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Flocculation gene variability in industrial brewer's yeast strains

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Abstract The brewer's yeast genome encodes a 'Flo' flocculin family responsible for flocculation. Controlled floc formation or flocculation at the end of fermentation is of great importance in the brewing industry since it is a cost-effective and environmental-friendly technique to separate yeast cells from the final beer. *FLO* genes have the notable capacity to evolve and diverge many times faster than other genes. In actual practice, this genetic variability may directly alter the flocculin structure, which in turn may affect the flocculation onset and/or strength in an uncontrolled manner. Here, 16 ale and lager yeast strains from different breweries, one laboratory *Saccharomyces cerevisiae* and one reference *Saccharomyces pastorianus* strain, with divergent flocculation strengths, were selected and screened for characteristic *FLO* gene sequences. Most of the strains could be distinguished by a typical pattern of these *FLO* gene markers. The *FLO1* and *FLO10* markers were only present in five out of the 18 yeast strains, while the *FLO9* marker was ubiquitous in all the tested strains. Surprisingly, three strongly flocculating ale yeast strains in this screening also share a typical 'lager' yeast *FLO* gene marker. Further analysis revealed that a complete *Lg-FLO1* allele was present in these ale yeasts. Taken together, this explicit genetic variation between flocculation genes hampers attempts to understand and control the flocculation behavior in industrial brewer's yeasts.

Keywords Flocculation · *Saccharomyces cerevisiae* · Adhesin · Tandem repeats · Brewery fermentations

Introduction

At the end of beer fermentation, when all fermentable sugars are converted into ethanol and carbon dioxide, yeast cells clump together and sediment at the bottom of the fermentation tank, a process called flocculation. Natural flocculation provides brewers with a cost-efficient way to separate yeast cells from green beer. The yeast sediment obtained after fermentation can be used for repitching into subsequent brews (Smart and Whisker 1996; Powell and Diacetis 2007). Correct floc formation at the end of fermentation is a vital phenomenon for brewers (Verstrepen et al. 2003). Late or weak flocculation makes it necessary to employ expensive filter or centrifugation systems more intensively and complicates repitching of the yeast cells. On the other hand, too early flocculation will cause attenuation problems.

Historically, brewer's yeast has been classified in two groups based on their ability to form flocs that will either

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rise to the surface of the liquid medium or sediment to the bottom of the fermentation tanks (Dengis and Rouxhet 1997). Besides this distinction, top-fermenting (ale) and bottom-fermenting (lager) yeasts exhibit very different physiological traits which result in beers with their own characteristic taste. Lager yeast has been classified as *Saccharomyces pastorianus*, a natural hybrid of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* in which chromosome sets from both parental species are present (Yamagishi and Ogata 1999; Rainieri et al. 2006; Dunn and Sherlock 2008; Ogata et al. 2008; Nakao et al. 2009). Dunn and Sherlock (2008) further found that two different, but closely related, ale yeasts were the donors for the *S. cerevisiae* portion of the hybrid genome. Ale yeasts were predominantly classified as *S. cerevisiae*. The fact that some ale yeast strains were discovered to be *S. cerevisiae* x *Saccharomyces kudriavzevii* hybrids suggests that an important fraction of brewer's strains originally classified as *S. cerevisiae* may also correspond to hybrids (Gonzalez et al. 2008; Querol and Bond 2009). Within each yeast type, long-term selection by scientists and/or brewers has lead to yeast strains with a personal and unique genomic make-up (Schacherer et al. 2007; Borneman et al. 2008; Liti et al. 2009).

The sequenced S288C laboratory strain contains five *FLO* genes, four located nearby the chromosome telomeres *FLO1*, *FLO5*, *FLO9*, and *FLO10* and one located neither at the centromere nor the telomeres: *FLO11* (Teunissen and Steensma 1995; Caro et al. 1997). These genes encode lectin-like proteins which are also known as adhesins, zymolectins, or flocculins. Yeast adhesins share a common three-domain structure (Dranginis et al. 2007; Goossens and Willaert 2010). The C-terminal domain is modified by the secretion machinery of the cells and finally anchors the adhesin to the yeast cell wall (Pittet and Conzelmann 2007). The central serine- and threonine-rich repeated region functions as a spacer that improves the accessibility of the N-terminal binding domain outside of the yeast cell wall (Bony et al. 1997; Breinig and Schmitt 2002). Moreover, this region forms amyloids which may be directly involved in flocculation (Ramsook et al. 2010). The widely accepted model for yeast flocculation describes flocculation as the result of the interaction between adhesins and mannans, polysaccharides built up of mannose residues, present on mannoproteins in the cell wall (Miki et al. 1982; Stratford 1989). In most laboratory *S. cerevisiae* strains, added mannose will block the 'Flo1-type' adhesin-binding sites and thus inhibits flocculation by preventing the adhesins to bind to mannans present on neighboring cells (Stratford 1989). A very similar adhesin of considerable industrial importance, Lg-flo1, responsible for the mannose-, glucose-, and maltose-sensitive 'newflo'-type of lager yeast strains, was discovered in a *S. pastorianus* strain (Kobayashi et al. 1998). The most important characteristic of 'Lg-flo1'-based flocculation is its inhibition during fermentation when the concentration of typical wort carbohydrates such as maltose and glucose is sufficiently high. In this case, competitive binding of such carbohydrates by this adhesin occur and will thus ensure that flocculation will only occur at the appropriate moment, when all fermentable carbohydrates are depleted (Kobayashi et al. 1998; Verstrepen et al. 2003). The molecular basis behind the distinction in carbohydrate recognition of yeast adhesins is situated in the N-terminal binding domain. More specifically, pentapeptides in *S. cerevisiae* S288C and *S. pastorianus* KBY001, respectively, the amino acid motifs VSWGT and KVLAR located at positions 226–230 and 199–203 in respectively Flo1 and Lg-Flo1, were discovered to have carbohydrate-binding properties (Zupancic et al. 2008). They also exactly match the carbohydrate-binding sequences described in previous studies (Kobayashi et al. 1998; Bayly et al. 2005).

A major requirement for flocculation is that adhesins are decorating the cell wall at the end of fermentation when flocculation should preferentially occur (Govender et al. 2008; Van Mulders et al. 2009). The transcription of flocculation genes in laboratory yeast strains is tightly regulated through at least three signal pathways to sense and respond to their environment: the cAMP/PKA-, MAPK-, and glucose-repression pathways (Gagiano et al. 2002; Verstrepen and Klis 2006). Yet, knowledge about the contribution of each pathway in industrial yeast strains remains scarce.

Besides the direct adhesin–carbohydrate interactions, other factors are presumably involved in the onset of yeast flocculation such as cell surface hydrophobicity (Rhymes and Smart 2000; Verstrepen et al. 2001; Speers et al. 2006; Strauss et al. 2006; Mortensen et al. 2007; Govender et al. 2008; Van Mulders et al. 2009), medium composition (Verstrepen et al. 2003; Sampermans et al. 2005; Claro et al. 2007; Smukalla et al. 2008), aerobic and anaerobic conditions (Lawrence and Smart 2007; Lawrence et al. 2009), and cell age (Powell et al. 2003; Powell and Diacetis 2007). The recent findings that an identical adhesin present on different yeast strain cell walls may have opposite adhesion phenotypes further support the role of indirect factors influencing yeast flocculation (Bayly et al. 2005; Douglas et al. 2007). Together with the fact that *Saccharomyces diastaticus* Flo11 adhesin targets are presumably the targets on the adhesin itself, which is called a homotypic binding, this highlights the importance of particular adhesin conformations on the yeast cell wall for both appropriate binding and receptor functions (Douglas et al. 2007; Smukalla et al. 2008).

Some brewers report that yeast strains that showed perfect flocculation properties 1 day, can swiftly change

and evolve towards too early or late flocculation onset, or even lose the ability to flocculate altogether. A plethora of studies described the presence of mutated sequences present in the *FLO* genes (Sato et al. 2001; Sato et al. 2002; Ogata et al. 2008). The role of tandem repeats in the frequent recombination events observed in flocculation genes has been elucidated (Verstrepen et al. 2005). These unstable repeats drive replication slippage reactions within and between *FLO* genes and pseudogenes, leading to the constant generation of novel *FLO* alleles and pseudogenes (Verstrepen et al. 2004; Verstrepen et al. 2005). The mutational frequency of tandem repeats, which is at least 100 times higher than normal point mutations (Rando and Verstrepen 2007), explains that industrial yeast strains may thus have their personal small reservoir of different adhesin-encoding genes that differs from the *FLO* gene family described in the sequenced *S. cerevisiae* S288C strain (Verstrepen et al. 2005; Damas-Buenrostro et al. 2008).

Frequent intragenic recombination events will typically result in the net loss or gain of tandem repeat units. Expansion of the *FLO1* tandem repeat domain size results in a stronger flocculation (Verstrepen et al. 2005). Remarkably, a recent study reported a switch from flo1-type to newflo-type flocculation based on tandem repeat number variation (Liu et al. 2007). Intergenic recombination events between *FLO5* and *YAL065c* may have lead to the generation of *Lg-FLO1* (Kobayashi et al. 1998; Ogata et al. 2008). Tandem repeat-independent mutations, for example within the genetic pathways regulating *FLO* gene expression, occur at much lower rates, but are also shown to be involved in yeast flocculation variability (Liu et al. 1996; Fichtner et al. 2007). In addition to genetic mechanisms, epigenetic mechanisms add another level of complexity in the control of the expression of the *FLO* genes and consequently, in the control of yeast flocculation (Fleming and Pennings 2001; Halme et al. 2004; Dietvorst and Brandt 2008; Dietvorst and Brandt 2009).

The genetic variability of flocculation genes may have important consequences for studies and applications targeting these genes in industrial yeast strains with unknown genomes. Here, we designed primers to detect specific flocculation gene markers in an industrially relevant brewer's yeast strain collection. Strong variance between the presence and absence of the different flocculation gene markers could readily be observed. A typical 'lager' yeast flocculation gene, *Lg-FLO1*, was discovered in three strongly flocculating ale yeasts. Our results reveal that considerable genetic variability occurs in the subtelomeric chromosomal regions where flocculation genes are located. Therefore, flocculation gene expression research and industrial applications of flocculation should be adapted to the specific flocculation gene families present in industrial yeast strains.

Material and methods

Yeast strains

In this study, 16 strains from the Centre for Malting and Brewing Science (CMBS) yeast collection were selected. Yeast strains were selected based on their brewing-technical properties. Ale and lager yeast strains, flocculating and non-flocculating, with low and high phenyl off flavors activity were included and are listed in Table 1. These strains were isolated from breweries in different countries. *S. cerevisiae* BY4742 (Brachmann et al. 1998) and *S. pastorianus* CBS1513 were used as reference strains.

Differentiation of ale and lager yeasts

To verify the brewing–technical background information about the strains, such as the ale or lager characteristics, a growth test at 37 °C and a growth test on melibiose were used according to the methods specified by the American Society of Brewing Chemists (ASBC 1992). In order to confirm that the yeast strains were selected without bias and were not all related to each other, the improved interdelta analysis of Legras and Karst (2003) was used. This routinely used polymerase chain reaction (PCR)-based technique allows yeast strain discrimination based on delta elements, which are repeated sequences at various positions in yeast genomes.

Construction of knockout strains

In this study, a *FLO* gene knockout collection was constructed to facilitate *FLO* primer verification. For that purpose, the Cre/LoxP technique described by Güldener et al. (1996, 2002) was used with primers listed in Supplementary Table 1. The G418 resistance marker *kanMX* from the pUG6 plasmid (except for *FLO11*: phleomycin-resistance marker *ble* from the pUG66 plasmid) was selected to delete *FLO1*, *FLO5*, *FLO9*, *FLO10*, and *FLO11*. After the transformations, the marker was rescued by introducing the Cre-recombinase plasmid (pSH47) and by inducing the expression of the Cre-recombinase gene. The presence and removal of the deletion cassette, as well as the correct integration at the desired *FLO* locus were verified using standard PCRs. Quantitative real time polymerase chain reaction (qRT-PCR) experiments were performed to detect *FLO* gene mRNA using primers and high-specificity Taqman probes, as described previously (Van Mulders et al. 2009).

Table 1 *Saccharomyces* strains used in this study. Ale and lager characteristics as well as origin are listed according to the brewing-technical background information from the Centre for Malting and Brewing Science yeast database

Yeast strain name	Ale or lager yeast/genotype	Origin
BY4742	*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Brachmann et al. (1998)
BY4742 *flo1*Δ	Like BY4742, but *FLO1*::*kanMX*	This study
BY4742 *flo5*Δ	Like BY4742, but *FLO5*::*kanMX*	This study
BY4742 *flo9*Δ	Like BY4742, but *FLO9*::*kanMX*	This study
BY4742 *flo10*Δ	Like BY4742, but *FLO10*::*kanMX*	This study
BY4742 *flo11*Δ	Like BY4742, but *FLO11*::*kanMX*	This study
CMBSVM6	Ale	Isolated from a Belgian brewery
CMBSVM11	Ale	Isolated from a English brewery
CMBSVM12	Ale	Isolated from a Belgian brewery
CMBSVM20	Ale	Isolated from a Belgian brewery
CMBSVM21	Ale	Isolated from a Belgian brewery
CMBSVM22	Ale	Isolated from a Belgian brewery
CMBSVM23	Ale	Origin unknown
CMBSVM24	Ale	Origin unknown
CMBSVM25	Ale	Isolated from a Belgian brewery
CMBSVM33	*Saccharomyces cerevisiae*	Isolated from a Brazilian ethanol plant
CMBSVM34	*Saccharomyces cerevisiae*	Isolated from a Brazilian ethanol plant
CMBSVM3	Lager	Isolated from a Dutch brewery
CMBSVM13	Lager	Isolated from a German brewery
CMBSVM14	Lager	Isolated from a Belgian brewery
CMBSVM16	Lager	Isolated from a Belgian brewery
CMBSVM17	Lager	Isolated from a Czech brewery
CBS1513	Type strain of *Saccharomyces carlsbergensis (S. pastorianus)*	Centraal Bureau voor Schimmelculturen (CBS)

Verification of characteristic *FLO* gene sequences

To map the presence or absence of flocculation genes in industrial yeast strains, primers able to detect characteristic *FLO* gene sequences ranging from 50 to 150 bp for each of the five S288C *FLO* genes (*FLO1*, *FLO5*, *FLO9*, *FLO10*, and *FLO11*), S288C *FLO* pseudogenes (*YAR061W* and *YAR062W*) and for *S. pastorianus Lg-FLO1*, were designed (Table 2). These primers were first tested for their selectivity by a standard PCR using genomic DNA from the *FLO* gene knockout strains as template. The temperature program for the mapping of flocculation genes consisted of an initial denaturation for 2 min at 94 °C, and 25 cycles with a denaturation step at 94 °C during 10 s, a primer hybridization step at 60 °C during 15 s, and an elongation step at 72 °C during 45 s.

Flocculation tests

To determine the flocculation characteristics of the yeast strains, cells were inoculated into test tubes containing 5 ml 12 °P all-malt hopped wort and grown aerobically during 48 h at 25 °C. Next, cells were counted, diluted to 5×10^6 cells mL^{-1}, and used for the inoculation of a new test tube with 5 mL 12 °P all-malt hopped wort. Cells were grown anaerobically during 72 h at 25 °C (Anaerocult® A, Merck, Darmstadt, Germany). The medium was centrifuged and the cells resuspended into 1 mL ethylenediaminetetraacetic acid (EDTA; 50 mM, pH 7). Cells were counted, diluted to a concentration of 100×10^6 cells mL^{-1} and the EDTA (50 mM, pH 7) was discarded. Next, 1 mL of EDTA (50 mM, pH 7) was used to wash the cells. EDTA (50 mM, pH 7) was discarded and the samples were resuspended into 1 mL flocculation buffer (82.9 mM $NaCH_3COO$; 67.4 mM CH_3COOH; pH 4.5) without $CaSO_4$ (Lawrence and Smart 2007). Only for carbohydrate inhibition experiments, 500 μM glucose or 500 μM mannose were added to the flocculation buffer. This volume was subdivided into two 500 μL volumes which were transferred to a microcentrifuge tube. Fifty microliter was sampled and transferred to a multiwell plate containing 50 μL EDTA (50 mM, pH 7). Afterwards, 10 μL $CaCl_2$ (500 mM) was added to the microcentrifuge tubes with screw caps. The tubes were shaken horizontally at 100 rpm during 15 min. After 10 min sedimentation time in a vertical position, 50 μl samples were taken under the meniscus and diluted into 50 μL EDTA (50 mM, pH 7). The relative absorbance of the wells ($OD_{600\ nm}$) before and after adding $CaCl_2$ is a measure for the flocculation intensity.

Table 2 Oligonucleotides used for the identification of characteristic *FLO* gene sequences and for the q(RT-)PCR analyses. Primer-binding sites relative to the ORF initiation ATG are also given. The second part includes oligonucleotides used for the 'ale' and 'lager' *Lg-FLO1* sequencing and for the interdelta analysis

Primer name	Primer sequence (5′ to 3′)	Primer binding site (relative to ORF initiation ATG)
ACT1-qPCR-FW	CGTCTGGATTGGTGGTTCTA	[+1014; +1033]
ACT1-qPCR-RV	GTGGTGAACGATAGATGGAC	[+1097; +1116]
FLO1-qPCR-FW	TAGCTGCTGAGACGATTACCAA	[+4268; +4289]
FLO1-qPCR-RV	GCGTGATTAGATCTTGAAAGCGAA	[+4326; +4349]
FLO11-qPCR-FW	GTTCAACCAGTCCAAGCGAAA	[+3038; +3058]
FLO11-qPCR-RV	GTAGTTACAGGTGTGGTAGGTGAAGTG	[+3078; +3104]
FLO10-qPCR-FW	CGTTTCGACAGCCACTGCTA	[+3051; +3070]
FLO10-qPCR-RV	GTGGGCTCCATGTGGAATAAA	[+3092; +3112]
YAR062W-qPCR-FW	AAGTTCTTACACGTTCAGGTTTGCTAAG	[+30; +57]
YAR062W-qPCR-RV	CACAACATTCGAACGCAACGTT	[+91; +112]
YAR061W-qPCR-FW	CTTATCACTATTTATTTTTGGCACTCTTCA	[+3; +34]
YAR061W-qPCR-RV	TGCTTGTGTACTTCCTGAAACAACAT	[+53; +78]
LgFLO1-qPCR-FW	GTACGCCGGTTACTATTACCCGAT	[+549; +572]
LgFLO1-qPCR-RV	GGAAGCCTAGCCAAGACTTTAGCAT	[+590; +614]
FLO5-qPCR-FW	GCACACCACTGCATATTTTTGGTAA	[+10; +34]
FLO5-qPCR-RV	GTAAGCACGCCTCTGTGGCT	[+72; +91]
FLO9-qPCR-FW	TTATTGTTTACTACTAGCCATCGTCACA	[+15; +42]
FLO9-qPCR-RV	AAGTTTACATTCATACCATTCTTCCTTGA	[+100; +128]
LgFLO1-128-FW	GTACGCCGGTTACTATTACCCGAT	
LgFLO1-265-FW	GCTCTGCAGTAAATTCCG	
LgFLO1-261-RV	GGTGTGCTACGAGGCTGTT	
LgFLO1-263-RV	CAGGTGAAGTAGTTTCGGAAC	
LgFLO1-361-RV	GGAAGCCTAGCCAAGGCTTTAGCAT	
LgFLO1-431-RV	GAAACAAAATAATGCAAAGCCG	
LgFLO1-432-RV	AAAACACAGATACCTCGAAAC	
delta12	TCAACAATGGAATCCCAAC	
delta21	CATCTTAACACCGTATATG	

qRT-PCR

CMBSVM11 cells in mid-exponentially growth phase were transferred to fresh YP medium with a minimal concentration of glucose [0.1% (*w*/*v*)] and sampled after 0, 3, 5, and 8 h. Lg-*FLO1*, *YAR062W*, *FLO9*, and *FLO11* expression levels were monitored as described previously (Van Mulders et al. 2009).

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper have been deposited at GenBank with accession number HM358276.

Results

Design and verification of *FLO* gene primer sequences

Based on the S288C genome (*Saccharomyces* Genome Database), primers were designed to selectively amplify characteristic sequences for the five different *FLO* genes and two *FLO* pseudogenes, *YAR061W* and *YAR062W*. The high similarity between the different *FLO* genes required a thorough verification of primer specificity, which was initiated with a BLAST analysis of all S288C *FLO* genes. Next, knockout strains for each of the five *FLO* genes were constructed using the primers listed in Supplementary Table 1. Genomic DNA of these knockout strains was used as a template for the amplification of the characteristic *FLO* gene sequences. In this analysis, each primer set was clearly shown to amplify a characteristic fragment in the wild-type strain and every knockout strain, except for the knockout strain in which the intended *FLO* gene was deleted (Fig. S1). qRT-PCR experiments confirmed the total absence of any *FLO* mRNA in the corresponding knockout strains (Fig. S2). Consequently, the S288C-derived primers can be used to selectively detect S288C-type *FLO* gene markers in other yeast strains. Primers were also designed for *Lg-FLO1*. *Lg-FLO1*, which is a *FLO* gene originally detected in a *S. pastorianus* strain, could not be detected in the S288C strain, but was clearly present in the *S. pastorianus* CBS1513 strain.

Flocculation properties within the brewer's yeast strain collection

In a next step, we selected different yeast strains from the CMBS collection. In this selection, both flocculating and non-flocculating lager and ale yeast strains were included, as deduced from the CMBS collection data about these strains. Interdelta analysis was used to discriminate yeast strains based on specific delta element positions in the yeast genomes. Relatedness between yeast strains is correlated with the similarity of the resulting electrophoretical patterns. Significant differences and parallels between the selected strains can clearly be observed (Fig. 1). The strains were subjected to a flocculation test based on the method used by Lawrence and Smart (2007). The flocculation characteristic was shown to be calcium-dependent for all flocculent strains (results not shown). A wide range of flocculation strengths could be observed as listed in Table 3. Additionally, glucose and mannose specificity was assessed to divide these strains in flo1 (mannose sensitive) or newflo (mannose and glucose sensitive) flocculation-type groups. This distinction was less straight forward than for laboratory strains expressing adhesins such as Flo1 (Van Mulders et al. 2009). Repeatedly, only intermediate inhibition of flocculation could be observed with 500 μM glucose or mannose, which does not allow a clear distinction (results not shown).

Flocculation gene abundance within the brewer's yeast strain collection

Genomic DNA from this yeast strain collection was isolated and used as a template for *FLO* gene identification by means of abovementioned primer sets listed in Table 2. In this experimental setup, we can only show the presence or absence of the characteristic *FLO* gene sequences between 50 and 150 bp (Fig. S3). The absence of an amplified fragment indicates either the presence of mutations at the primer binding sites or the absence of a partial or entire *FLO* gene sequence. We cannot conclude whether the *FLO* gene is expressed and if so, whether the Flo adhesin is responsible for flocculation. Considerable variation between the yeast strains was observed for all *FLO* loci except for *FLO9* and *FLO11* (see Table 3). In the examined flocculating lager and, more remarkably, also several ale type yeast strains, the characteristic *Lg-FLO1* sequence could be detected. On the other hand, in other ale strains, both flocculating and non-flocculating, no *Lg-FLO1* sequence could be detected. As a positive control, the *ACT1* gene marker could routinely be detected in all yeast strains.

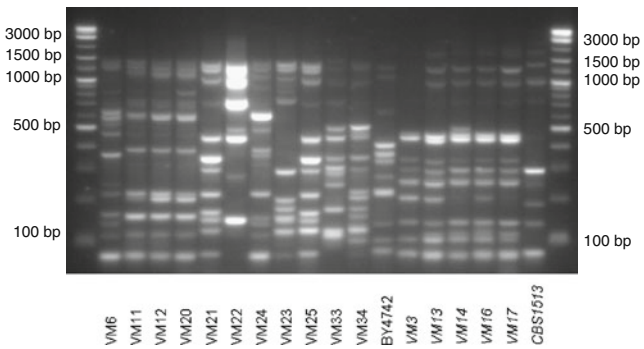

Fig. 1 Interdelta analysis of the brewer's yeast collection. Electrophoretical patterns obtained for different yeasts with delta12-delta21 primers as described by Legras and Karst (2003)

Lg-flo1-type adhesin in ale yeast strains

The three ale yeast strains with the characteristic lager yeast *Lg-FLO1* sequence, CMBSVM11, CMBSVM12, and CMBSVM20, clustered together in the interdelta analysis (Fig. 1), which indicates possible relatedness between them. This previously unreported 'ale' *Lg-FLO1* sequence leads to the question whether these yeasts were actually belonging to the ale-type. A first growth test at 37 °C was positive, and a second growth test on melibiose was negative, confirming that these three strains indeed were corresponding to the brewer's ale type. Nevertheless, the precise genetic background of these strains remains unknown, which impedes the classification within the *Saccharomyces sensu stricto* group.

To further investigate whether the characteristic *Lg-FLO1* sequence found in ale yeast strains belongs to a complete open reading frame (ORF), we amplified and sequenced in a first step the 5′ *Lg-FLO1* domain (accession nos. HM358276), and compared it to the known *Lg-FLO1* sequences from CBS1513 (also confirmed in this study), BF1 and KBY001 (Kobayashi et al. 1998; Ogata et al. 2008), as well as to *Lg-FLO1* from CMBSVM3, CMBSVM13, CMBSVM16, and CMBSVM17 which were sequenced in this study (Fig. 2). The *Lg-FLO1* 5′ domain for the three ale yeast strains was 100% identical and had a nucleotide divergence of approximately 3% with respect to the investigated lager yeasts *Lg-FLO1*. The industrial lager yeasts *Lg-FLO1 5′* domains in this study, which were 100% identical, only had one point mutation compared to *Lg-FLO1* in the bottom-fermenting yeast, BF1 (Ogata et al. 2008), but differed 0.5% with respect to CBS1513. Therefore, CMBSVM11 was used as representative ale strain and CMBSVM3 as representative lager strain for the alignment in Fig. 2. Remarkably, the pentapeptide KVLAR involved in ligand binding of the *S. pastorianus* KBY001 adhesin (Kobayashi et al. 1998; Zupancic et al. 2008), was not identified in CBS1513; the four tested lager yeast strains nor the three ale strains with *Lg-FLO1* (Fig. 3). Instead, a point mutation at nucleotide position 599

Table 3 Schematic overview of the flocculation gene abundance. Lager yeasts are shown in italics. For each yeast strain, characteristic sequences for each of the flocculation genes were detected using selective primers. The presence (*black*) or absence (*white*) of an amplicon is illustrated. Amplicons with a deviating length are depicted in *gray*. The results for the growth on melibiose and growth at 37 °C as well as the flocculation intensity of the strains (n=4) are summarized in the last three columns

Yeast strain	*ACT1*	*FLO1*	*FLO5*	*FLO9*	*FLO10*	*FLO11*	*YAR062W*	*YAR061W*	Lg-*FLO1*	Growth on melibiose	Growth at 37°C	Flocculation intensity
BY4742										No	Yes	1% ± 5%
CMBSVM6										No	Yes	59% ± 29%
CMBSVM11										No	Yes	96% ± 1%
CMBSVM12										No	Yes	97% ± 1%
CMBSVM20										No	Yes	90% ± 3%
CMBSVM21										No	Yes	27% ± 9%
CMBSVM22										No	Yes	44% ± 4%
CMBSVM23										No	No	58% ± 10%
CMBSVM24										No	Yes	77% ± 7%
CMBSVM25										No	Yes	29% ± 4%
CMBSVM33										No	Yes	43% ± 3%
CMBSVM34										No	Yes	25% ± 13%
CMBSVM3										Yes	No	1% ± 5%
CMBSVM13										Yes	No	37% ± 9%
CMBSVM14										Yes	No	37% ± 31%
CMBSVM16										Yes	No	87% ± 1%
CMBSVM17										Yes	No	50% ± 11%
CBS1513										Yes	No	20% ± 15%

changed the valine into an alanine. From this result, it can be concluded that the Lg-Flo1 KALAR motif is more conserved between the examined yeast strains in this study.

To completely sequence the ale yeast *Lg-FLO1* gene, it was assumed that the 3′ sequence, which encodes the glycosylphosphatidylinositol (GPI)- anchor site, is sufficiently conserved. Using forward primers LgFLO1-128-FW and LgFLO1-265-FW in the 5′ domain of the ale yeast *Lg-FLO1* and several putative reverse primers in the 3′ domain, LgFLO1-261-RV and LgFLO1-263-RV, we succeeded in amplifying fragments for the three ale yeasts. The successful PCR and subsequent sequencing indicate the presence of a 3′ GPI anchor site coding sequence and therefore most likely a complete and functional ORF coding for an adhesin. Finally, using two putative reverse primers downstream of the *Lg-FLO1* ORF, LgFLO1-431-RV and LgFLO1-432-RV, the complete 3′ part of the ORF could be amplified. The ORF length for CMBSVM11 is approximately 3,700 bp.

Lg-FLO1 expression in ale-type yeast strains

To investigate whether the identified *Lg-FLO1* gene is expressed, CMBSVM11 cells growing exponentially were transferred to fresh YP medium with a minimal concentration of glucose [0.1% (*w*/*v*)]. It was assumed that carbon limitation would induce flocculation gene expression (Sampermans et al. 2005). *Lg-FLO1* expression levels of flocculent cells were monitored after 0, 3, 5 and 8 h and were fluctuating between 10% and 20% of *ACT1* levels (see Fig. 4). Previous studies showed that high expression levels of *FLO1* (at least 1% of *ACT1* levels), generally correspond to a strong flocculation phenotype (>85%; Smukalla et al. 2008). Therefore, we can assume that *Lg-FLO1* is highly expressed in the CMBSVM11 strain. *FLO11* also showed expression levels above 1% of *ACT1* levels in CMBSVM11. The other tested *FLO* genes present in CMBSVM11, *FLO9* and *YAR062W* (see Table 3), showed expression levels lower than 1% of *ACT1* levels.

Discussion

Top-fermenting and bottom-fermenting brewer's yeast strains are characterized by different genomic make-ups. However, brewers expect yeast strains from different origins or with different brewing technical properties to flocculate preferentially at a well-defined time point, when the fermentable carbohydrates are depleted. The yeast slurry may then be decanted in order to use for a subsequent fermentation. Compared to other brewing technical properties of yeasts, flocculation is one of the most variable (Sato et al. 2001). The impressive *FLO* gene variation within a brewer's yeast strain collection could be visualized in this study by detecting the presence or absence of characteristic flocculation gene sequences. The specific markers for *FLO* genes, which are located near the telomeres, except for the *FLO9* marker, were shown to vary significantly within the strain collection. The marker for the

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CMBSVM3_Lg-FLO1      ATGACAATTGCACACCACTGCATATTTTTGGTAATCTTGGCCTTTCTGGAGCTACTTAAC 60
BF1_Lg-FLO1          ATGACAATTGCACACCACTGCATATTTTTGGTAATCTTGGCCTTTCTGGAGCTACTTAAC 60
KBY001_Lg-FLO1       ATGACAATTGCACACCACTGCATATTTTTGGTAATCTTGGCCTTTCTGGAGCTACTTAAC 60
CBS1513_Lg-FLO1      ATGACAATTGCACACCACTGCATATTTTTGGTAATCTTGGCCTTTCTGGAGCTACTTAAC 60
CMBSVM11_Lg-FLO1     ATGACAATTGCACACCACTGCATATTTTTGGTAATCTTGGCCTTTCTGGAGCTACTTAAC 60
                     ************************************************************

CMBSVM3_Lg-FLO1      GTAGCATCAGGAAGTACACAAGCATGCCTGCCAGTGGGCTCGAGGAAAAATGGGATGAAT 120
BF1_Lg-FLO1          GTAGCATCAGGAAGTACACAAGCATGCCTGCCAGTGGGCTCGAGGAAAAATGGGATGAAT 120
KBY001_Lg-FLO1       GTAGCATCAGGAAGTACACAAGCATGCCTGCCAGTGGGCTCGAGGAAAAATGGGATGAAT 120
CBS1513_Lg-FLO1      GTAGCATCAGGAAGTACACAAGCATGCCTGCCAGTGGGCTCGAGGAAAAATGGGATGAAT 120
CMBSVM11_Lg-FLO1     GTAGCATCAGGAAGTACACAAGCATGCCTGCCAGTGGGCCCGAGGAAAAACGGAATGAAC 120
                     ************************************ ********** ** *****

CMBSVM3_Lg-FLO1      GTCAACTTTTATAAATACTCATTACAGGATTCAACAACGTATTCCGACCCGCAATATATG 180
BF1_Lg-FLO1          GTCAACTTTTATAAATACTCATTACAGGATTCAACAACGTATTCCGACCCGCAATATATG 180
KBY001_Lg-FLO1       GTCAACTTTTATAAATACTCATTACAGGATTCAACAACGTATTCCGACCCGCAATATATG 180
CBS1513_Lg-FLO1      GTCAACTTTTATAAATACTCATTACAGGATTCAACAACGTATTCCGACCCGCCATATATG 180
CMBSVM11_Lg-FLO1     ATCAACTTTTATGAATACTCATTAGATGATTCAACAACGTATTCCGACCCGCAATATATG 180
                      *********** *********** * ************************ *******

CMBSVM3_Lg-FLO1      GCCTATAAATACTCCGATACAAAGAAGTTAGGTTCCGTTAGCGGACAGACCCATCTCTCC 240
BF1_Lg-FLO1          GCCTATAAATACTCCGATACAAAGAAGTTAGGTTCCGTTAGCGGACAGACCCATCTCTCC 240
KBY001_Lg-FLO1       GCCTATAAATACTCCGATACAAAGAAGTTAGGTTCCGTTAGCGGACAGACCCATCTCTCC 240
CBS1513_Lg-FLO1      GCCTATAAATACTCCGATACAAAGAAGTTAGGTTCCGTTAGCGGACAGACCCATCTCTCC 240
CMBSVM11_Lg-FLO1     GCCTATAAATACTCCGATACAAAGAAGTTAGGTTCCGTTAGCGGACAGACCCATCTCTCC 240
                     ************************************************************

CMBSVM3_Lg-FLO1      ATATACTATGGCCCAAATACTGCCTTTTGGAATACTGCCTCTTGGAGTTCTGATCTTTTT 300
BF1_Lg-FLO1          ATATACTATGGCCCAAATACTGCCTTTTGGAATACTGCCTCTTGGAGTTCTGATCTTTTT 300
KBY001_Lg-FLO1       ATATACTATGGCCCAAATACTGCCTTTTGGAATACTGCCTCTTGGAGTTCTGATCTTTTT 300
CBS1513_Lg-FLO1      ATATACTATGACCTAAATACTGCCTTTTGGAATACTGCCTCTTGGAGTTCTGATCTTTTT 300
CMBSVM11_Lg-FLO1     ATATACTATGACCCAAATACTGGCTTTTGGAATACTGCCTCTTGGAGTTCTGATCTTTTT 300
                     ********** ** ******** ************************************

CMBSVM3_Lg-FLO1      GGTTTCTATACTACTCCAACTAATGTAACTGTGGAAATGACAGGGTACTTTTTACCACCA 360
BF1_Lg-FLO1          GGTTTCTATACTACTCCAACTAATGTAACTGTGGAAATGACAGGGTACTTTTTACCACCA 360
KBY001_Lg-FLO1       GGTTTCTATACTACTCCAACTAATGTAACTGTGGAAATGACAGGGTACTTTTTACCACCA 360
CBS1513_Lg-FLO1      GGTTTCTATACTACTCCAACTAATGTAACTGTGGAAATGACAGGGTACTTTTTACCACCA 360
CMBSVM11_Lg-FLO1     GGTTTCTATACTACTCCAACTAATGTAACTGTGGAAATGACAGGGTACTTTTTACCACCA 360
                     ************************************************************

CMBSVM3_Lg-FLO1      CAGACGGGTTCTTACACATTCAAGTTTGCTACAGTTGACGACTCTGCAATTTTATCGGTT 420
BF1_Lg-FLO1          CAGACGGGTTCTTACACATTCAAGTTTGCTACAGTTGACGACTCTGCAATTTTATCGGTT 420
KBY001_Lg-FLO1       CAGACGGGTTCTTACACATTCAAGTTTGCTACAGTTGACGACTCTGCAATTTTATCGGTT 420
CBS1513_Lg-FLO1      CAGACGGGTTCTTACACATTCAAGTTTGCTACAGTTGACGACTCTGCAATTTTATCGGTT 420
CMBSVM11_Lg-FLO1     CAGACGGGTTCTTACACGTTTTTTTTTCCTACAGTTGACGACTCTGCAATTTTATCGGTT 420
                     ***************** **    *** ************************************

CMBSVM3_Lg-FLO1      GGTGGTAGCATTGCGTTCGAATGTTGTGCACAAGAACAACCTCCTATCACATCAACGGAT 480
BF1_Lg-FLO1          GGTGGTAGCATTGCGTTCGAATGTTGTGCACAAGAACAACCTCCTATCACATCAACGGAT 480
KBY001_Lg-FLO1       GGTGGTAGCATTGCGTTCGAATGTTGTGCACAAGAACAACCTCCTATCACATCAACGGAT 480
CBS1513_Lg-FLO1      GGTGGTAGCATTGCGTTCGAATGTTGTGCACAAGAACAACCTCCTATCACATCAACGGAT 480
CMBSVM11_Lg-FLO1     GGTGGTAACGTTGCGTTCGAATGTTGTGCACAAGAACAACCTCCTATCACATCAACGGAT 480
                     ******* * ************************************************

CMBSVM3_Lg-FLO1      TTCACTATTAACGGTATTAAACCATGGGACGCAGCTGCACCTACCGACATAAAGGGGTCA 540
BF1_Lg-FLO1          TTCACTATTAACGGTATTAAACCATGGGACGCAGCTACACCTACCGACATAAAGGGGTCA 540
KBY001_Lg-FLO1       TTCACTATTAACGGTATTAAACCATGGGACGCAGCTGCACCTACCGACATAAAGGGGTCA 540
CBS1513_Lg-FLO1      TTCACTATTAACGGTATTAAACCATGGGGCGCAGCTGCACCTACCGACATAAAGGGGTCA 540
CMBSVM11_Lg-FLO1     TTCACTGTTAACGGTGTTAAATCATGGGGCGCAGCTGCACCTACCGACATAAAGGGGTCA 540
                     ****** ******** ***** ****** ******* ************************

CMBSVM3_Lg-FLO1      ACGTACATGTACGCCGGTTACTATTACCCGATCAAAATTGTTTATTCAAATGCTAAAGCC 600
BF1_Lg-FLO1          ACGTACATGTACGCCGGTTACTATTACCCGATCAAAATTGTTTATTCAAATGCTAAAGCC 600
KBY001_Lg-FLO1       ACGTACATGTACGCCGGTTACTATTACCCGATCAAAATTGTTTATTCAAATGCTAAAGTC 600
CBS1513_Lg-FLO1      ACGTACATGTACGCCGGTTACTATTACCCGATCAAAATTGTTTATTCAAATGCTAAAGCC 600
CMBSVM11_Lg-FLO1     ACGTACATGTACGCCGGTTACTATTACCCGATCAAAATTGTTTATTCAAATGCTAAAGCC 600
                     ************************************************************ *

CMBSVM3_Lg-FLO1      TTGGCTAGG 609
BF1_Lg-FLO1          TTGGCTAGG 609
KBY001_Lg-FLO1       TTGGCTAGG 609
CBS1513_Lg-FLO1      TTGGCTAGG 609
CMBSVM11_Lg-FLO1     TTGGCTAGG 609
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Fig. 2 Multiple alignment of the conserved 5′ nucleotide sequence of CMBSVM11 *Lg-FLO1*, CMBSVM3 *Lg-FLO1*, and CBS1513 *Lg-FLO1* (this study), the BF1 *Lg-FLO1* (Ogata et al. 2008), the KBY001 *Lg-FLO1* (Kobayashi et al. 1998). The numbers on the right of each sequence represent the location of the sequence in the open reading frame. Asterisks below the sequences indicate the positions at which the same nucleotide is present in each *FLO* gene

FLO11 allele, which is located neither at the telomeres nor at the centromere of chromosome IX, was conserved with the exception of strains CMBSVM22 and CMBSVM23. Intrinsic properties of the flocculation genes such as the presence of tandem repeats and their (sub-) telomeric location are likely to be responsible for this variation (Verstrepen et al. 2005). The reason why the marker for the *FLO9* allele, which is located at the left arm of chromosome I, was shown not to differ between all the tested yeast strains is unknown. Flocculation gene variation can significantly affect adhesion phenotypes in brewer's yeast strains, but also in other yeasts. For example in 'Flor

Fig. 3 Multiple alignment of the amino acid sequence of the S288C (BY4742) Flo1, Flo5, Flo9, Flo10 and Flo11, the KBY001 Lg-flo1 (Kobayashi et al. 1998), the CBS1513 Lg-flo1 (Ogata et al. 2008; confirmed in this study) and the CMBSVM11 Lg-Flo1 N-terminal parts involved in sugar recognition as shown by Kobayashi et al. (1998). The amino acids indicated with a *gray cross* contribute to sugar recognition as described by Kobayashi et al. (1998). The *gray box* indicates the pentapeptide region of the adhesins involved in glycan specificity as described by Zupancic et al. (2008)

yeasts', typically used in sherry wine fermentations, a specific 'buoyancy' phenotype is conferred through mutations in the *FLO11* promoter and an intragenic event in which tandem repeat number increased and tandem repeat order changed (Fidalgo et al. 2006). These two changes result in a higher expression of a more hydrophobic Flo11 adhesin and show that pronounced phenotypic changes can easily occur by means of tandem-repeat mutations (Fidalgo et al. 2008).

An intergenic event between very similar sequences on different chromosomes might have generated the *Lg-FLO1* gene, which is located on the *S. cerevisiae* (SC-) chromosome VIII of bottom-fermenting yeast at the same position as the *FLO5* gene of the laboratory yeast S288C (Ogata et al. 2008). The *Lg-FLO1* 5′ non-coding region indeed shows high similarity to the corresponding region of *FLO5* (SC-chromosome VIII) and the 3′ non-coding region high similarity to the corresponding region of *YAL065c* (SC-chromosome I; Kobayashi et al. 1998). Besides *Lg-FLO1*, the presence of *ILF1* (inactivated *Lg-FLO1*) on chromosome VIII has also been observed in lager yeasts (Sato et al. 2002; Ogata et al. 2008). *ILF1* presumably originates from a translocation between *Lg-FLO1* on the SC-chromosome VIII and *YIL169c* on the SC-chromosome IX. The remarkable observation of *Lg-FLO1* in a group of three ale type brewer's strains in this study raises several questions. The theory that these three ale type brewer's strains actually were lager yeasts was rejected by discriminating them based on growth temperature and growth on melibiose. Dunn and Sherlock (2008) provide evidence that interspecific hybrid formation among the *Saccharomyces sensu stricto* group has occurred in breweries multiple times, and that the *S. cerevisiae* parents of the hybrid lager yeasts were likely to be ale yeasts. This phenomenon implies that the strict distinction between ale and lager yeasts may be artificial in certain cases, in particular at a genomic level. In breweries using different yeast strains at high cell densities, together with the unavoidable presence of contaminant yeasts, interspecific hybrid formation may be a continuous process leading to yeast strains with a genomic make-up as well as brewing technical properties ranging in between the 'typical' ale and lager yeasts. For example, CMBSVM23 does not assimilate melibiose and does not grow at 37 °C, which put these strain outside of the traditional ale/lager classification. Additionally, the presence of Lg-flo1 with a 'typical' glucose-mannose binding site, in CMBSVM11, CMBSVM12 and CMBSVM13 can be an explanation for the newflo-type flocculation in some ale yeast strains, which was not completely understood (Jin and Speers 2000; Sampermans et al. 2005; Speers et al. 2006; Rando and Verstrepen 2007). Several attempts were made to understand the functional role of Lg-Flo1 by deleting the *Lg-FLO1* allele in CMBSVM11, CMBSVM12, or CMBSVM20. This did not succeed using replacement cassettes with homologous tails up to 85 bp. Possible reasons are that DNA fragment transplacement is strain-dependent and that tail regions should be

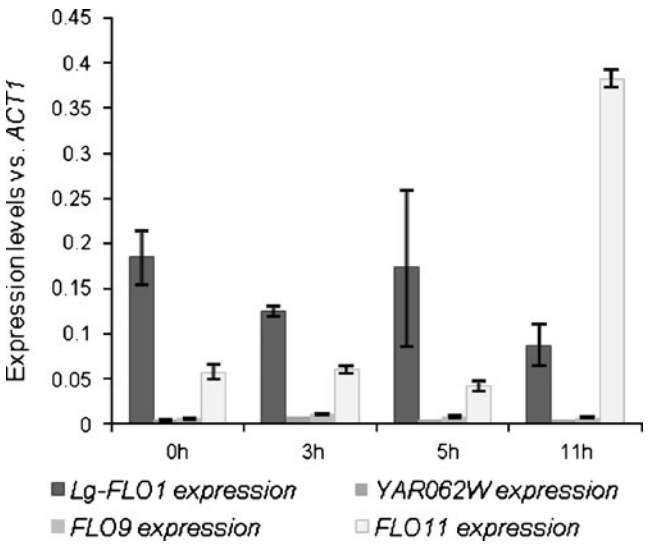

Fig. 4 CMBSVM11 cells growing mid-exponentially were transferred to fresh YP medium with a minimal concentration of glucose (0.1% *w/v*). *Lg-FLO1*, *YAR062W*, *FLO9*, and *FLO11* expression levels were monitored after 0, 3, 5, and 8 h. Gene expression levels were normalized with respect to *ACT1* expression levels. These experiments were executed in duplicate

extensive for high transformation efficiencies in industrial yeasts (Manthey et al. 2004; Govender et al. 2010). Nevertheless, the significant *Lg-FLO1* expression levels compared to other *FLO* genes in CMBSVM11 under low fermentable carbohydrate concentrations may indicate a potential role for this adhesin in ale yeast flocculation.

Each brewer, winemaker, and yeast biotechnologist has carefully selected yeast strains during years to be highly suited for specific industrial applications. This has lead to genetically distinct yeasts in these industries. During this selection, flocculation gene variation has been catalyzed enormously by their intrinsic properties such as tandem repeats and their (sub-) telomeric location. This extensive variation complicates the knowledge needed to control yeast flocculation at a genetic level. Moreover, flocculation gene expression studies need a strain-by-strain approach, specifically designed towards the particular strain *FLO* gene family. To circumvent *FLO* gene expression-related flocculation variation, natural promotors were replaced by inducible promotors (Verstrepen et al. 2001; Govender et al. 2008), which was recently achieved in industrial wine strains with an unknown genotype for genes with sufficient similarity to S288C flocculation genes (Govender et al. 2010). However, this can be problematic in yeast strains with flocculation genes without sufficient similarity to S288C. Next-generation sequencing technologies will enable biotechnologists to uncover the unique stretches of DNA that characterize their personal industrial yeast strains (Borneman et al. 2008). This will open the path towards targeted genetic techniques for the optimization of the brewer's yeast flocculation pattern.

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