylases, Hst1p and Hst2p.

Here, we describe a detailed phenotypic characterization of the full array of five *FLO* genes in the strain S288C. All *FLO* genes are transcriptionally silent in this strain, providing an excellent opportunity to activate each *FLO* allele one by one and investigating the resulting adhesion characteristics. A considerable diversity in both cell–cell interactions and cell–surface interactions is observed, demonstrating how this small reservoir of *FLO* genes confers a surprisingly wide variation in adhesion phenotypes.

Materials and methods

Microbial strains and culturing conditions

All yeast strains and plasmids used in this study are listed in Table 1. Yeast cultures were routinely pregrown overnight at 30 °C in YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and 4% (w/v) glucose], orbitally shaken at 150 r.p.m. in Erlenmeyer flasks. Before the fermentation experiments, EDTA (50 mM, pH 7) was used to deflocculate the yeast cells to allow reliable measurement of the $OD_{600\,nm}$. Afterwards, a calculated amount of yeast cells was washed twice with sterile isotonic saline [0.85% (w/v) NaCl] and inoculated. Adhesive growth and invasive growth on agar were assessed by the plate washing assay (Roberts & Fink, 1994; Braus *et al.*, 2003). The visualization of invasive cells was performed by photographing the agar with a Nikon SMZ800 stereomicroscope equipped with a DN100 camera.

Selection of the overexpression strains was performed on YPD+geneticine medium (100 μg mL^{-1}, Difco). Selection of the strains transformed with an intact *FLO8* and *URA3* was performed on minimal synthetic defined medium containing 1.7 g L^{-1} yeast nitrogen base without amino acids and without ammonium (Difco), 5 g L^{-1} $(NH_4)_2SO_4$ and 20 g L^{-1} glucose (Sigma-Aldrich, Steinheim, Germany), supplemented with 0.77 g L^{-1} complete supplement mixture-Ura (Bio 101 Inc. Systems, Vista, CA).

Construction of the overexpression strains and the intact *FLO8* strain

Construction of the overexpression strains was performed as described by Janke *et al.* (2004) using the primers listed in Table 2. The geneticine-resistant colonies were analysed by PCR to confirm correct genomic integration of the translation elongation factor 1 (*TEF1*) overexpression construct (Expand Long Range DNA-polymerase, Roche Applied Science, Mannheim, Germany). In order to further confirm and distinguish the overexpression strains, specific *FLO* gene expression was measured by quantitative real-time PCR (qRT-PCR).

The plasmid pHL1, containing an intact version of *FLO8*, was linearized with BglII restriction enzyme (New England Biolabs, Ipswich, MA) and transformed into the BY strain as described by Liu *et al.* (1996). *URA3* was amplified from pHL1 and inserted at the *FLO8* locus to construct a reference BY4741 strain without uracil auxotrophy.

Table 1. Yeast strains and plasmids used in this study

Strains and plasmids	Genotype/description	Background
Saccharomyces cerevisiae		
BY4741 (WT)*	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	S288C
BY4742 (WT)*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	S288C
BY4741 [URA3]†	*MAT a his3Δ1 leu2Δ0 met15Δ0 URA3*	S288C
BY4741 [FLO8]†	*MAT a his3Δ1 leu2Δ0 met15Δ0 URA3 FLO8*	S288C
BY4742 [FLO1]†	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 kanMX4:P_{TEF1}-FLO1*	S288C
BY4742 [FLO5]†	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ kanMX4:P_{TEF1}-FLO5*	S288C
BY4742 [FLO9]†	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 kanMX4:P_{TEF1}-FLO9*	S288C
BY4742 [FLO10]†	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 kanMX4:P_{TEF1}-FLO10*	S288C
BY4742 [FLO11]†	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 kanMX4:P_{TEF1}-FLO11*	S288C
Σ1278b†	*MATα leu2Δ0 ura3Δ0*	Σ1278b
Plasmids		
pHL1	4.3-kb insert with *FLO8* on pRS316	Liu *et al.* (1996)
pYM-N18	*TEF* promotor *kanMX4*	EUROSCARF (Frankfurt, Germany)

*Brachmann *et al.* (1998).
†This study.

qRT-PCR

The levels of *FLO* gene expression in the overexpression strains were determined using qRT-PCR. Yeast cells were inoculated in 50 mL of YPD (40 g L^{-1} glucose) to an $OD_{600\,nm}$ of 0.5 and grown until the late-exponential growth phase was reached. Extraction of the RNA of pelleted cells

Table 2. Oligonucleotides used for the construction of the overexpression strains (5′ → 3′)

Oligonucleotide	Sequence (5′ → 3′)
FLO1_TEF_FW	TTCCGGGTTCTTATTTTTAATTCTTGTCACCAGTAAACAGAACATCCAAAAATG**CGTACGCTGCAGGTCGAC**
FLO1_TEF_RV	AGTGCCAGAAGTGTAAAGACTGCCAAAAACATATAGCGATGAGGCATTGTCAT**CGATGAATTCTCTGTCG**
FLO5_TEF_FW	CAGTAAATTCCGCAAATGATTTTCTTTAAATTGATTAGCACCACTAAAAAAAATG**CGTACGCTGCAGGTCGAC**
FLO5_TEF_RV	TAATTAGTGCCAGAAAGGCCAAGATTACCAAAAATATGCAGTGGTGTGCAATTGTCAT**CGATGAATTCTCTGTCG**
FLO9_TEF_FW	GCTCTTTAAATTGCAATTTAAAAAGAACAATTGTACAATAAAAGCCCCAAAAATG**CGTACGCTGCAGGTCGAC**
FLO9_TEF_RV	TAGTTAATCCCAGCAATGTGACGATGGCTAGTAGTAAACAATAATGTGCCAGAGACAT**CGATGAATTCTCTGTCG**
FLO10_TEF_FW	TTACGTTGAAGATTTGTTTTAGGGTGCTTAATCAAAGAACAACAAATAAAAAATG**CGTACGCTGCAGGTCGAC**
FLO10_TEF_RV	TAGCTACAGATAGCAAAAATAGGCCGGTCAAAAATATATATCGAGCAGCCACAGGCAT**CGATGAATTCTCTGTCG**
FLO11_TEF_FW	TTCTAATTAAAATATACTTTTGTAGGCCTCAAAAATCCATATACGCACACTATG**CGTACGCTGCAGGTCGAC**
FLO11_TEF_RV	AAGCCGAGTTAAATAGAAGCGAAAGGACCAAATAAGCGAGTAGAAATGGTCTTTGCAT**CGATGAATTCTCTGTCG**

The sequences shown in bold are the overexpression plasmid binding sites.

was performed with Trizol (Invitrogen), used according to the manufacturer's instructions. For each sample, 1 μg of total RNA was subject to reverse transcription (RT) using an RT system (A3500; Promega, Madison). Concentrations were determined, and samples were diluted to obtain a concentration of 100 ng μL^{-1}. The 25 μL PCR mixture consisted of 12.5 μL Power SYBR® Green (Applied Biosystems, Warrington, UK) or 12.5 μL Taqman® Universal PCR Master mix (Applied Biosystems) and 1.25 μL of each primer (500 nM). Additionally, the Taqman® assay contained 2.5 μL of an MGB probe (250 nM, 5′-FAM, 3′-MGB, Applied Biosystems). Five microlitres of cDNA (100 ng μL^{-1}) was added to each reaction mixture. The two-step PCR program on the ABI Prism 7500 instrument (Applied Biosystems) consisted of an initial denaturation for 10 min at 95 °C and amplification using 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The actin gene (*ACT1*) was used as the reference gene, because the expression of this gene was found to be relatively stable in this setup. A standard curve of each gene was constructed with genomic DNA. The PCR primers were all designed with the PRIMER EXPRESS software (Applied Biosystems, Ceshire, UK) according to the Applied Biosystems guidelines. The primer and probe sequences used for qRT-PCR analysis (5′–3′) are listed in Table 3. The expression levels were analysed with SDS software (Applied Biosystems). The levels of expression of the different genes were all normalized with respect to the levels of *ACT1* expression.

Table 3. Primers and probes used for the qRT-PCR and the verification of the transformants by normal PCR (KANMX_FW and FLO$_x$_TAQ_RV or SYBR_RV)

Oligonucleotide	Sequence (5′ → 3′)
KANMX_FW	TGATTTTGATGACGAGCGTAAT
FLO1_TAQ_FW	ATCGCTATATGTTTTTGGCAGTCTTTA
FLO1_TAQ_RV	GTAAGCACGCCTCTGTGGCT
FLO1_MGB_FW	ACTTCTGGCACTAACTAGT
FLO5_TAQ_FW	GCACACCACTGCATATTTTTGGTAA
FLO5_TAQ_RV	GTAAGCACGCCTCTGTGGCT
FLO5_MGB_FW	CCTTTCTGGCACTAATT
FLO9_TAQ_FW	TTATTGTTTACTACTAGCCATCGTCACA
FLO9_TAQ_RV	AAGTTTACATTCATACCATTCTTCCTTGA
FLO9_MGB_FW	CATGCCTGCCAGCAA
FLO8_SYBR_FW	TTTGACGCCCTCAAAAATTCAA
FLO8_SYBR_RV	AATTCTCGTTTGGTGTTCTATTGTTAGA
FLO10_SYBR_FW	CGTTTCGACAGCCACTGCTA
FLO10_SYBR_RV	GTGGGCTCCATGTGGAATAAA
FLO11_SYBR_FW	GTTCAACCAGTCCAAGCGAAA
FLO11_SYBR_RV	GTAGTTACAGGTGTGGTAGGTGAAGTG
ACT1_SYBR_FW	CGTCTGGATTGGTGGTTCTA
ACT1_SYBR_RV	GTGGTGAACGATAGATGGAC
ACT1_TAQ_FW	CTCCACCACTGCTGAAAGAGAA
ACT1_TAQ_RV	CCAAGGCGACGTAACATAGTT
ACT1_MGB_FW	TTGTCCGTGACATCAA

Flocculation tests

Standard flocculation tests were performed by the method of D'Hautcourt & Smart (1999) with some modifications. Cells were harvested in the (late-) exponential phase and washed with 50 mM EDTA (pH 7). The deflocculated cell suspension was moved to an Eppendorf tube, harvested and resuspended in 1 mL of a 3.75-mM $CaSO_4$ solution. Subsequently, the liquid was discarded and 5 mL flocculation buffer (3.75 mM $CaSO_4$; 82.9 mM $NaCH_3COO$; 67.4 mM CH_3COOH; pH 4.5) was added to the cells, moved to a glass tube, to a final $OD_{600\,nm}$ of 10. The cell suspension was vortexed for 30 s at maximum speed, inverted 15 times, and after 1 min, an aliquot of 50 μL was taken below the meniscus and suspended into 50 mM EDTA (pH 7). The relative difference in $OD_{600\,nm}$ between the initial deflocculated cell suspension and the sample after treatment is the flocculation percentage. Carbohydrates were added at the appropriate concentrations (1 M, 500 mM, 100 mM and 20 mM) to the flocculation buffer to determine the carbohydrate recognition characteristics of the adhesins. This was performed using the method described above, but with a gently rotating incubation step during 15 min instead of the

vortexing step to include the analysis of unstable flocculation phenotypes.

Hydrophobicity – MATH test

Cells were harvested in the stationary phase and washed with 50 mM EDTA (pH 7) in order to ensure deflocculation before determining $OD_{600\,nm}$. A calculated amount of yeast cells was washed once and resuspended in 2 mL isotonic saline to achieve an $OD_{600\,nm}$ of *c.* 0.8. The yeast suspension was overlaid by 0.4 mL of a hydrophobic hydrocarbon, octane, and vortexed at maximum speed for 60 s. After a phase separation period of 10 min, the absorbance of the water layer was measured. The relative difference between the absorbance of the water layer before and after vortexing is considered as the hydrophobicity percentage (Rosenberg, 1984).

Polystyrene adhesion

Adhesion to plastics was tested in 96-well flat bottom plates (Microtest^{TM} Flat bottom 96-well plate, Becton Dickinson Labware, NJ) according to Reynolds & Fink (2001) with some minor modifications. One hundred and fifty microlitres of YPD medium (40 g L^{-1} dextrose) was inoculated at an $OD_{600\,nm}$ of 0.5. Plates were incubated on an orbital shaker (150 r.p.m.) at 25 °C during 40 h. To quantify mat formation, the wells were washed twice in phosphate-buffered saline (PBS). The remaining attached yeast cells were stained for 30 min with 150 μL of a filtered 0.1% (w/v) crystal violet in an isopropanol–methanol–PBS solution (1 : 1 : 18 v/v) (Lebeer *et al.*, 2007). After washing with PBS, the remaining crystal violet was solubilized in 150 μL of absolute ethanol. The OD of each well was measured at 570 nm using a Powerwave 340 (Biotek, Winooski, VT). Each strain was tested sixfold. The experiment was performed in duplicate.

Results

Construction of the overexpression strains

In this study, the strong and constitutive *TEF1* promoter was used to drive the expression of each copy of the five *FLO* genes in the strain S288C. Primers, with 5′-flanking regions containing *c.* 50 bp homologous to the intended chromosomal loci, were designed to amplify the *KanMX* marker and *TEF1* promoter (Table 2). Because of the considerable sequence similarity of the *FLO* genes and their flanking regions, different rounds of transformations were needed to obtain transformants for each *FLO* gene, *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*. The resulting transformants were verified by PCR using a forward primer in the *KanMX* marker and a reverse primer in the *FLO* ORF. However, because of the extensive sequence similarity, PCR did not allow faithful discrimination between *FLO1*, *FLO5* and *FLO9*. For these genes, quantitative Taqman PCR using primers and MGB probes were designed to bind specifically to unique loci in the *FLO* ORFs (Table 3). In this way, *FLO* transcripts could be distinguished from each other and quantified, on the one hand, in the *FLO1*-, *FLO5*- and *FLO9*-overexpression strains and, on the other hand, in the *FLO10*- and *FLO11*-overexpression strains as shown in Fig. 1a and b. Additionally, we quantified the expression of *FLO* genes in the strain containing an intact copy of the *FLO8* transcriptional regulator (Fig. 1c). It was shown that only the expression levels of *FLO1* and *FLO11* significantly increased upon activation by Flo8, respectively, by 7.2-fold and 2.1-fold. For each *FLO* gene, the expression level was higher in the overexpression strains than in the strain with the intact *FLO8* gene. Finally, the growth rates of at least three independent transformants for each constructed strain were assessed in test tube fermentations at a high rotation speed (150 r.p.m.) to avoid mass transfer limitations because of floc formation. It was shown that, in this experimental setup, the growth rates of the constructed strains and the wild-type (WT) strain were similar (data not shown).

Cell–cell adhesion types (flocculation)

The activation of the expression of various adhesins resulted in the formation of macroscopic flocs ranging in diameter from around 100 micrometers to several millimetres (Fig. 2). Only the WT strain and the *FLO11*-overexpression strain did not form macroscopic aggregates. A secondary effect of the strong flocculation observed for the *FLO1*- and *FLO9*-overexpressing strains was a reduction in growth rate in a gently mixed liquid culture (data not shown). Possibly, mass transfer limitation of substrates and fermentation products caused the decrease in growth rate. Remarkably, the *FLO5*-overexpression strain did not show this growth reduction. Interestingly, even for strains showing a reduced growth rate, the glucose consumption rate was not always reduced (data not shown). Only the *FLO1*-overexpression strain reached the glucose depletion phase significantly later than the other transformants. Possibly, these observations may be explained by the fact that almost all cells overexpressing *FLO1* were incorporated in a floc, whereas overexpression of other *FLO* genes always left some fraction of planktonic cells. The percentage of cells that was not incorporated in the flocs, based on a spectrophotometric measurement ($OD_{600\,nm}$) of the top layer of a liquid culture after a sedimentation time of 5 min, was *FLO10* (5.3%) > *FLO5* (2.1%) > *FLO9* (1.7%) > *FLO1* (0.4%) (data not shown).

Two distinct visual observations have been made when comparing the flocculation strength of the transformant strains that expressed the diverse Flo proteins. The first group, including the *FLO1*-, *FLO5*- and *FLO9*-overexpression strains and the intact *FLO8* strain, showed a strong

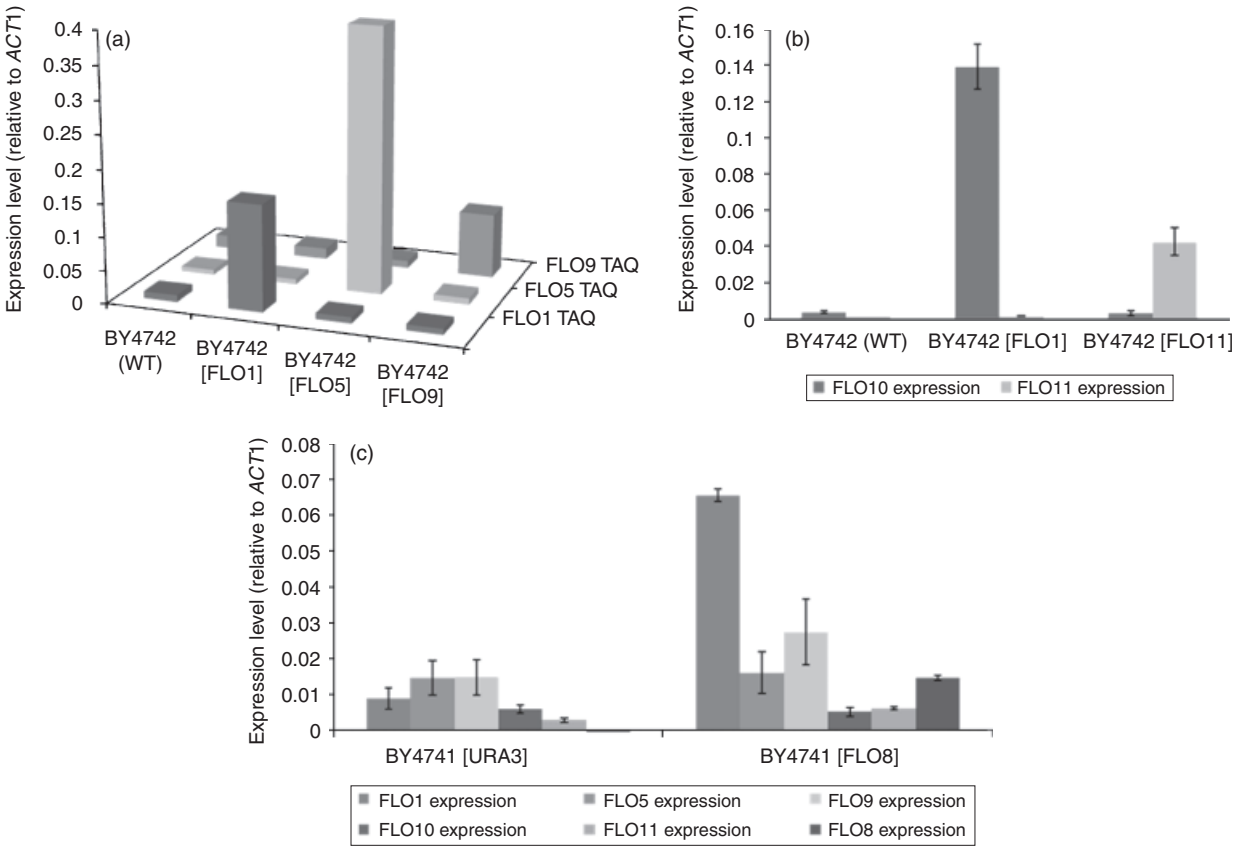

Fig. 1. Verification of the constructed yeast strains by qRT-PCR. (a) Detection of *FLO1*, *FLO5* and *FLO9* transcripts in the WT and overexpression strains was performed using primers and Taqman MGB probes directed to *FLO1*, *FLO5* and *FLO9* (see Table 3). SDs were within 20% of the measured values. (b) *FLO10* and *FLO11* transcripts were detected using primers and SYBR green chemistry (see Table 3). (c) Detection of *FLO* transcripts in the WT strain and the strain containing an intact *FLO8* gene. In each case, gene expression was normalized with respect to *ACT1* expression levels. These experiments were carried out in duplicate.

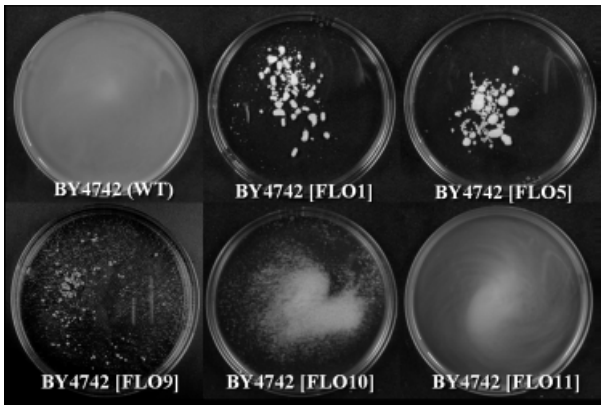

Fig. 2. Photographs of the WT and overexpression strains. Petri dishes were filled with equivalent volumes of YPD medium originating from a liquid cell culture and photographed after an incubation period of 1 h on an orbital shaker (100 r.p.m.).

flocculation phenotype, with flocs that do not break upon vortexing and strong shaking (Fig. 3). In the second group, the *FLO10*-overexpression strain grew in unstable macroscopic flocs when gently mixed, but dissolved to microclumps after vigorous vortexing. Afterwards, the cells needed some gentle agitation to recover the aggregation into macroscopic flocs. This indicates that the binding forces of Flo10-induced flocculation are much lower than those associated with the other *FLO* genes. In either case, however, the flocculation was calcium dependent (data not shown). Interestingly, Flo11 did not cause cell–cell adherence on a macroscopic level, although microflocs consisting of 6–30 cells could be observed under the microscope.

In order to determine the carbohydrate recognition of the various adhesins, we added carbohydrates at different concentrations to the flocculation cultures and investigated whether these sugars could disrupt the flocs through competitive inhibition of the Flo proteins. We observed that Flo1 was only inhibited by mannose at a concentration of 1 M

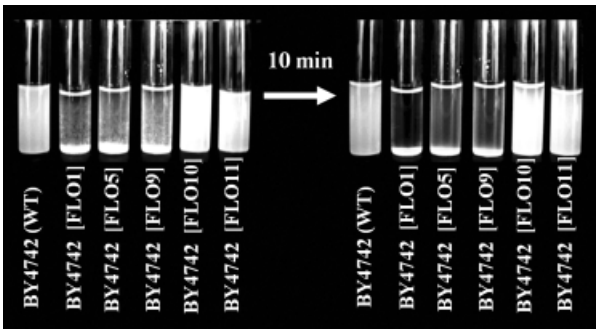

Fig. 3. Sedimentation test of the WT and overexpression strains in the flocculation buffer described in Materials and methods. Pictures were taken immediately after shaking the tubes at high speed and after a sedimentation period of 10 min.

(Fig. 4). Moreover, even at such high mannose concentrations, inhibition was not complete. Remarkably, Flo5-dependent flocculation was almost completely inhibited by mannose and only very slightly by sucrose, maltose and glucose. This was also the case for Flo9, except for its higher sensitiveness towards maltose and glucose. Flo10, on the other hand, was strongly inhibited by mannose, sucrose, glucose and maltose (Fig. 4). The strain bearing the intact *FLO8* gene showed partial inhibition only by mannose (data not shown), reinforcing the theory that *FLO8* induces flocculation by activating the *FLO1* adhesin-encoding gene in the BY4741 strain (Fig. 1c). The significantly larger percentage of planktonic cells that was present in the intact *FLO8* strain when compared with the *FLO1* overexpression strain can be attributed to the differences in gene expression

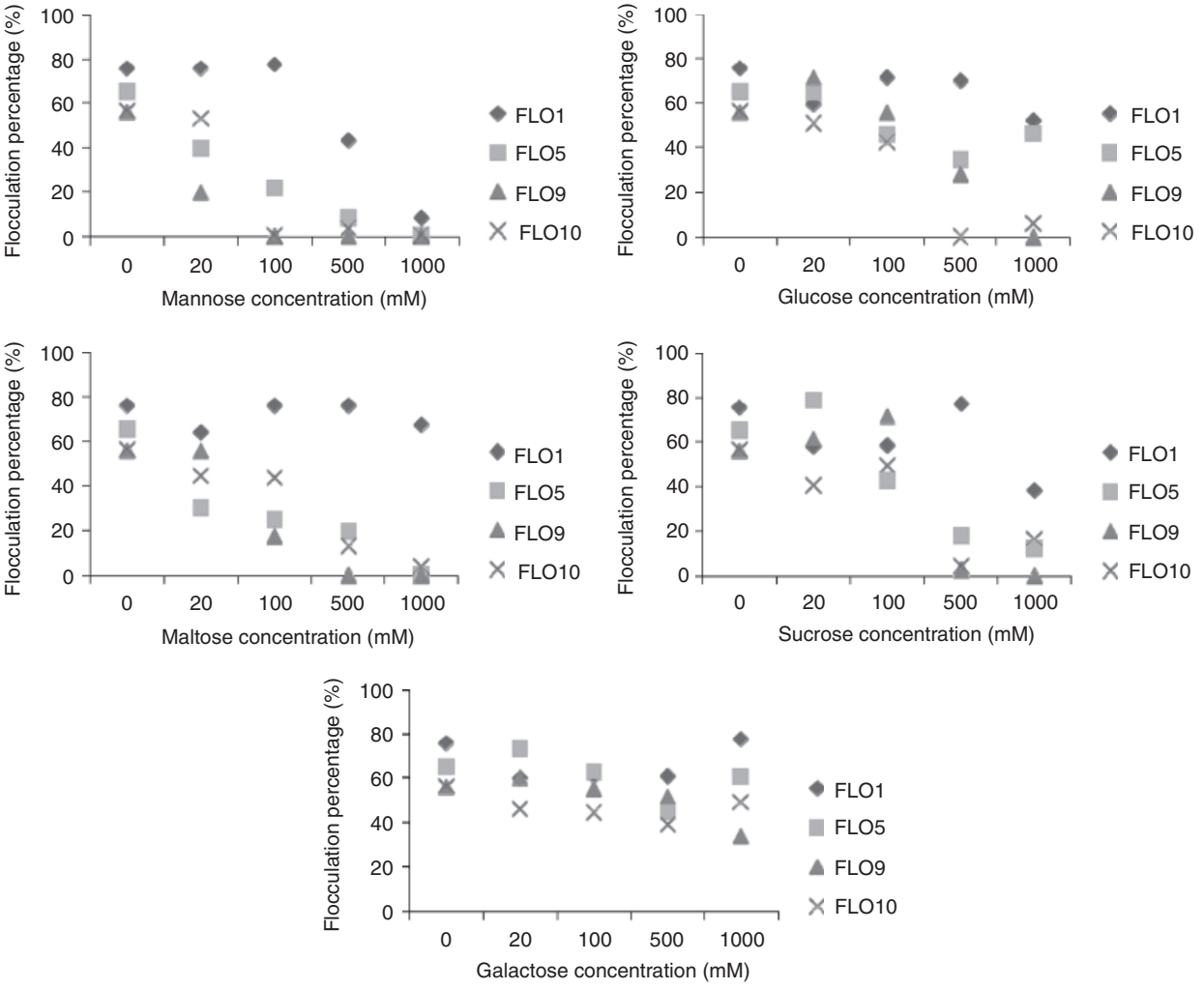

Fig. 4. Flocculation percentages of the different flocculating overexpression strains: FLO1, BY4742 [FLO1]; FLO5, BY4742 [FLO5]; FLO9, BY4742 [FLO9]; FLO10, BY4742 [FLO10]. Increasing concentrations of sugars (0, 20, 100, 500 and 1000 mM) were added to the flocculation buffer to determine the inhibition profile of the expressed adhesins. This is a typical example of an experiment performed twice sedimentation period of 10 min.

levels (Fig. 1). Galactose could not inhibit flocculation in either overexpression strain, even at high concentrations.

Adhesive and invasive growth

A standard plate-washing assay showed particular differences between the adherence on agar of the different overexpression strains (Fig. 5a). In the case of *FLO1*, *FLO5* and *FLO9*, yeast flocs were washed off the agar plates after gentle rinsing. Frequently, a small amount of floc-like structures remained on the agar because of their macroscopic size. The *FLO10*- and *FLO11*-overexpression strains adhered more strongly to the agar as stronger rinsing and even rubbing were needed to remove the cells from the agar surface. After wiping off all superficial cells, no remaining cell layer was observed in the agar for the different overexpression strains (Fig. 5b). For the

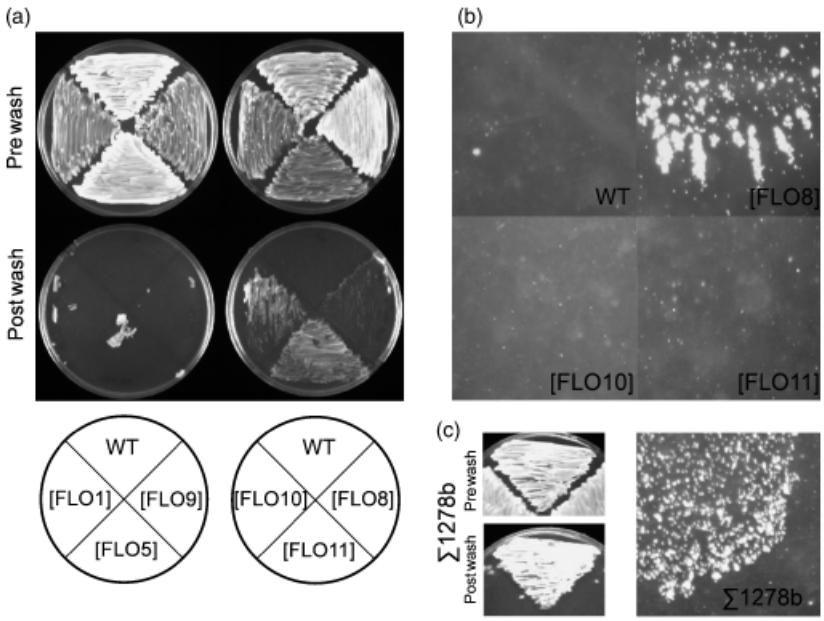

Fig. 5. (a) Determination of the adhesive growth of the WT and overexpression strains. The indicated yeast strains were streaked on the agar and incubated for 6 days at 25 °C. Photographs were taken after incubation (prewash) and directly after intense washing under a stream of water (postwash). (b) Invasive growth was assessed by sweeping off intensively the superficial cells from the agar. Invasive cells that remained in the agar were photographed under a stereomicroscope. (c) As a positive control, adhesive and invasive growth was also assessed in a Σ1278b strain.

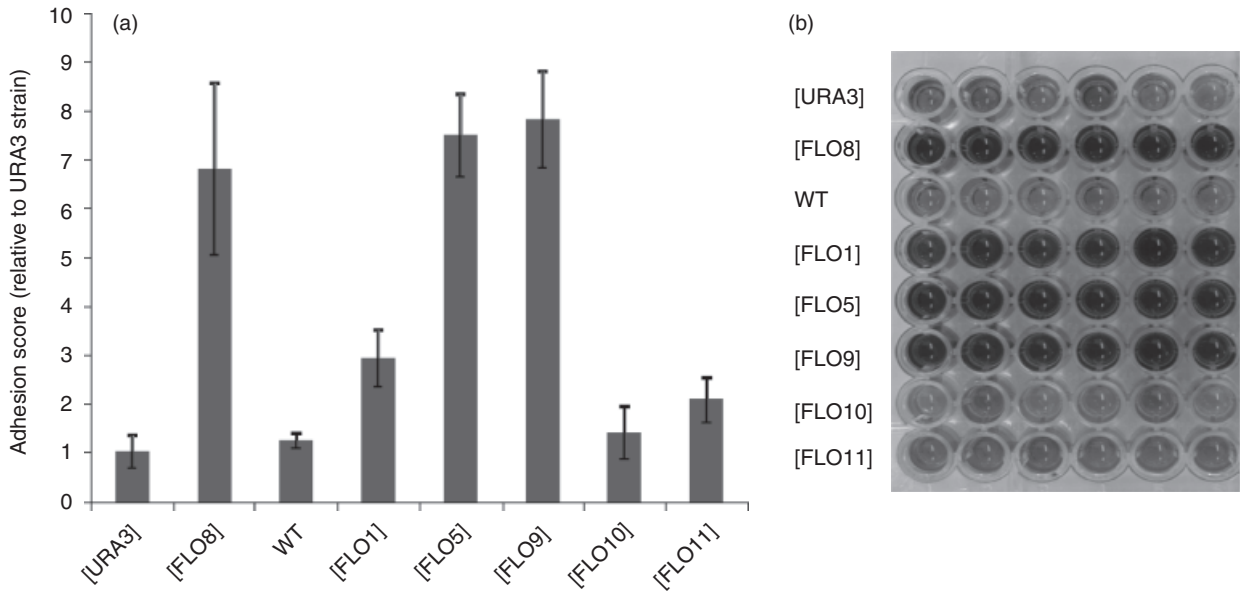

Fig. 6. Quantification (a) and visualization (b) of mat formation in polystyrene plates. The intensity of the crystal violet staining ($OD_{570\,nm}$) is a measure for mat formation. The adhesion score represents the measured $OD_{570\,nm}$ relative to the WT strain. The error bars represent SDs of six biological experiments. The data shown are representative of three independent experiments.

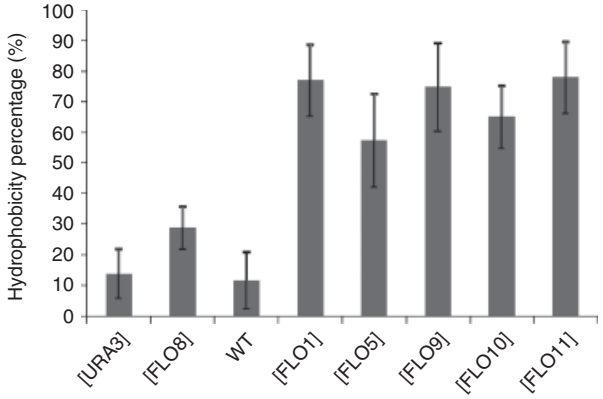

Fig. 7. Quantification of cell wall hydrophobicity by means of a biphasic partitioning assay using octane as hydrophobic solvent and water. The error bars represent the SDs of four experiments.

FLO8 intact strain, on the other hand, a thin layer of cells could not be washed off the agar plate, even after intense wiping and rubbing. Upon close inspection, it became clear that these cells had invaded the agar surface. This invasive growth was as pronounced as in the Σ1278b strain (Fig. 5c). In contrast, the Σ1278b strain adhered more strongly to the agar than the *FLO8* intact strain and stuck as a thick cell layer on the agar surface, even after vigorous washing.

Polystyrene adhesion of *S. cerevisiae* cells

The ability of pathogenic yeasts, such as *Candida albicans* and *Candida glabrata*, to bind to plastic devices, such as medical prostheses and catheters, is of crucial importance in hospital-acquired infections (Douglas, 2003). *Saccharomyces cerevisiae* can also adhere to surfaces, a process in which Flo11 was shown to play a major role (Reynolds & Fink, 2001). In our study, the tendency to form such biofilms (or 'mats', as these adherent cell layers lack some typical biofilm properties, such as increased resistance to antibiotics and the presence of extracellular material) differed strongly between the overexpression strains (Fig. 6). On the polystyrene plates, the most intense mats were observed with the intact *FLO8* strain (6.8 times more than WT) and the *FLO5*- and *FLO9*-overexpression strains (7.49 and 7.8 times more than WT, respectively). The *FLO1*- and *FLO11*-overexpression strains had a slightly higher tendency to form adherent mats than the WT strains and the *FLO10*-overexpression strain.

Hydrophobicity of the cell wall is influenced by the presence of adhesins

Hydrophobic forces often play a general role in the primary interaction between biological ligands and receptors (van Oss, 1995). Moreover, hydrophobic interactions are also believed to be responsible for the adhesion of cells to abiotic materials such as plastics. In our study, we found that each Flo protein in *S. cerevisiae* influences adhesion in two ways: through direct interaction between a binding site and a receptor, and indirectly by dramatically increasing cell wall hydrophobicity (Fig. 7). Statistically, no differences in hydrophobicity were observed between the different overexpression strains as measured by the affinity of the cells to octane (see Materials and methods for details): Flo1 (77%), Flo5 (57%), Flo9 (75%), Flo10 (65%) and Flo11 (78%). The strain with an intact *FLO8* showed an intermediate hydrophobicity percentage (29%).

Discussion

Here, we describe the comprehensive phenotypic characterization of a nonsexual adhesin family in *S. cerevisiae*. The five members of the *FLO* adhesin family in the S288C background, *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*, were similarly overexpressed by chromosomal integrations of the constitutive *TEF1* promoter directly upstream of the *FLO* ORFs. Notwithstanding the fact that several members of the *FLO* adhesin family share up to 96% of similarity, we succeeded in selectively overexpressing each *FLO* gene. Highly selective Taqman MGB probes targeted to unique sites in the *FLO* ORFs were designed to confirm this statement. A possible drawback of this approach is that the high expression levels caused by the constitutive and strong *TEF1* promoter can cause secondary adaptations because of the strong flocculation, such as mutations to restore the normal growth rate observed in planktonic cultures. When considering the expression levels of the overexpression strains (relative to *ACT1*) in our study, it can be observed that they are of the same magnitude as the *FLO*-expression level of haploid derivatives of the feral strain EM93 (relative to *ACT1*) (Smukalla *et al.*, 2008). We shed light on the possible secondary adaptations by performing test tube fermentations at a high rotation speed (150 r.p.m.) to avoid mass transfer limitations. Here, we could observe that the growth rates of the different independent transformants were similar, reinforcing the fact that no important mutations occurred. Nevertheless, when considering more static fermentations, reductions in growth rate can be observed between planktonic and flocculating cells as discussed previously.

In the S288C background, adhesive phenomena such as flocculation, pseudohyphal growth and invasive growth are completely absent because of a naturally occurring nonsense mutation at nucleotide position 608 in the transcriptional activator gene, *FLO8* (Liu *et al.*, 1996). Introduction of an active copy of *FLO8* in the S288C background resulted in the activation of *FLO1* and, to a lesser degree, *FLO11*. However, for unknown reasons, no increase in *FLO5*, *FLO9* and *FLO10* transcripts could be detected in our study. This finding was

also observed in the study performed by Fichtner *et al.* (2007). As a consequence, all *FLO* genes are transcriptionally silent, providing a unique opportunity to evaluate the adhesion characteristics of each individual *FLO* gene. Interestingly, invasive growth is restored in a *FLO8* intact strain, but remains absent in a *FLO11*-overexpression strain, confirming the observations of Purevdorj-Gage *et al.* (2007). These findings indicate that *FLO8* has an additional role in the triggering of specific factors leading to the invasive phenotype. It is hypothesized that *FLO8* plays a role in the invasiveness of yeast controlling other downstream targets that in turn alter the budding pattern, cell shape, cell polarization or other cell–surface or extracellular proteins (Kobayashi *et al.*, 1999). Indeed, under inducing conditions, diploid pseudohyphal cells often show polarized staining of Flo11, which is required for pseudohyphal growth (Guo *et al.*, 2000).

With respect to the number, size and strength of the flocs, different flocculation types could be distinguished. The observed substantial variation was even more remarkable when considering the carbohydrate recognition profiles of the overexpressed adhesins. Our results showed that Flo1, which is only inhibited by mannose, is the most specific lectin-like Flo adhesin in the S288C strain. Flo5 is not only strongly inhibited by mannose, but also very weakly by a range of other sugars. Less-selective adhesins such as Flo9 and Flo10 were strongly inhibited by mannose and weakly inhibited by a range of sugars. It was already demonstrated that Flo1 and, contrary to our results, already suggested on the basis of sequence homology that Flo5 and Flo9 were mannose-binding flocculins (Teunissen & Steensma, 1995). Govender *et al.* (2008) recently showed that Flo1 and Flo5 both exhibit a mannose-specific flocculation phenotype. Possibly, reduced expression levels of the adhesin-encoding *FLO*5 gene revealed the slight inhibition by other sugars in our experiment. Our results thus indicate that the classification in flo1- and newflo-type adhesins is not stringent for Flo5, Flo9 and Flo10. In the Σ1278b strain, Flo1 was inhibited by the presence of mannose and Flo10 by mannose, maltose, sucrose and glucose (Guo *et al.*, 2000). Remarkably, the sugar specificity of Flo10 in both the Σ1278b strain and the S288C strain remained the same, despite the fact that the length and sequence of the intragenic repetitive domain of both proteins are different as shown by Verstrepen *et al.* (2005). This observation reinforces the statement that the central part of the adhesin only influences the strength of the cell–cell interaction and not the specificity. On the other hand, other researchers do find changes in sugar specificity that correlate with changes in the middle repetitive domain of Flo proteins (Liu *et al.*, 2007). Clearly, this is a major unresolved issue; it would be interesting to investigate if and how both the N-terminal and middle Flo domains contribute to sugar binding and specificity. Another captivating issue is the strain specificity of Flo11-mediated flocculation. It was already shown that Flo11 in *S. diastaticus* was responsible for mannose-specific cell–cell adherence (Bayly *et al.*, 2005; Douglas *et al.*, 2007). The target for this adhesin is also Flo11, which thus acts as a receptor (Douglas *et al.*, 2007). Remarkably, it was shown that both Flo11 from *S. cerevisiae* and *S. diastaticus* were able to cause cell adherence to *S. diastaticus* cells when covalently bound to beads, and thus when decoupled from their host organism (Douglas *et al.*, 2007). Possibly, the (carbohydrate) receptors for cell–cell adherence are different in both strains, which highlights the importance of the genetic background in which adhesins are studied.

Yeast adhesins share a common three-domain structure: the C-terminal domain anchors the adhesin to the yeast cell wall while the central intramolecular Ser/Thr-rich regions function as a spacer that improves the accessibility of the N-terminal carbohydrate or peptide-binding domains (Breinig & Schmitt, 2002; Dranginis *et al.*, 2007). Recently, Zupancic *et al.* (2008) identified a pentapeptide involved in sugar recognition in the N-terminal ligand-binding domain of the *C. glabrata EPA* (epithelian adhesin) family. Multiple alignment of the Flo1, Flo5, Flo9, Flo10, Lg-Flo1 and Flo11 N-terminal binding domains containing this pentapeptide (VSWGT/KVLAR motifs in Flo1/Lg-Flo1) is shown in Fig. 8. The tryptophan residue at position 228, which is present in all the studied adhesins with the exception of Lg-Flo1, was shown to be involved in mannose recognition through binding with the C-2 hydroxyl group of mannose (Kobayashi *et al.*, 1998; Bayly *et al.*, 2005), which is in accordance with our observations. The leucine residue at the same position in the Lg-Flo1 protein binds neither to

Fig. 8. Multiple alignment of the amino acid sequence of the N-terminal part of the Flo1, Flo5, Flo9, Flo10, Lg-flo1 and Flo11 adhesins involved in sugar recognition as shown by Kobayashi *et al*. (1998). Nonconserved amino acid residues are shaded in grey. The amino acids indicated with a grey cross contribute to sugar recognition as described by Kobayashi *et al*. (1998). The black line indicates the region of the adhesins involved in glycan specificity as described by Zupancic *et al*. (2008).

mannose nor to glucose, and is thus not involved in sugar recognition (Kobayashi *et al.*, 1998). At position 202, a proline residue is observed in Flo1, which possibly sterically and aspecifically blocks sugar entrance. Together with the tryptophan residue at position 228, this may explain the specific but incomplete inhibition of Flo1 by mannose, even at high concentrations (1 M). Remarkably, Flo10 and Lg-Flo1 are identical in the region between position 200 and 240 with the exception of the pentapeptide motifs (VSWGT/KVLAR in Flo10/Lg-Flo1). Consequently, they share a threonine residue at position 202, which is shown to bind to the C-4 hydroxyl group of mannose and glucose but not to the inversely oriented hydroxyl group of galactose (Kobayashi *et al.*, 1998). Flo5 and Flo9 share an aspartate residue at the same position, which possibly also allows the binding of nongalactose sugars, but to a lesser extent than Flo10. In contrast to Flo1, which has one sugar recognition site, Flo5, Flo9 and Flo10 may therefore possess two recognition sites, explaining the more pronounced inhibition by mannose. Together with the aspecific binding by threonine at position 202, this may also explain the more relaxed sugar specificity of these adhesins. Taken together, both the specificity and the strength of sugar inhibition can be explained by this model, although it may seem possible that other domains may also play a role. Further research has to clarify whether the number and sequence of intragenic repeats also play a role in the sugar inhibition profiles as suggested by Liu *et al.* (2007).

The combined action of hydrophobic interactions and cell–cell interactions in the *FLO5*- and *FLO9*-overexpression strains likely explains the tendency to form the strongest mats on plastics. Remarkably, the *FLO11*-overexpression strain showed a slightly more pronounced mat formation than the WT. This observation conflicts with the important role of Flo11 in plastic adhesion in the Σ1278b strain (Reynolds & Fink, 2001), but can possibly be related to the presence of hydrophilic groups after surface treatment of the polystyrene that has been used in the experimental setup (Mortensen *et al.*, 2007a). Fichtner *et al.* (2007) already suggested that, unlike Flo11 for the Σ1278b strain, Flo1 is the major adhesin for substrate interaction of S288C. In addition, we showed that Flo5 and Flo9 further enhance this substrate interaction. Remarkably, the strain with an intact *FLO8* enhances mat formation on plastics even more than the *FLO1*-overexpression strain, notwithstanding the lower *FLO1* expression. This shows that other factors involved in the interaction between polystyrene surfaces and the yeast cell wall are linked to *FLO8*.

The hydrophobicity conferred by Flo11 is in accordance with the results from Reynolds & Fink (2001), Purevdorj-Gage *et al.* (2007), Mortensen *et al.* (2007b) and Govender *et al.* (2008). Moreover, different studies describe the increased hydrophobicity when Flo1 or Flo5 is present in the cell wall (Rhymes & Smart, 2000; Verstrepen *et al.*, 2001; Govender *et al.*, 2008). In our study, we generalize this point of view and suggest that every hydrophobic cell surface molecule might promote nonspecific adhesion to certain surfaces by increasing cell wall hydrophobicity. In the strain bearing an intact *FLO8* gene, intermediate hydrophobicity was observed, which can be attributed to the reduced expression of *FLO1* and *FLO11* when compared with the overexpression strains. These insights may be an important factor in our understanding of the adhesion step of pathogenic fungi (Doyle, 2000).

In conclusion, our results offer a clear and 'clean' insight into the remarkable capacity of *S. cerevisiae* to display several adhesion phenotypes, each of which may be fine-tuned to a specific environment. Moreover, our results represent a step forward in the elucidation of the molecular mechanism behind the carbohydrate recognition of adhesins. A knowledge of the relation between adhesin structure and function will be of valuable importance in both a medical and an industrial context.

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References

Bayly JC, Douglas LM, Pretorius IS, Bauer FF & Dranginis AM (2005) Characteristics of Flo11-dependent flocculation in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **5**: 1151–1156.

Bester MC, Pretorius IS & Bauer FF (2006) The regulation of *Saccharomyces cerevisiae FLO* gene expression and Ca^{2+}-dependent flocculation by Flo8p and Mss11p. *Curr Genet* **49**: 375–383.

Bony M, Thines-Sempoux D, Barre P & Blondin B (1997) Localization and cell surface anchoring of the *Saccharomyces cerevisiae* flocculation protein Flo1p. *J Bacteriol* **179**: 4929–4936.

Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P & Boeke JD (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**: 115–132.

Braus GH, Grundmann O, Brückner S & Mösch H-U (2003) Amino acid starvation and Gcn4p regulate adhesive growth and *FLO11* gene expression in *Saccharomyces cerevisiae*. *Mol Biol Cell* **14**: 4272–4284.

Breinig F & Schmitt MJ (2002) Spacer-elongated cell wall fusion proteins improve cell surface expression in the yeast *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **58**: 637–644.

Caro LHP, Tettelin H, Vossen JH, Ram AFJ, Van Den Ende H & Klis FM (1997) *In silico* identification of glycosyl-phosphatidylinositol-anchored plasma-membrane and cell wall proteins of *Saccharomyces cerevisiae*. *Yeast* **13**: 1477–1489.

Ceccato-Antonini SR & Sudbery PE (2004) Filamentous growth in *Saccharomyces cerevisiae*. *Braz J Microbiol* **35**: 173–181.

Cullen PJ & Sprague GF (2000) Glucose depletion causes haploid invasive growth in yeast. *P Natl Acad Sci USA* **97**: 13619–13624.

da Silva PC, Horii J, Miranda VS, Brunetto HG & Ceccato-Antonini SR (2007) Characterization of industrial strains of *Saccharomyces cerevisiae* exhibiting filamentous growth induced by alcohols and nutrient deprivation. *World J Microbiol Biot* **23**: 697–704.

D'Hautcourt O & Smart KA (1999) Measurement of brewing yeast flocculation. *J Am Soc Brew Chem* **57**: 123–128.

Dickinson JR (2005) Are yeasts free-living unicellular eukaryotes? *Lett Appl Microbiol* **41**: 445–447.

Douglas LJ (2003) *Candida* biofilms and their role in infection. *Trends Microbiol* **11**: 30–36.

Douglas LM, Li L, Yang Y & Dranginis AM (2007) Expression and characterization of the flocculin Flo11/Muc1, a *Saccharomyces cerevisiae* mannoprotein with homotypic properties of adhesion. *Eukaryot Cell* **6**: 2214–2221.

Doyle RJ (2000) Contribution of the hydrophobic effect to microbial infection. *Microb Infect* **2**: 391–400.

Dranginis AM, Rauceo JM, Coronado JE & Lipke PN (2007) A biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions. *Microbiol Mol Biol R* **71**: 282–294.

Fichtner L, Schulze F & Braus GH (2007) Differential Flo8p-dependent regulation of *FLO1* and *FLO11* for cell–cell and cell–substrate adherence of *S. cerevisiae*. *Mol Microbiol* **66**: 1276–1289.

Gagiano M, Bauer FF & Pretorius IS (2002) The sensing of nutritional status and the relationship to filamentous growth in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **2**: 433–470.

Gancedo JM (2001) Control of pseudohyphae formation in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **25**: 107–123.

Gognies S, Ait Barka E, Gainvors-Claisse A & Belarbi A (2006) Interactions between yeasts and grapevines: filamentous growth, endopolygalacturonase and phytopathogenicity of colonizing yeasts. *Microb Ecol* **51**: 109–116.

Govender P, Domingo JL, Bester MC, Pretorius IS & Bauer FF (2008) Controlled expression of the dominant flocculation genes *FLO1*, *FLO5*, and *FLO11* in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **74**: 6041–6052.

Guo B, Styles CA, Feng Q & Fink GR (2000) A *Saccharomyces cerevisiae* gene family involved in invasive growth, cell–cell adhesion, and mating. *P Natl Acad Sci USA* **97**: 12158–12163.

Hahn MW, De Bie T, Stajich JE, Nguyen C & Cristianini N (2005) Estimating the tempo and mode of gene family evolution from comparative genomic data. *Genome Res* **15**: 1153–1160.

Halme A, Bumgarner S, Styles CA & Fink GR (2004) Genetic and epigenetic regulation of the FLO gene family generates cell-surface variation in yeast. *Cell* **116**: 405–415.

Harrison P, Kumar A, Lan N, Echols N, Snyder M & Gerstein M (2002) A small reservoir of disabled ORFs in the yeast genome and its implications for the dynamics of proteome evolution. *J Mol Biol* **316**: 409–419.

Janke C, Magiera MM, Rathfelder N *et al.* (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **21**: 947–962.

Kobayashi O, Hayashi N, Kuroki R & Sone H (1998) Region of Flo1 proteins responsible for sugar recognition. *J Bacteriol* **180**: 6503–6510.

Kobayashi O, Yoshimoto H & Sone H (1999) Analysis of the genes activated by the *FLO8* gene in *Saccharomyces cerevisiae*. *Curr Genet* **36**: 256–261.

Lebeer S, Verhoeven TLA, Velez MP, Vanderleyden J & De Keersmaecker SCJ (2007) Impact of environmental and genetic factors on biofilm formation by the probiotic strain *Lactobacillus rhamnosus* GG. *Appl Environ Microbiol* **73**: 6768–6775.

Lipke PN & Ovalle R (1998) Cell wall architecture in yeast: new structure and new challenges. *J Bacteriol* **180**: 3735–3740.

Liu H, Styles CA & Fink GR (1996) *Saccharomyces cerevisiae* S288C has a mutation in FLO8, a gene required for filamentous growth. *Genetics* **144**: 967–978.

Liu N, Wang D, Wang ZY, He XP & Zhang B (2007) Genetic basis of flocculation phenotype conversion in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7**: 1362–1370.

Lo W-S & Dranginis AM (1998) The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by *Saccharomyces cerevisiae*. *Mol Biol Cell* **9**: 161–171.

Masy CL, Henquinet A & Mestdagh MM (1992) Flocculation of *Saccharomyces cerevisiae*: inhibition by sugars. *Can J Microbiol* **38**: 1298–1306.

Mortensen HD, Dupont K, Jespersen L & Arneborg N (2007a) The Flo11p-deficient *Saccharomyces cerevisiae* strain background S288C can adhere to plastic surfaces. *Colloid Surface B* **60**: 131–134.

Mortensen HD, Dupont K, Jespersen L, Willats WGT & Arneborg N (2007b) Identification of amino acids involved in the FLO11p-mediated adhesion of *Saccharomyces cerevisiae* to a polystyrene surface using phage display with competitive elution. *J Appl Microbiol* **103**: 1041–1047.

Palkova Z (2004) Multicellular microorganisms: laboratory versus nature. *EMBO Rep* **5**: 470–476.

Pittet M & Conzelmann A (2007) Biosynthesis and function of GPI proteins in the yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1771**: 405–420.

Prusty R, Grisafi P & Fink GR (2004) The plant hormone indole acetic acid induces invasive growth in *Saccharomyces cerevisiae*. *P Natl Acad Sci USA* **101**: 4153–4157.

Purevdorj-Gage B, Orr ME, Stoodley P, Sheehan KB & Hyman LE (2007) The role of *FLO11* in *Saccharomyces cerevisiae* biofilm development in a laboratory based flow-cell system. *FEMS Yeast Res* **7**: 372–379.

Reynolds TB & Fink GR (2001) Bakers' yeast, a model for fungal biofilm formation. *Science* **291**: 878–881.

Reynolds TB, Jansen A, Peng X & Fink GR (2008) Mat formation in *Saccharomyces cerevisiae* requires nutrient and pH gradients. *Eukaryot Cell* **7**: 122–130.

Rhymes MR & Smart KA (2000) The relationship between flocculation and cell surface physical properties in a *FLO1* ale yeast. *Brewing Yeast Fermentation Performance* (Smart KA, ed), pp. 152–159. Blackwell Science, Oxford.

Roberts RL & Fink GR (1994) Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genes Dev* **8**: 2974–2985.

Rosenberg M (1984) Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. *FEMS Microbiol Lett* **22**: 289–295.

Rupp S, Summers E, Lo H-J, Madhani H & Fink GR (1999) MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast *FLO11* gene. *EMBO J* **18**: 1257–1269.

Sampermans S, Mortier J & Soares EV (2005) Flocculation onset in *Saccharomyces cerevisiae*: the role of nutrients. *J Appl Microbiol* **98**: 525–531.

Smukalla S, Caldara M, Pochet N *et al.* (2008) FLO1 is a hyper-variable green beard gene that drives a transition to multicellularity in budding yeast. *Cell* **135**: 726–737.

Stanhill A, Schick N & Engelberg D (1999) The yeast ras/cyclic AMP pathway induces invasive growth by suppressing the cellular stress response. *Mol Cell Biol* **19**: 7529–7538.

Teunissen AWRH & Steensma HY (1995) Review: the dominant flocculation genes of *Saccharomyces cerevisiae* constitute a new subtelomeric gene family. *Yeast* **11**: 1001–1013.

van Oss CJ (1995) Hydrophobicity of biosurfaces – origin, quantitative determination and interaction energies. *Colloid Surface B* **5**: 91–110.

Verstrepen KJ & Klis FM (2006) Flocculation, adhesion and biofilm formation in yeasts. *Mol Microbiol* **60**: 5–15.

Verstrepen KJ, Derdelinckx G, Delvaux FR, Winderinckx J, Thevelein JM, Bauer FF & Pretorius IS (2001) Late fermentation expression of *FLO1* in *Saccharomyces cerevisiae*. *J Am Soc Brew Chem* **59**: 69–76.

Verstrepen KJ, Derdelinckx G, Verachtert H & Delvaux FR (2003) Yeast flocculation: what brewers should know. *Appl Environ Microbiol* **61**: 197–205.

Verstrepen KJ, Reynolds TB & Fink GR (2004) Origins of variation in the fungal cell surface. *Nat Microbiol Rev* **2**: 533–540.

Verstrepen KJ, Jansen A, Lewitter F & Fink GR (2005) Intragenic tandem repeats generate functional variability. *Nat Genet* **37**: 1–5.

Vopálenská I, Hulková M, Janderová B & Palkova Z (2005) The morphology of *Saccharomyces cerevisiae* colonies is affected by cell adhesion and the budding pattern. *Res Microbiol* **156**: 921–931.

Zaragoza O & Gancedo JM (2000) Pseudohyphal growth is induced in *Saccharomyces cerevisiae* by a combination of stress and cAMP signalling. *Antonie Van Leeuwenhoek* **78**: 187–194.

Zupancic ML, Frieman M, Smith D, Alvarez RA, Cummings RD & Cormack BP (2008) Glycan microarray analysis of *Candida glabrata* adhesin ligand specificity. *Mol Microbiol* **68**: 547–559.