Differential Flo8p-dependent regulation of FLO1 and FLO11 for cell–cell and cell–substrate adherence of S. cerevisiae S288c

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Summary

Cell–cell and cell–surface adherence represents initial steps in forming multicellular aggregates or in establishing cell–surface interactions. The commonly used Saccharomyces cerevisiae laboratory strain S288c carries a flo8 mutation, and is only able to express the flocculin-encoding genes FLO1 and FLO11, when FLO8 is restored. We show here that the two flocculin genes exhibit differences in regulation to execute distinct functions under various environmental conditions. In contrast to the laboratory strain Σ1278b, haploids of the S288c genetic background require FLO1 for cell–cell and cell–substrate adhesion, whereas FLO11 is required for pseudohyphae formation of diploids. In contrast to FLO11, FLO1 repression requires the Sin4p mediator tail component, but is independent of the repressor Sfl1p. FLO1 regulation also differs from FLO11, because it requires neither the KSS1 MAP kinase cascade nor the pathways which lead to the transcription factors Gcn4p or Msn1p. The protein kinase A pathway and the transcription factors Flo8p and Mss11p are the major regulators for FLO1 expression. Therefore, S. cerevisiae is prepared to simultaneously express two genes of its otherwise silenced FLO reservoir resulting in an appropriate cellular surface for different environments.

Introduction

Natural fungal populations respond to appropriate environmental conditions by cell to cell adherence, cell to substrate adherence, or the formation of biofilms. Morphological changes such as flocculation or biofilm formation are important for various biotechnological processes. Adherence to human tissue and to plastic devices are of medical relevance because they represent initial steps in the establishment of pathogenic fungal–host interactions which can result in access to internal organs for the fungus. The budding yeast Saccharomyces cerevisiae has been used as a fungal model organism to explore cell–substrate and cell–cell adhesion. Diploid yeast strains are dimorphic and can therefore switch between a single celled and a filamentous pseudohyphal growth form with elongated cells. Diploid pseudohyphae formation depends on sufficient supply of fermentable carbon sources like glucose and simultaneous limitation of nitrogen sources such as ammonium ions (Gimeno and Fink, 1992; Gimeno et al., 1992; Mösch and Fink, 1997). In haploid yeast cells, adhesive growth, adherence to surfaces (Roberts and Fink, 1994; Guo et al., 2000) and formation of biofilms (Roy et al., 1991; Cappellaro et al., 1994; Reynolds and Fink, 2001) can be induced on rich media when carbon sources become limiting.

All adherence events require the expression of specific cell surface glycoproteins, which are encoded by the FLO gene family. In S. cerevisiae Σ1278b, which has been primarily used for such studies, only FLO11 is expressed and is activated under specific environmental conditions (Guo et al., 2000; Halme et al., 2004; Verstrepen et al., 2004). Four additional FLO genes (FLO1, FLO5, FLO9 and FLO10) are epigenetically silenced by different histone deacetylases (HDAC) (Halme et al., 2004). The active FLO11 gene, which is also named MUC1 (Lambrechts et al., 1996), encodes a glycosylphosphatidylinositol-linked glycoprotein similar to adhesins of pathogenic fungi (Lo and Dranginis, 1996).

The inducible FLO11 gene carries one of the largest promoters of the yeast genome (Rupp et al., 1999). Various transcription factors, which perceive multiple distinct external signals from specific signal transduction cascades (Banuett, 1998; Lengeler et al., 2000). During the yeast form of growth, FLO11 expression is inhibited by HDAC silencing and by repressors such as Sfl1p (Pan and Heitman, 2002) or Nrg1p/Nrg2p (Kuchin et al., 2002) which interact with the FLO11 promoter. The corepressor Tup1p which has multiple functions in yeast acts in

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concert with Sfl1p and also affects the Nrg repressors (Berkey et al., 2004). Various components of the RNA polymerase II mediator complex including Sin4p (Conlan and Tzamarias, 2001), Srb8p or Ssn8p, have been identified as additional repressors for FLO11 expression (Palecek et al., 2000).

Glucose starvation in haploids or nitrogen starvation in diploids overcomes FLO11 repression, and aromatic alcohols have been identified as inducing signals (Chen and Fink, 2006).

Several signalling pathways, including the mitogen-activated protein kinase (MAPK) cascade with Kss1p as specific MAP kinase or the cAMP-dependent protein kinase A (PKA) pathway activate FLO11 expression (Roberts and Fink, 1994; Mösch et al., 1999; Rupp et al., 1999; Cullen and Sprague, 2000; Palecek et al., 2000). Kss1p activates the transcription factors Ste12p and Flo8p (Köhler et al., 2002; Brückner et al., 2004; Chou et al., 2004). Mss11p represents another transcriptional activator that plays an essential role at the convergence of the MAPK and PKA pathways (Gagiano et al., 1999; van Dyk et al., 2005). The repressor Sfl1p and the activator Flo8p antagonistically control the expression of FLO11 by binding to a common promoter element. Sfl1p and Flo8p are direct molecular targets of the PKA catalytic subunit Tpk2p. Phosphorylation by PKA promotes Flo8p binding and activation of the FLO11 promoter and relieves repression by prohibiting dimerization and DNA binding by Sfl1p (Pan and Heitman, 2002). Low glucose in haploid and low nitrogen in diploid yeasts also activate the protein kinase Snf1p which positively regulates FLO11 expression by antagonizing the two repressors Nrg1p and Nrg2p (Kuchin et al., 2002).

FLO11 expression can also respond to other forms of nutritional limitations including amino acid starvation in haploid as well as in diploid cells. This response depends on the transcription factor Gcn4p which is regulated by the general control of amino acid biosynthesis pathway and its sensor kinase Gcn2p (Hinnebusch, 1997; Hinnebusch and Natarajan, 2002; Braus et al., 2003). The large FLO11 promoter is also affected by additional factors which support adaptation to changing environmental conditions including the transcription factors Sok2p (Pan and Heitman, 2000; Vachova et al., 2004), Phd1p (Gimeno and Fink, 1992) and the product of the MSN1 (also known as PHD2, MSS10 or FUP4) gene (Lorenz and Heitman, 1998). In addition to the transcriptional regulation, there is also evidence for control of FLO11 expression on a post-transcriptional level (Strittmatter et al., 2006).

In industrial yeasts, including flocculent bottom-fermenting yeast strains, another gene of the FLO family, FLO1, has been shown to be active and regulated by FLO8. It is considered to play an important role in mannose-specific flocculation, which is inhibited by mannose but not by glucose (Kobayashi et al., 1996; 1998; 1999). In S. cerevisiae Σ1278b, FLO1 is silenced and has only artificially been activated by inserting the GAL1 promoter upstream of the FLO1 open reading frame. Induced expression of this engineered GAL1-FLO1 strain in galactose medium also resulted in enhanced flocculation (Guo et al., 2000; Halme et al., 2004; Verstrepen et al., 2004). The most commonly used S. cerevisiae laboratory strain S288c is impaired in haploid adhesion, biofilm formation and diploid pseudohyphal growth. This has been at least partially attributed to the acquisition of a nonsense mutation in the FLO8 gene encoding one of the key transcriptional activators of FLO genes (Liu et al., 1996). Restoration of FLO8 resulted in the activation of transcription of two FLO genes, FLO11 and FLO1, suggesting a similar regulatory mechanism for both promoters (Kobayashi et al., 1999). In addition, overexpression of the transcription factor encoding MSS11 (Bester et al., 2006) or the GST1 gene for a Sfl1p repressor interacting protein (Shen et al., 2006) induce FLO1 transcription in flo8-deficient yeasts.

We have studied the regulation and the function of FLO11 and FLO1 in more detail in a FLO8-restored S. cerevisiae strain S288c. We find that Flo11p is primarily required to establish a cell–substrate interaction of the initial cell layer, whereas Flo1p is essential to support cell–cell interactions of the following cell layers of the colony. In addition, we find that the FLO1 promoter is significantly less complex than the FLO11 promoter. Laboratory conditions favour yeast strains which are less adhesive, because a detailed analysis of the FLO1 locus of the Σ1278b strain revealed that the Flo8p binding site is missing in the promoter which is presumably the reason why only the FLO11 gene can be activated in this yeast.

Results

FLO1 and FLO11 of S288c play distinct roles in adhesion, pseudohyphae formation and flocculation

Haploid invasive growth of S. cerevisiae Σ1278b due to the induction of FLO11 can be achieved in rich yeast peptone dextrose (YPD) medium after glucose becomes limiting which requires six or more days (Roberts and Fink, 1994; Guo et al., 2000). A comparison of different S288c [FLO8] derivatives revealed subtle differences in the intensity of adhesion, which are achieved by a cooperation of the two adhesins Flo1p and Flo11p (Fig. 1A). S288c [FLO8] cells carrying only a fIo11 deletion were completely washed off the agar surface as fluffs after a short time of gentle washing. Most of the corresponding fIo11A derivative cells were also easily washed off the plate; however, a layer of yeast cells remained directly on

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the agar surface and could be washed off neither by a strong stream of water (Fig. 1A) nor by rubbing the surface (not shown). Strains lacking both flocculin genes, FLO1 and FLO11, are completely washed off the agar without any remaining cell layer on the surface. This suggests that under these glucose limiting conditions both flocculins are required and fulfill distinct functions for S288c [FLO8]. Flo11p seems to be required for cell–substrate interaction for anchoring the yeast population to the agar surface. Flo1p allows cell–cell interaction of different yeast cell layers, which resembles its function during flocculation. The Flo11p substrate interaction function is not necessary for adhesion to synthetic complete (SC) medium, to plastic surfaces and for flocculation where Flo1p alone is crucial and sufficient. In Σ1278b, such a functional diversity of flocculins has not been described, and all known aspects of adhesive growth are contributed exclusively to Flo11p.

FLO11 is also required for pseudohyphal formation of diploid yeast Σ1278b, which can be induced when sufficient carbon sources are available but ammonium ions are limiting. Pseudohyphal formation of S288c requires an intact FLO8 gene (Fig. 1B). When we analysed the formation of pseudohyphal of diploid S288c [FLO8] derivatives carrying homozygous flo11 deletions, we found that they showed the same morphological phenotypes as described for Σ1278b mutant strains (Gimeno and Fink, 1992; Mösch and Fink, 1997). Like in the Σ1278b background, diploid S288c [FLO8] strains deleted for both copies of FLO11 are not able to form pseudohyphae under inducing conditions. However, S288c [FLO8] derivatives carrying homozygous flo1 or flo10 deletions are not impaired in pseudohyphal formation.

We then analysed adhesion on plastic surfaces in SC medium of several haploid S288c [FLO8] derivatives to further explore the role of FLO1 for haploid adhesive growth of this strain in more detail (Fig. 2B). Neither the deletion of FLO11 nor the deletion of another FLO gene, FLO10, abolished the capability for adhesion of a S288c strain with an intact FLO8 gene. However, the deletion of FLO1 resulted in non-adherent yeasts in spite of an intact FLO8 which is comparable to all S288c derivatives without intact FLO8 genes. In contrast to Σ1278b, this further corroborates that under these conditions FLO1 and not FLO11 is the major adhesin for substrate interaction of S288c [FLO8].

These pronounced differences in the induction of haploid adhesive growth between S288c [FLO8] and Σ1278b prompted us to a further comparative analysis. Induction of adhesion of Σ1278b can be achieved in SC medium by amino acid starvation; therefore, the addition of the histidine analogue 3-amino-triazole (3AT) results in the expression of FLO11 in Σ1278b (Braus et al., 2003). Σ1278b only shows the adherence phenotype in SC medium in the presence of 3AT whereas cultivation of S288c [FLO8] on SC media with or without supplementation of 10 mM 3AT resulted in adhesion (Fig. 2C). We transformed a Σ1278b strain with additional copies of FLO8 (pHL1) to exclude any dosage effect of the transcriptional activator gene. However, the transformed Σ1278b strain is still unable to grow adhesively in the absence of 3AT. These data further suggest that FLO1-dependent adhesion of S288c [FLO8] is differently regulated in comparison with FLO11-regulated adhesion of Σ1278b.

We analysed several S288c [FLO8] derivatives to further address differences in FLO regulation. S288c [FLO8] strains deleted for FLO1 (flo1Δ) failed to grow adhesively under every condition tested. Correspondingly, flo10 or flo11 deletion strains exhibited a similar phenotype as S288c [FLO8] and therefore do not seem to play a major role during haploid adhesion under these conditions. These data further corroborate that in contrast to the yeast Σ1278b, Flo1p plays a prominent and
Fig. 2. Induction of FLO genes of haploid S. cerevisiae S288c [FLO8] in comparison with haploid S. cerevisiae Σ1278b.
A. FLO1 and FLO11 transcript levels of yeast S288c, S288c [FLO8] (pHL11) and Σ1278b respectively. RNAs of the various strains grown on SC media were isolated and compared after RT-PCR followed by semiquantitative PCR using Taq polymerase. The PCR reactions were compared by 1% TAE-agarose gel electrophoresis. ACT1 transcript levels served as control.

B. Adhesion to plastic surfaces of S. cerevisiae S288c derivatives. Ninety-six well plate with indicated yeast strains grown for 24 h in liquid SC media are documented before and after staining and washing. After staining cells with crystal violet and subsequent washing, biofilm formation on the plastic surface is indicated by a remaining cell-layer (dark wells).

C. Yeast S288c, S288c [FLO8], Σ1278b (adhesive control) and Σ1278b fio11 (non-adhesive control) are compared with S288c fio10Δ with or without intact FLO8 gene (first row), with S288c fio1Δ with or without intact FLO8 gene (second panel) and with S288c fio11Δ with or without intact FLO8 gene (third row). The fourth panel compares adhesive growth of indicated Σ1278b strains with or without an extra copy of the FLO8 gene (pHL1). Adhesion was assayed after 1 day of growth on SC medium without histidine and on amino acid starvation-inducing SC medium caused by the addition of the histidine analogue 3-amino-triazole (3AT). Plates were documented before and after non-adhesive cells were washed off the agar.
specific role for haploid adherence of S288c [FLO8] yeast cells.

The importance of FLO1 was further analysed by flocculation assays in liquid culture of haploid S288c [FLO8] derivatives carrying deletions in flo1Δ, flo10Δ and flo11Δ respectively, in comparison with Σ1278b (Table 1). Flocculation reflects the potential for cell–cell interactions. In the Σ1278b background, a haploid flo11Δ strain does not flocculate. For Flo8p-dependent flocculation of S288c [FLO8], FLO1 is exclusively essential, whereas flo10Δ and flo11Δ strains flocculated similarly as the S288c [FLO8] control. This suggests an additional crucial role of Flo1p for cell–cell interactions in haploid yeast S288c [FLO8].

In summary, these results demonstrate that the haploid yeast S288c [FLO8] requires FLO1 for adhesive growth on substrates and cell–cell interactions during flocculation, two functions which are fulfilled by FLO11 in Σ1278b. In addition, the amino acid starvation experiments suggest differences in the regulation of FLO1 and FLO11. For diploid pseudohyphae formation in S288c, like in Σ1278b, Flo11p is the only essential cell–surface protein.

FLO1 and FLO11 are differentially regulated in the commonly used S. cerevisiae S288c [FLO8] strain

As shown before, FLO1 and FLO11 of the commonly used S. cerevisiae S288c can only be activated when the defective allele for the transcription activator Flo8p is restored (Liu et al., 1996; Kobayashi et al., 1999). Haploid adhesion to substrates and diploid filamentous growth can be regained by FLO8, whereas biofilm formation cannot be restored even in high copy number (Fig. 3A). It had been proposed that FLO1 might be similarly regulated as FLO11 (Kobayashi et al., 1999). To compare the regulation of both genes in more detail, we performed a genetic screen for suppressor mutations of flo8, resulting in haploid adhesive growth of S288c.

Individual knockout strains of the ordered S288c yeast deletion collection (Brachmann et al., 1998) were grown in liquid SC medium in microtiter plates and assayed for their ability to adhere to the plastic surface. Six mutant strains were able to suppress the adherence defect of yeast S288c on plastic surfaces (Fig. 3B) and were verified by assaying adherence on SC agar for cells growing on Petri dishes (Fig. 3C). The mrpl28Δ mutant strain was not included into further analyses, because it showed a significantly weaker adhesion phenotype than the others. Whereas the sfl1Δ mutation resulted only in partial suppression, the tup1Δ, ssn8Δ, sin4Δ and srb8Δ deletions were strong suppressor mutations of adhesive growth (Fig. 3B and C). Sfl1p is bridged to Tup1p and requires the mediator tail Sin4p to be one of the major specific repressors of the adhesin gene FLO11 of S. cerevisiae Σ1278b (Conlan and Tzamarias, 2001). Srb8p and Ssn8p are additional mediator components which repress FLO11 expression (Palecek et al., 2000). We were surprised that in S288c, the impact of tup1Δ or the three mediator genes on adhesive growth was significantly more pronounced than the impact of a sfl1Δ deletion (Fig. 3B and C). This suggests that Tup1p and the mediator components might have additional Sfl1p-independent functions in repressing adherence of S288c.

For a more detailed analysis, we wanted to know which FLO gene is affected by the five suppressor mutations that restore adhesive growth of S. cerevisiae S288c. Figure 3D shows that integration of an intact FLO8 activator gene induces FLO11 and FLO1 in S288c as expected, whereas strain Σ1278b is only able to activate FLO11 (Fig. 2A). None of the suppressors resulted in the induction of the silent FLO5, FLO9 or FLO10 (data not shown). Deletion of SFL1 in the flo8 genetic background, which only partially restores adhesive growth, resulted only in the induction of FLO11. However, defects in the gene for the mobile repressor Tup1p resulted in the induction of both FLO11 and FLO1. This suggests that Tup1p is involved not only in Sfl1p-mediated FLO11 repression but also in Sfl1p-independent FLO1 repression. Defects in the mediator genes for Srb8p and Ssn8p also resulted in the induction of both FLO genes, suggesting a similar mode of repression for both genes. Interestingly, the deletion of the strong suppressor gene sin4Δ encoding a part of the mediator tail resulted only in the induction of FLO1 but not of FLO11 (Fig. 3D). The result that the sin4 mutation represents a significantly stronger suppressor gene of the adherence defect of S288c than the sfl1 deletion points to
a more prominent role of \textit{FLO1} than \textit{FLO11} under these conditions for S288c adhesion.

In summary, these data further support that \textit{FLO1} is even more important than \textit{FLO11} for haploid adherence under specific growth conditions in the yeast strain S288c, which carries a restored \textit{FLO8} gene. \textit{FLO1} and \textit{FLO11} regulation share similarities but there are also significant differences in regulation, because \textit{FLO1} depends on not only an active mediator but also the mediator tail protein Sin4p. In addition, induction of \textit{FLO1} in yeast S288c is independent of Sfl1p but not Tup1p.

In S1278b yeast strains, Flo8p is activated by the cAMP-dependent PKA pathway and is impaired by a deletion of the \textit{tpk2}\Delta gene encoding the catalytic subunit of the PKA complex (Roberts and Fink, 1994; Mösch \textit{et al.}, 1999; Rupp \textit{et al.}, 1999; Cullen and Sprague, 2000). Here we show that a \textit{tpk2}\Delta deletion also prevents Flo8p-mediated haploid adhesive growth of S288c strains on agar (Fig. 4A), plastic (Fig. 4B) and rich medium after glucose starvation (Fig. 4C) respectively. Even \textit{FLO8} overexpression from a high copy number plasmid could not suppress the \textit{tpk2}\Delta-mediated adherence defect and therefore the dependence for activation of the pathway by Tpk2p (Fig. 4B). Therefore, \textit{FLO1} expression of S288c shares a similar control to \textit{FLO11} by the PKA signal transduction pathway which activates the transcription factor Flo8p.

In parallel to the PKA pathway, the MAPK cascade has been shown to be crucial for \textit{FLO11}-mediated haploid and diploid filamentous growth of S1278b (Roberts and Fink, 1994; Mösch \textit{et al.}, 1999; Rupp \textit{et al.}, 1999; Cullen and Sprague, 2000). However, neither the deletion of the gene for MAPK, \textit{kss1}\Delta, nor the deletion of \textit{tec1}\Delta encoding the corresponding \textit{FLO11}-activating transcription factor impaired haploid invasive growth of S288c [\textit{FLO8}] (Fig. 4). This suggests a less prominent role for the MAPK...
pathway in controlling \( FLO1 \) and \( FLO11 \) in S288c in comparison with \( \Sigma 1278b \).

Deletion analysis also showed that Flo8p-mediated adhesive growth of S288c requires neither the gene for the repressor of meiosis encoded by \( RME1 \) (Gagiano et al., 2003) nor the transcription factor encoded by \( MSN1 \) (Lorenz and Heitman, 1998) which have been both described to regulate \( FLO11 \) expression of \( \Sigma 1278b \). The deletions \( \Delta me1 \) or \( \Delta msn1 \) did not impair haploid adhesive growth of S288c [\( FLO8 \)]. These data further corroborate differences in the regulation of \( FLO1 \) and \( FLO11 \).

The \( \Sigma 1278b \) yeast strain requires the general control transcription factor of amino acid biosynthesis to activate \( FLO11 \) (Braus et al., 2003). The results described above (Fig. 2) already showed that \( FLO1 \) expression of S288c [\( FLO8 \)] is not dependent on the presence or absence of sufficient amount of amino acids. Consistently, the results of Fig. 4 verify that in contrast to \( \Sigma 1278b \), which requires \( GCN4 \) for \( FLO11 \) expression, a \( gcn4\Delta \) deletion does not impair haploid adhesive growth under all tested conditions, suggesting that it is not required for \( FLO1 \) expression.

Another transcription factor, \( Msns11p \) (van Dyk et al., 2005), is essential for adhesive growth of S288c [\( FLO8 \)]. The \( msn1\Delta \) deletion prevented haploid adhesive growth completely under all tested conditions on agar and on plastic (Fig. 4). This impairment cannot be suppressed by overexpression of \( FLO8 \). The adhesion assay upon glucose limitation on YPD media demonstrates a special role of \( Msns11p \) for S288c adherence. The two non-adherent S288c [\( FLO8 \)] derivatives \( msn1\Delta \) and \( \Delta tpk2 \) show subtle but significant differences in phenotype upon glucose starvation (Fig. 4C). Whereas the \( \Delta tpk2 \) strain still shows a remaining layer of cells on the agar indicating a

Fig. 4. Haploid adhesive growth of \( S. cer e va iae \) S288c [\( FLO8 \)] derivatives is impaired in various gene deletions of the filamentous growth pathway.

A. Haploid adhesive growth on SC agar plates. Indicated yeast strains were grown for 1 day on SC media. The plates were documented before and after non-adhesive cells were washed off the agar.

B. Haploid adhesive yeast growth in liquid cultures. Ninety-six well plates with indicated yeast strains were grown for 24 h in liquid SC media (pre-wash) and the same 96-well plate were washed after cell-staining with crystal violet (post-wash). Adhesive growth is indicated by a remaining cell-layer (dark wells) after washing.

C. Haploid adhesive yeast growth after glucose starvation. Indicated S288c [\( FLO8 \)] (pHL11) derivatives were patched on YPD plates and incubated for 3 days at 30°C and then stored for three more days at room temperature to induce glucose starvation. The plates were documented before non-adhesive cells were washed off the agar as described in Fig. 3. Strains able to invade the agar show a remaining cell-layer on the agar surface even after the final hard washing step. An example of a 50x magnification of cells on the agar surface after the last washing step is shown for yeast S288c [\( FLO8 \)] \( \Delta tpk2 \) as an example in contrast to the \( msn1\Delta \) cells which are completely washed away.

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low level of FLO11 transcription, the mss11Δ cells are entirely washed away indicating repression of both genes, FLO1 and FLO11 (Fig. 4C). Therefore, Mss11p is essential for the expression of both adhesin encoding genes.

Transcript analyses confirmed the stronger impact of the mss11Δ in comparison with the tpk2Δ deletion on adherence of S288c [FLO8] (Fig. 5). A low but significant FLO11 expression and no detectable transcript of FLO1 can be observed in the S288c [FLO8] derivative tpk2Δ. The total abolishment of adherence in the mss11Δ strain correlates with no detectable transcripts of FLO1 and FLO11.

In summary, these data suggest that FLO1 of S288c [FLO8] is primarily under the control of the PKA pathway and the transcription factors Flo8p and Mss11p, whereas other control mechanisms which are known for FLO11 are missing for activation of FLO1.

Differential regulation and function of FLO1 and FLO11 in S288c compared with Σ1278b are reflected by differences in the corresponding promoters and coding sequences

The distinct regulations and functions of FLO1 and FLO11 in S. cerevisiae S288c with an intact regulator Flo8p prompted us to compare these genes to the corresponding genes of strain Σ1278b, which also carries an intact Flo8p combined with active FLO11 (GenBank accession EF670006) but inactive FLO1 (GenBank accession EF670005). As expected, the sequences of the two active FLO11 genes of both strains did not show extraordinary differences. In the Σ1278b FLO11 promoter there are two small insertions at position –407 (TCTTT) and –1976 (AAGAGATGTCGC) in comparison with S288c. The coding region of Σ1278b FLO11 shows several single amino acid exchanges spread over the gene, an insertion of 15 amino acids at position 118 and small deletions at several positions (Fig. S1A). Interestingly, the repetitive sequences are differently arranged in Σ1278b including five more large and four more short repeats respectively (Fig. S1B). Complementation analyses using Σ1278b FLO11 revealed complementation of S288c flo11Δ and partial complementation of S288c flo1Δ in wash tests, whereas the S288c FLO1 is unable to complement Σ1278b Δflo11 (data not shown).

The situation is completely different for the two FLO1 DNA sequences representing an active gene in S288c [FLO8] and an inactive FLO1 locus in Σ1278b. The Flo8 binding site of the FLO1 promoter in Σ1278b is mutated in four positions in comparison with S288c AAAACCTTAT TCTACGGAAAAACCTTATT at position –751 to –724 of S288c (Kobayashi et al., 1999) to AAAACCTTACGGAAAAACCTTATT (Fig. 6). Furthermore, there are two major deletions from codon 347–526 and codon 680–774 within the FLO1 open reading frame of Σ1278b. These deletions result in the loss of six tandem repeats, which have been shown to be important for adhesion (Verstrepen et al., 2005). In addition, we found several minor changes in the downstream part of the gene
(Fig. S2). We assume that the change in the Σ1278b FLO1 promoter and the lack of overall 267 amino acids play a major role that this strain has developed a primarily Flo11p based strategy for adhesion.

Discussion

The baker’s yeast S. cerevisiae accompanies human culture in bread making or brewing of alcoholic beverages since several millennia. In addition, yeast strains have been cultivated since many decades after the first isolation of a pure culture which still was showing dimorphism in 1883 by Emil Chr. Hansen (Hansen, 1883) in numerous laboratories and have been selected for non-adherent and non-flocculating phenotypes. A major target of this selection seems to be the FLO8 transcriptional activator gene, which is mutated in the commonly used S. cerevisiae S288c resulting in a completely non-adherent yeast (Liu et al., 1996). In this study we analysed the differences in the regulation mechanisms and the distinct functions of the two flocculin encoding genes, FLO1 and FLO11, which can be activated when FLO8 is restored.

In addition, our data suggest that even S. cerevisiae Σ1278b has been selected to impair complete Flo8p activator action, because its FLO1 gene, which cannot be activated, has accumulated mutations in the Flo8p promoter binding site (Fig. 6). Σ1278b had been the research object which allowed the rediscovery of dimorphism including adherence and filamentation of S. cerevisiae (Gimeno et al., 1992), although this phenomenon had been originally described in the 19th century (Hansen, 1883). In this strain the transition from a unicellular to a multicellular organism including the analysis of diploid pseudohyphae and haploid adhesive growth has been primarily studied. Σ1278b carries only one expressed FLO gene (FLO11), which is responsible for all necessary functions within the haploid as well as the diploid yeast life cycle in different environments. The Σ1278b FLO11 has rearranged and reshaped its repetitive region in comparison with S288c resulting in five more long and four more short repeats (Fig. S1). The repetitive region is important for the efficiency of adhesion and flocculation and the changes might partially reflect the development of a one gene based adhesion strategy (Verstrepen et al., 2005).

Four other FLO genes including FLO1 are silent in Σ1278b and can only be re-activated by mutation or genomic rearrangements (Verstrepen et al., 2004; 2005). The inactive Σ1278b FLO1, encoding a protein that is significantly truncated by 267 amino acids when compared with S288c FLO1 (Fig. S2), might also be a consequence of a genomic reorganization. Another example for the importance of genomic rearrangements for the adaptation of the yeast cell–surface is the foam forming AWA1 gene product. This protein is present in S. cerevisiae strains used for sake production, and is presumably a chimeric protein corresponding to parts of two genes of the commonly used S288c (Alexandre et al., 2000; Shimoi et al., 2002). Accordingly S288c is unable to form foams or biofilms, even after the restoration of FLO8 (Fig. 1A).

Cell–surface diversity, which can be the key to virulence in a host–pathogen relationship, is primarily the result of differentially expressed genes for surface proteins. An additional level of adaptation to the environment can be mediated by stochastic processes that result in a variegated expression of surface proteins in an otherwise homogeneous population (Halme et al., 2004). In naturally fluctuating environments stochastic switching patterns might be more effective than sensing mechanisms (Kussell and Leibler, 2005). The diversity of cell–surfaces in pathogen populations is an important strategy to become less accessible for hosts. Many organisms have developed strategies to model their appearance by recombining, silencing or activating different genes for their cell–surface proteins (Esser and Schoenbechler, 1985; Kyes et al., 2001). Pathogenic yeasts such as Candida albicans or Candida glabrata express multiple agglutinin-like ALS (Hoyer, 2001) and epithelial adhesion EPA genes (De Las Penas et al., 2003) respectively. The three morphological Candida forms, single cell yeasts, pseudohyphae and true hyphae, significantly differ in their cell–surface and are also partially regulated by Flo8p (Lopez-Ribot, 2005; Nobile and Mitchell, 2005; Ramage et al., 2005). S. cerevisiae represents a yeast with a significant less complex arsenal of variant surface proteins compared with Candida. It consists of only two active genes of the five members of the FLO family in FLO8-restored strain S288c, which has been used in this study. However, a limited number of flocculin genes have already enough potential to cause a significant impact on different habitats. One example is the S. cerevisiae outbreak of fungemia among intensive care unit patients that has been reported (Cassone et al., 2003). In S288c [FLO8] the two flocculin genes FLO1 and FLO11 are kept in a metastable state and can be either silenced and repressed or activated differently. The activator Flo8p which cooperates with other factors like Mga1p and Mss11p (Bester et al., 2006; Borneman et al., 2006) is able to activate both genes, but is only one of many genes and their products which are involved in the control of FLO1 and FLO11.

The molecular mechanisms controlling FLO1 and FLO11 share similarities as well as significant differences (Fig. 6). Repression and/or silencing of FLO1 as well as of FLO11 depends on an intact mediator complex of the RNA polymerase II, which has different functions including chromatin remodelling. FLO1 and FLO11 repression requires Srp8p and Ssn8p, both components of the Cdk8 mediator subcomplex. It is yet unknown whether there is
a connection between Srp8p and Ssn8p and the epigenetic control of FLO silencing by different histone-deacetylases as it has been described (Halme et al., 2004). Cdk8 is also involved in phosphorylation of the RNA–polymerase C-terminal-domain and therefore is part of the transcriptional repression control (Liao et al., 1995; Holstege et al., 1998). In addition, Cdk8 phosphorylates and therefore destabilizes Ste12p or Gcn4p which are transcriptional activators of FLO11 (Chi et al., 2001; Nelson et al., 2003). It is yet unclear which of these functions is necessary to turn off the two FLO genes. In addition to the common function of the mediator components Srb8p and Ssn8p in repressing FLO1 and FLO11, there is an additional specific function of Sin4p, which is only required for the repression of FLO1 but not for FLO11. Sin4p is part of the mediator tail interacting with various transcriptional activators (Pan and Heitman, 1999; Park et al., 2000), and it remains to be elucidated which interactions are important for the specific effect of Sin4p on FLO1. Sfl1p, the key repressor of FLO11, shares its promoter binding site with the Flo8p activator (Pan and Heitman, 2002). However, the finding that Sfl1p is not required for FLO1 repression represents another important difference in the regulation of both FLO genes. Tup1p, the corepressor of Sfl1p in the FLO11 promoter (Conlan and Tzamarias, 2001), is known as a corepressor for different DNA-binding proteins and is also required for FLO1 repression. It remains to be elucidated which protein is the partner in FLO1 repression. The FLO1 and FLO11 expression depends essentially on the Mss11p transcription factor (van Dyk et al., 2005). A deletion of MSS11 can be suppressed neither by overexpression of activators nor by deletion of repressors. This suggests Mss11p as being a key player for yeast cell–cell and cell–surface interactions. FLO1 and FLO11 are regulated by the cAMP–PKA cascade including the Tpk2p catalytic subunit of PKA (Mösch et al., 1999; Cullen and Sprague, 2000; Sengupta et al., 2007) and the transcription factor Flo8p. Other regulators of FLO11 (Fig. 6) including the MAPK (KSS1) pathway are not relevant for FLO1 expression, suggesting significant differences in the regulation mechanism of both genes.

The two active FLO genes of S288c carrying a restored FLO8 correspond to only partially overlapping and mostly distinct functions. FLO1 is primarily important for haploid and allows cell–cell interactions, which corresponds to the finding that it is expressed in several industrial yeasts (Kobayashi et al., 1996; 1998; 1999). In industry, flocculation can be a desirable property, allowing easy separation of products and biomass. Flo1p also supports cell–substrate interactions of haploids under specific environmental conditions. The other active FLO gene of S288c, FLO11, encodes the typical surface marker of diploids. The carefully regulated FLO11 is essential for the developmental programme, which results in the formation of diploid pseudohyphae with their distinct features (Kron et al., 1994). Interestingly, both S288c FLO genes are required for an appropriate morphological response in a specific environment. An example of such a response is the growth of haploid S288c cells on a surface during glucose starvation. Flo11p functions as a first adhesin to establish the initial cell–substrate interaction of the first layer of cells. Flo1p is responsible for the second step and increases the population at this specific spot of the habitat by adding additional layers of cells due to its cell–cell interaction potential.

Our studies have revealed several differences in the regulation and function of the two Flo8p regulated genes, FLO1 and FLO11, of the budding yeast. It will be interesting to analyse in the future, if natural S. cerevisiae wild-type yeasts even express a still larger portion of the FLO gene reservoir and what additional surface protein encoding genes have been created by reshaping the genome for additional functions in specific environmental conditions.

**Experimental procedures**

**General methods, yeast strains and plasmids**

Cultivations of S. cerevisiae in SC or rich YPD media and yeast methods including genetic crosses and transformation were carried out as described previously (Gietz et al., 1992; Sherman, 1991). The yeast strains used in this study (Table 2) are either derivatives of S. cerevisiae strain Σ1278b also known as MB1000 and MB758-5b (Brandriss and Magasanik, 1979; Siddiqui and Brandriss, 1988), or of the S288c strain-derived BY-series (Brachmann et al., 1998). Deletions of FLO1 and FLO11 in strains Y06870 (flo1Δ) and Y05953 (flo11Δ) were confirmed by PCR. Plasmids of the YCplac and YEpplac series used for complementation of auxotrophic marker alleles were described previously (Gietz and Sugino, 1988). FLO8 carrying plasmids pH1 (ARS-CEN, low copy), pH1L (integrative) and pH1L35 (2 μ, high copy) which were used for complementation of S288c flo8, were described previously (Liu et al., 1996). Amino acid starvation was induced by the histidine analogue 3AT. Strains used for 3AT adhesion tests were reversed to histidine prototrophy by integrating a HIS3 1.7 kb BamHI fragment from pBR322-Sc2676 (Struhl and Davis, 1980) at its original locus.

**Growth tests**

All strains used in the different growth tests were either diploid (pseudohyphae) or MATa. Tests for adhesive growth on agar were performed as described (Roberts and Fink, 1994; Braus et al., 2003). Adhesion to plastic surface was assayed in 96 well flat bottom plates (Reynolds and Fink, 2001). Pseudohyphal growth was assayed on synthetic low ammonium dextrose (SLAD) medium (Gimeno and Fink, 1992) using diploid strains. For assaying flocculation, yeast strains were grown overnight in SC media and their ability to flocculate was judged from the presence of visible cell aggregates...
in the media and quantified as described before (Kobayashi et al., 1996) using the equation FA = 1 - B/A, where A is OD_{600} in the absence and B in the presence of 0.1% CaCl_{2}. To assay the formation of biofilms, mat formation was monitored on YPD plates with 0.3% (w/v) agar and 0.2% glucose (Reynolds and Fink, 2001) and photographed after 4 days of incubation at 25°C.

**Screening yeast deletion collection**

We performed flo8 suppressor screening using a Freedom Evo robot (TECAN) and the systematic MATa yeast deletion collection (Brachmann et al., 1998). The 4895 individual collection deletion strains were grown in 96 well microtiter plates in liquid SC media for 24 h. Cells were stained by crystal violet and non-adhesive cells were washed off the plates (Reynolds and Fink, 2001). Wells containing adhesive deletion strains were further analysed.

**Gene transcription analyses**

Cells were grown in SC media to an OD_{600} of 0.7, total RNA was isolated (Cross and Tinkelenberg, 1991) and samples were treated afterwards with RNase free DNase (Qiagen). RT-PCR experiments involved equal amounts of total RNA (1 µg) subjected to first-strand cDNA synthesis with the Reverse Aid™ kit (MBI Fermentas) according to the manufacturer’s recommendations. After first-strand synthesis, 1/20 of the cDNA was used for semiquantitative PCR (Frohloff et al., 2001). Oligonucleotide primers used for specific amplification were as follows: FLO1 (RT1A: 5′-CTCATCGCTATATGTTTTGG-3′, RT1B: 5′-CGAGTTAAA GAACTTTATTGG-3′), FLO11 (RT11A: 5′-CTCATCGCTATATGTTTTGG-3′, RT11B: 5′-CGAGTTAAA GAACTTTATTGG-3′), ACT1 (RTactA: 5′-TTGGTACCGG-3′, RTactB: 5′-TACAACGG-3′), FLO5 (RT5A: 5′-GGTTGA CTTATTTGG-3′, RT5B: 5′-GCAAACCATTGGTACCGG-3′), FLO9 (RT9A: 5′-GGTTGA CTTATTTGG-3′, RT9B: 5′-GCAAACCATTGGTACCGG-3′), FLO10 (RT10A: 5′-GGTTGA CTTATTTGG-3′, RT10B: 5′-GCAAACCATTGGTACCGG-3′). The specific annealing of FLO1 and FLO11 was performed as follows: FLO1 (RT1A: 5′-CTCATCGCTATATGTTTTGG-3′, RT1B: 5′-CGAGTTAAA GAACTTTATTGG-3′, RT1actA: 5′-TTGGTACCGG-3′, RT1actB: 5′-TACAACGG-3′), FLO11 (RT11A: 5′-CTCATCGCTATATGTTTTGG-3′, RT11B: 5′-GCAAACCATTGGTACCGG-3′, RT11actA: 5′-GTGGGA CTTATTTGG-3′, RT11actB: 5′-GTGGGA CTTATTTGG-3′). The specific annealing of FLO1 and FLO11 was performed by the Sequence Laboratories Göttingen GmbH (SEQLAB). We thank Sven Krappmann, Oliver Valerius and Nirmala Padmanabhan for comments on the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, the Volkswagen-Stiftung and the Research Center Molecular Physiology of the Brain (CMPB).

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**Supplementary material**

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