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

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**Keywords**

flocculation; *Saccharomyces cerevisiae*; adhesin; invasive growth; hydrophobicity; biofilm.

**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; Vopalenská 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., 2007; 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 adhesion and inhibit flocculation are consumed. Consequently, this results in a cost-effective and environmentally 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 transcription 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 Ira1 mutants, the expression of FLO10 is also variegated by means of a distinct set of histone deacetylases, Hist1p and Hist2p.

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], orbital shakend 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 OD600nm. 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 DMS100 camera.

Selection of the overexpression strains was performed on YPD+ geneticine medium (100 μg/mL, Difco). Selection of the strains transformed with an intact FLO8 andURA3 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 (NH4)2SO4 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).

Table 1. Yeast strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Genotype/description</th>
<th>Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BY4741 (WT)</td>
<td>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>S288C</td>
</tr>
<tr>
<td>BY4742 (WT)</td>
<td>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</td>
<td>S288C</td>
</tr>
<tr>
<td>BY4741 (URA3)</td>
<td>his3Δ1 leu2Δ0 met15Δ0 URA3</td>
<td>S288C</td>
</tr>
<tr>
<td>BY4741 (FLO8)</td>
<td>his3Δ1 leu2Δ0 met15Δ0 URA3 FLO8</td>
<td>S288C</td>
</tr>
<tr>
<td>BY4742 (FLO1)</td>
<td>kanMX4:PTEF1-FLO1</td>
<td>S288C</td>
</tr>
<tr>
<td>BY4742 (FLO5)</td>
<td>kanMX4:PTEF1-FLO5</td>
<td>S288C</td>
</tr>
<tr>
<td>BY4742 (FLO9)</td>
<td>kanMX4:PTEF1-FLO9</td>
<td>S288C</td>
</tr>
<tr>
<td>BY4742 (FLO10)</td>
<td>kanMX4:PTEF1-FLO10</td>
<td>S288C</td>
</tr>
<tr>
<td>BY4742 (FLO11)</td>
<td>kanMX4:PTEF1-FLO11</td>
<td>S288C</td>
</tr>
<tr>
<td>Σ1278b</td>
<td>leu2Δ0 ura3Δ0</td>
<td>Σ1278b</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH1</td>
<td>4.3-kb insert with FLO8 on pRS316</td>
<td>Liu et al. (1996)</td>
</tr>
<tr>
<td>pYM-N18</td>
<td>TEF promoter kanMX4</td>
<td>EUROSCARF (Frankfurt, Germany)</td>
</tr>
</tbody>
</table>

Table 2. The geneticine-resistant colonies were analysed by PCR to confirm correct genomic integration of the translation elongation factor 1 (TEFI) 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 pH1, 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 pH1 and inserted at the FLO8 locus to construct a reference BY4741 strain without uracil auxotrophy.

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 OD600nm of 0.5 and grown until the late-exponential growth phase was reached. Extraction of the RNA of pelleted cells
Five microlitres of cDNA (100 ng were diluted, 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 mM 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 microliters 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.

**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 CaSO₄ solution. Subsequently, the liquid was discarded and 5 mL flocculation buffer (3.75 mM CaSO₄; 82.9 mM NaCH₃COO; 67.4 mM CH₃COOH; pH 4.5) was added to the cells, moved to a glass tube, to a final OD₆₀₀nm 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 OD₆₀₀nm 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

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

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLO1_TEF_FW</td>
<td>TTTCCGGTGTCTATTTTTATCTTGTACCAAGTAACAGAATCTCCAAAATAATCGGTACGCTGACGTGACCTGAC</td>
</tr>
<tr>
<td>FLO1_TEF_RV</td>
<td>AGTGGCAAGAATGGAAGTCGCCAAAATACATAGCAGATGAGGATGCTGACCAAGATATTCAGCTGTAGCATG</td>
</tr>
<tr>
<td>FLO5_TEF_FW</td>
<td>CAGTAAATCTTCCATTTTTATATTGAGGCAGACTATATAGCTGACGACGTGACCTGAC</td>
</tr>
<tr>
<td>FLO5_TEF_RV</td>
<td>TAATAGTGGCAAGAAAGGGCCAGATACACAAAAATAGCGTGTGGCAGATTGTCTGACAGATG</td>
</tr>
<tr>
<td>FLO9_TEF_FW</td>
<td>GCTCTTTTATATGGCAAAAAAGGAAAGGTGTAACATAAAAGCCAAAAAGGGAATGTGAGAGGAC</td>
</tr>
<tr>
<td>FLO9_TEF_RV</td>
<td>TAGTGGCAAGAAAGGGCCAGATACACAAAAATAGCGTGTGGCAGATTGTCTGACAGATG</td>
</tr>
<tr>
<td>FLO10_TEF_FW</td>
<td>TTACGTTGAAGATTTGTTTACTGGTAGTAACAAAATATGTCAGACGACATGATGATTCTGACAGATG</td>
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<tr>
<td>FLO10_TEF_RV</td>
<td>CGTACGCTGACGTGAC</td>
</tr>
<tr>
<td>FLO11_TEF_FW</td>
<td>TTCTATATATATATTTTGTGACACTATATAGCTGACGACGTGACCTGAC</td>
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<tr>
<td>FLO11_TEF_RV</td>
<td>AAGCCGAGTIAAATAGGCAAGAAGGACAAATAAGGCGAGTGGAGAGGAC</td>
</tr>
</tbody>
</table>

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

**Table 3. Primers and probes used for the qRT-PCR and the verification of the transformants by normal PCR (KANMX_FW and FLO5_TAQ_RV or SYBR_RV)**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’→3’)</th>
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</thead>
<tbody>
<tr>
<td>KANMX_FW</td>
<td>TGATTTTGATGACGAGACGTAAT</td>
</tr>
<tr>
<td>FLO1_TAQ_FW</td>
<td>ATGCCATATGTTTTGAGCTTCTTAA</td>
</tr>
<tr>
<td>FLO1_TAQ_RV</td>
<td>GAAAGACAGCCTTCTGGCTT</td>
</tr>
<tr>
<td>FLO1_MGB_FW</td>
<td>ACTCTGGCAGACTAACTAGT</td>
</tr>
<tr>
<td>FLO5_TAQ_FW</td>
<td>GCACACACTCATTTTTGTGAA</td>
</tr>
<tr>
<td>FLO5_TAQ_RV</td>
<td>GAAAGACAGCCTTCTGGCTT</td>
</tr>
<tr>
<td>FLO5_MGB_FW</td>
<td>CCTTTCTGGCAGACTAA</td>
</tr>
<tr>
<td>FLO9_TAQ_FW</td>
<td>TTATGTTTACTACAGCATTGACGTCACA</td>
</tr>
<tr>
<td>FLO9_TAQ_RV</td>
<td>AAGTTACATTACATACATTCTCTTGG</td>
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<tr>
<td>FLO9_MGB_FW</td>
<td>CATGCTGTCGGAC</td>
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<tr>
<td>FLO8_SYBR_FW</td>
<td>TTGACGCGCTCATAAAATTCA</td>
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<td>FLO8_SYBR_RV</td>
<td>AAATCTCGTTTGTGTTCTTCTGTAAG</td>
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<td>CGTACGCTGACGACGTAAT</td>
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<td>FLO11_SYBR_FW</td>
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<td>FLO11_SYBR_RV</td>
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</tr>
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<td>ACT1_SYBR_FW</td>
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<td>ACT1_SYBR_RV</td>
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<td>ACT1_TAQ_FW</td>
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</tr>
<tr>
<td>ACT1_MGB_FW</td>
<td>GTTCCTGGACATCAA</td>
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</tbody>
</table>
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 OD₆₀₀nm. A calculated amount of yeast cells was washed once and resuspended in 2 mL isotonic saline to achieve an OD₆₀₀nm 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™ 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 OD₆₀₀nm 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 (OD₆₀₀nm) 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...
flocculation phenotype, with flocs that do not break upon vortexing and strong shaking (Fig. 3). In the second group, the \textit{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 \textit{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.
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).
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).

![Fig. 5](image.jpg)

**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 S1278b strain.

![Fig. 6](image.jpg)

**Fig. 6.** Quantification (a) and visualization (b) of mat formation in polystyrene plates. The intensity of the crystal violet staining (OD570nm) is a measure for mat formation. The adhesion score represents the measured OD570nm relative to the WT strain. The error bars represent SDs of six biological experiments. The data shown are representative of three independent experiments.
than the WT strains and the strains had a slightly higher tendency to form adherent mats WT, respectively). The FLO1-

\[\text{Flo family-mediated adhesion in Saccharomyces cerevisiae}\]

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure7.png}
\caption{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.}
\end{figure}

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 S1278b strain (Fig. 5c). In contrast, the S1278b 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.

\section*{Polystyrene adhesion of \textit{S. cerevisiae} cells}

The ability of pathogenic yeasts, such as \textit{Candida albicans} and \textit{Candida glabrata}, to bind to plastic devices, such as medical prostheses and catheters, is of crucial importance in hospital-acquired infections (Douglas, 2003). \textit{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.

\section*{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 \textit{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%).

\section*{Discussion}

Here, we describe the comprehensive phenotypic characterization of a nonsexual adhesin family in \textit{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 \textit{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 \textit{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
It is hypothesized that triggering of specific factors leading to the invasive phenotype. 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 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 adhesion (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 (epithelial 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 specifically 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 (VSWGTV/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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