Flocculation, Pseudohyphal Development and Invasive Growth in Commercial Wine Yeast Strains

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Flocculation of *Saccharomyces cerevisiae* cells at the end of alcoholic fermentation is an important phenomenon in winemaking, especially in the production of bottle-fermented sparkling wine. Most wine yeast strains do not flocculate during the fermentation process and it is unknown whether they contain the necessary genes to flocculate and whether these genes are expressed adequately under wine-making conditions. These genes include the *FLO1* flocculin gene which is one of the most important genes to confer the ability of yeast cells to flocculate and the *MUC1* gene (subsequently also cloned as *FLO11*) encoding a mucin-like protein which was previously shown not only to play a key role in pseudohyphal development and invasive growth, but also to be involved in flocculation in *S. cerevisiae*. Together with *MUC1*, the involvement of *FLO8* (encoding a transcriptional activator of *FLO1*) and *TUP1* in flocculation, pseudohyphal development and invasive growth indicates that these processes might somehow be linked. Therefore, in order to construct wine yeast strains that are able to flocculate, 25 commercial wine yeast strains were investigated for their ability to flocculate, form pseudohyphae and invade solid media. Twenty-one of these strains were able to penetrate into agar media and different degrees of pseudohyphal and invasive growth were observed. The average length of cells and pseudohyphae and the efficiency of invasive growth varied among these strains. Two of the strains are known to flocculate in wine, while three other strains could be induced to aggregate to a limited degree in glycerol-ethanol medium. Southern blot analyses revealed the presence of homologous DNA sequences in all of the 25 strains using DNA fragments of *FLO8, MSS10* and *MSS11* (encoding transcriptional activators of *MUC1*), *FLO1* and *MUC1* as probes. Using Northern blot analysis, *FLO1* transcripts were detected in only one of the strains that showed constitutive flocculation in all the growth media tested. *MUC1* transcripts of varying sizes could be detected in most of the strains. From these results it is clear that *MUC1* does not primarily confer the phenotype of flocculation and that *FLO1* (flocculation) and *MUC1* (pseudohyphal differentiation, invasive growth and flocculation) are not co-regulated. We therefore suggest that *MUC1*, as opposed to *FLO11*, be retained as the most appropriate designation of this gene encoding the *S. cerevisiae* mucin-like protein.

The yeast *Saccharomyces cerevisiae* is able to change its growth pattern under conditions of nutrient limitation. These changes include flocculation, pseudohyphal growth, the formation of hyphal-like structures and velum. The importance of flocculation of wine yeast strains to the wine industry, especially for the production of bottle-fermented sparkling wine, is widely recognized. At the end of alcoholic fermentation, some flocculent wine yeast strains aggregate spontaneously to form clumps of cells resulting in rapid sedimentation of these cells from the medium. The wine is then cleared in a short period of time resulting in lower production cost and optimization of wine quality due to reduced handling after fermentation.

True flocculation is defined as an asexual reversible aggregation process due to physical binding between lectins and lectin-receptors present on adjacent cells. Only cells with the ability to produce and secrete these lectins to the cell surface are able to flocculate. Calcium is a specific requirement for true flocculation to occur due to its influence on the specific conformation of the lectins (Straver *et al.*, 1993). The extent of flocculation is dependent on growth conditions, nutrient availability, cell concentration and agitation (Smit *et al.*, 1992; Bowen & Ventham, 1994).

Several dominant, semi-dominant and recessive genes (encoding structural proteins) are known to be involved in flocculation, namely *FLO1, FLO2, flo3, FLO4, FLO5, flo6, flo7, FLO9, FLO10* (for a recent review see Theunissen & Steensma, 1995) and *MUC1* (Lambrechts *et al.*, 1996), which was subsequently also cloned as *FLO11* (Lo & Dranginis, 1996). Of these flocculation genes, the *FLO1* gene is the most important flocculation gene, capable of inducing flocculation when transformed into non-flocculant cells (Russel *et al.*, 1980; Teunissen *et al.*, 1995a; Vezinhet *et al.*, 1991). Various degrees of homology exist among the coding regions of the various flocculation genes, whereas the promoter sequences are completely different from one another (Teunissen & Steensma, 1995). The gene product of *MUC1*,

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which has been shown to be involved in pseudohyphal development and invasive growth, is similar in overall structure to the flocculins, namely Flo1p, Flo5p, Flo9p and Flo10p (Lambrechts *et al.,* 1996; Lo & Dranginis, 1996). All these proteins comprise an amino-terminal domain containing a hydrophobic signal sequence and a carboxyl terminal domain with homology to the glycosyl-phosphatidyl-inositol-anchor-containing proteins separated by a central domain of highly repeated sequences rich in serine and threonine residues.

Regulatory genes involved in flocculation include *FLO8* and the *TUP1/SSN6* regulatory cascade (Teunissen *et al.,* 1995b; Lui *et al.,* 1996). Suppressor genes, such as *fsu1* and *fsu2,* may be responsible for the inability of cells possessing flocculation genes to flocculate (Sieiro *et al.,* 1993). Flo8p has been shown to be a putative transcriptional activator of *FLO1* (Liu *et al.,* 1996). Although this gene seems to be a prerequisite for flocculation in strains of the *FLO1* genotype, not all strains containing the *FLO8* gene are able to flocculate (Kobayashi *et al.,* 1996). It was suggested that Flo8p inactivates the *TUP1* and *CYC8/SSN6* cascade which represses flocculation in certain strains (Kobayashi *et al.,* 1996). Recently, both *TUP1* (Braun & Johnson, 1997) and *FLO8* (Lui *et al.,* 1996) have also been implicated in pseudohyphal formation and invasive growth.

The ability of yeast to interconvert between yeast-like and pseudohyphal growth modes, during which the daughter cells remain attached to the mother cell after budding, is triggered by several signals (for a recent review see Vivier *et al.,* 1997). This phenomenon has also been observed in brewing yeast strains, which are divided into four classes regarding their flocculation ability, of which class IV yeast strains are chain formers (Stratford, 1992b). Although regarded as unsuitable for brewing, these strains were found to be fairly common in a survey of ale strains. Stewart & Russel (1981) suggested that the latter phenomenon is due to the inability of daughter cells to separate from the mother cells, a mechanism also present during pseudohyphal development. One yeast strain may have the ability to either flocculate or form pseudohyphae or express both of these two phenotypes (Wilcocks & Smart, 1995). The extent of flocculation, pseudohyphal development and invasive growth is dependent on growth conditions, medium composition (nitrogen content and carbon source) and the ploidy of the cells (Gimeno & Fink, 1992; Smit *et al.,* 1992; Bowen & Ventham, 1994; Blacketer *et al.,* 1995; Lambrechts *et al.,* 1996).

During pseudohyphal development, cells become longer and thinner, facilitating the movement of vegetative cells into new substrates in search of nutrients. Pseudohyphae may also lead to invasive growth under nutrient-limiting conditions (Gimeno *et al.,* 1993). Although similar genes are involved in pseudohyphal development and invasive growth, these two phenomena appear to occur via separate pathways (Mösch and Fink, 1997). *MUC1* codes for a mucin-related, membrane-located protein (Lambrechts *et al.,* 1996). Apart from flocculation, Muc1p also plays an important role during pseudohyphal development and invasive growth (Lambrechts *et al.,* 1996). When present in multiple copies, the putative transcriptional activators of *MUC1*, Mss10p (also known as Msn1p, Fup1p and Phd2p) and Mss11p, induce pseudohyphal development, invasive growth and cell aggregation (Lambrechts *et al.,* 1996; Vivier *et al.,* 1997; Webber *et al.,* 1997; Gagiano *et al.,* 1998).

Filamentous invasive growth in *S. cerevisiae* is one of numerous responses to cues from the extracellular environment. These cues are then transduced from the cell surface to the interior of the cell, resulting in patterns of altered gene expression and protein activity, which result in a cellular response to the external environment (for a recent review see Banuette, 1998). In yeast cells, the MAPK cascade module is a key element in mediating the transduction of many signals generated at the cell surface to the nucleus. Pseudohyphal differentiation and invasive growth of *S. cerevisiae* requires multiple elements of the mitogen-activated protein kinase (MAPK) signaling cascade that are also components of the mating pheromone response pathway. Several of the *STE* genes (*STE7, STE11, STE12, STE20)* have been implicated as role-players with *STE12* and *TEC1,* both DNA-binding proteins, modulating these morphological changes (Madhani & Fink, 1997). At least one other pathway has also been implicated in this morphogenetic developmental switch (Lorenz & Heitman, 1997). Information on this signaling pathway is still fragmentary, although genes such as *MSS10, MSS11* and *FLO8* could possibly be part of it.

Only a few of the wine yeast strains that are available commercially are able to flocculate. In order to understand the flocculation phenotype and to eventually develop flocculating wine yeast strains, we screened for the presence of some of the structural and transcriptional activating genes playing an important role in flocculation, pseudohyphal development and invasive growth. In this study the presence and expression of *FLO1, FLO5, FLO9, FLO8, MUC1/FLO11, MSS10* and *MSS11* genes in 25 commercial wine yeast strains were investigated.

MATERIALS AND METHODS

Yeast strains and media: Twenty-five commercial active dried wine yeast strains most commonly used in the South African wine industry and four randomly selected laboratory strains were studied (Table 1). Four haploid laboratory strains (containing the following genes: *MUC1, MSS10, MSS11* and *FLO8*) were included as controls for testing for the presence of the relevant genes. According to information from the two yeast manufactures, Anchor Yeast and Lallemand, all commercial strains used in this study are diploid. Pure single cell isolates were obtained from the commercial dried Yeast and their identity verified by electrophoretic karyotyping. These pure cultures were used as inocula. The yeast growth media were: YPD (1% yeast extract, 2% peptone, 2% glucose), SLAD [2% glucose, 1,7% yeast nitrogen base (YNB) without amino acids and ammonium sulfate, 0,05 mM ammonium sulfate], YPGE (1% yeast extract, 2% peptone, 3% glycerol, 2% ethanol), SCGE (6,7% YNB without amino acids, 3% glycerol, 2% ethanol, 0,072% complete amino acid pool and SC (6,7% YNB without amino acids, 2% glucose, 0,072% complete amino acid pool). Chenin blanc grape juice supplemented with the necessary nutrients was used for fermentations. Solid media contained 2% agar. Yeast strains were routinely grown at 30°C.

TABLE 1

The presence of the flocculent, pseudohyphal and invasive growth characteristics in 25 commercial yeast strains.

NO	YEAST STRAIN	FLOCCULENCE	PSEUDOPHAL GROWTH **	INVASIVE GROWTH **	CELL LENGTH (mm) **f	SOURCE
1	Lalvin L2056	-	+	+++	7,5	Lallemand
2	Lalvin ICV D254	-	+	+++	15	Lallemand
3	Lalvin V1116	-	+	+	7,5	Lallemand
4	Lalvin L2226	-	+	+++	7,5 - 12,5	Lallemand
5	Lalvin DV10	-	+	+++	5	Lallemand
6	Lalvin EC1118	-	+	+++	7,5	Lallemand
7	Lalvin 71B	-	+	-	10	Lallemand
8	Lalvin Wadenswill 27	-	+	++	5	Lallemand
9	Lalvin ICV D47	-	+	++	7,5	Lallemand
10	Lalvin 016	+	+	+++	12,5	Lallemand
11	Enoferm Bordeaux Red	-	+	+++	7,5 - 10	Lallemand
12	Enoferm Assmanshausen	-	+	-	5	Lallemand
13	Enoferm M2	-	+	++	10 - 15	Lallemand
14	Uvaferm ALB	+	+	+++	5	Lallemand
15	Uvaferm Vinaroma	-	+	+++	6,25	Lallemand
16	Uvaferm PM	-	+	+++	5 - 7,5	Lallemand
17	Uvaferm BC	-	+	+	10	Lallemand
18	Uvaferm CGC62	-	+	++	7,5 - 10	Lallemand
19	VIN7	-	-	_	10 - 12,5	Anchor Yeast
20	VIN13	+	+	++	5	Anchor Yeast
21	N96	-	+	++	5	Anchor Yeast
22	WE372	-	+	++	7,5	Anchor Yeast
23	WE14	w	+	+++	10 - 12,5	Anchor Yeast
24	WE228	w	+	+++	5	Anchor Yeast
25	FLOR	-	+	+++	6,25 - 7,5	KWV
26	ISP12	nt	nt	nt	nt	This laboratory
27	ISP15	nt	nt	nt	nt	Lambrechts *et al.*, 1996
28	ISP17	nt	nt	nt	nt	This laboratory
29	ISP20	nt	nt	nt	nt	Gagiano *et al.*, 1998

\+ The presence of the characteristic (intensity corresponding to the number of symbols).
\- The absence of the characteristic.
nt Not Tested.
* Characteristic was tested in YPGE liquid medium.
w These strains showed aggregation, but not true flocculation.
** Characteristic was tested on SLAD solid medium.
f Data on the cell length should not be compared with the photogprahs of Fig. 1. The cell lengths reported in this table were measured microscopically as reported under Materials and Methods.

Pseudohyphal growth: Cells were grown aerobically in liquid YPD medium for 24 h. A diluted aliquote of the cells [absorbance at 600 nm (A_{600}) of 0,2] in sterile distilled water was spotted onto SLAD agar plates. These cultures were incubated for 3 days at 30°C. Small blocks of agar (0,5 cm x 0,5 cm) were cut, removed from a petri dish and placed on a subject glass, overlayed with a coverslip and photographed. A Nikon FDX-35 camera mounted on a Nikon Optiphot-2 Type 104 microscope at 40 X magnification was used. The average length of the cells was determined with an ocular micrometer after calibration with a stage micrometer. The ocular micrometer was mounted onto a Nikon SE Binocular microscope, calibrated and used at 40 X magnification.

Invasive growth: Cells were grown aerobically in YPD liquid medium for 24 h. A 20 µl undiluted suspension of this culture was spotted onto SLAD agar plates. These cultures were incubated for 7 days at 30°C. The plates were rinsed with slow running water and gently stroked with the finger to remove all cells that did not grow into the agar (Lambrechts *et al.,* 1996). The density of the spot remaining on the plates correlates with the ability of the strains to grow invasively. The invasive growth potential was photographed as described in the previous section. Strain ISP15 was routinely used as a reference strain.

Flocculation assay: Cells were cultured aerobically for 12 to 24 h in YPD medium containing 1% (w/v) calcium chloride. These cells were inoculated into sterilized grape juice, SLAD, SCGE, SC, YPD and YPGE medium containing either 0% or 1% calcium chloride. These cultures were grown shaking (160 rpm) for up to 5 days. If a precipitate was formed in the test tube used for growth, 250 mM EDTA was added. Cells in which the aggregation was reversed under these conditions were considered flocculent (Table 1) according to the definition of Johnston & Reader (1983).

Southern blot analysis: Chromosomal DNA samples were prepared according to the embedded-agarose procedure of Carle & Olson (1985). Intact chromosomal DNA was separated using contour clamped homogeneous electric field (CHEF) electrophoresis. A CHEF-DR II (Bio-Rad Laboratories, Richmond, USA) apparatus was used. The gel was prepared in 0,5 X TBE (5,4% Tris base, 2,75% boric acid, 10 mM EDTA, pH 8,0) buffer and electrophoresed in the same buffer at a set temperature of 4°C for 30,5 h at a constant voltage of 200 V. The pulse rate was set at 60 s for the first 17 h and 90 s for the last 13 h. Gels were stained with ethidium bromide, destained with 0,5 X TBE buffer, viewed and photographed on a transilluminator with a camera mounted to it. Gels were depurinated twice with 0,25 M HCl for 15 min at room temperature, denatured twice with a 0,5 M NaOH / 1,5 M NaCl solution for 30 min and neutralized twice with a 1 M Tris-HCl / 1,5 M NaCl / 10 mM EDTA solution for 30 min. The DNA was then transferred to nylon (Hybond N, Amersham) membranes using 20 X SSC (17,53% sodium chloride, 8,82% sodium citrate, pH 7,0). The hybridization and prehybridization techniques were performed according to Sambrook *et al.* (1989). Prehybridization was performed at 68°C for 4 to 6 h and hybridization was performed overnight at 68°C. The membranes were hybridized with the appropriate gene probes.

Northern blot analysis: Cells were precultured in YPD medium for 24 h and subsequently inoculated into a liquid culture of YPGE and grown until an OD_{600} of 1,5 was reached. RNA was isolated by using the Fast Prep kit (BIO101, CA). The RNA was separated in an agarose gel containing formaldehyde. Gels were washed three times with diethylpyrocarbonate (DEPC)-treated distilled water for 30 min, subjected for 25 min to 0,05 M NaOH and treated with 20 X SSC for 45 min. The RNA was transferred to nylon (Hybond N) membranes using 20 X SSC. Prehybridization and hybridization techniques were used as described by Sambrook *et al.* (1989). Prehybridization was performed at 42°C for 2 to 5 h in the presence of formamide. Hybridization with the appropriate DNA probes was carried out overnight at 42°C.

Probes: The restriction fragments used as probes for both the Southern and Northern blot analyses were *FLO1* (2,7-kb *Eco*RI - *Eco*RV fragment), *FLO8* (2,6-kb *Hind*III fragment), *MUC1* (1,9-kb *Bam*HI - *Hin*dIII fragment), *MSS10* (1,9-kb *Eco*RI - *Bam*HI fragment) and *MSS11* (0,77kb *Pst*I fragment). DNA probes were labeled with [α^{32}P]dATP, using the Random Primed DNA Labeling kit from Boehringer Mannheim (Randburg, South Africa).

RESULTS

Pseudohyphal growth: The difference in the ability of commercial wine yeast strains to form pseudohyphae is shown in Fig. 1. The cells differ among each other according to the length of the cells and the length of the pseudohyphae formed. There seems to be a positive correlation between the length of the pseudohyphae and the length of the cells comprising these pseudohyphae (Table 1).

Cell elongation during pseudohyphal growth seems to be a characteristic inherent to certain strains and is independent of the growth medium. This is based on comparable cell lengths of a specific strain when grown on different media like grape juice agar without any nitrogen supplementation (data not shown) and SLAD media. When the strains were incubated for periods longer than 7 days on SLAD media, the pseudohyphae became covered with ellipsoidal cells, making it impossible to observe further elongation of the pseudohyphal cells.

Invasive growth: The intensity of agar penetration (invasive growth) is shown in Fig. 2. The strains can be divided into three different groups, depending on the degree of agar penetration. Strains may be incapable of agar penetration, slightly invasive or may strongly invade the agar. Not all strains showing pseudohyphal growth have the ability to penetrate the agar. Although cells of strain VIN7 (no. 19) are strongly elongated when grown under these conditions, these elongated cells did not penetrate the agar, even after 20 days of growth. Incubation for periods of 20 days did not enhance invasive growth of the cells used in this study when compared to those incubated for only 7 days. Seven days were sufficient for the evaluation of the invasive phenotype.

Differences between the morphologies of the cells growing at the top of the colony and those at the surface of the nitrogen-deficient medium (SLAD) were investigated (data not shown). Three strains of which the cells did not elongate and five strains that

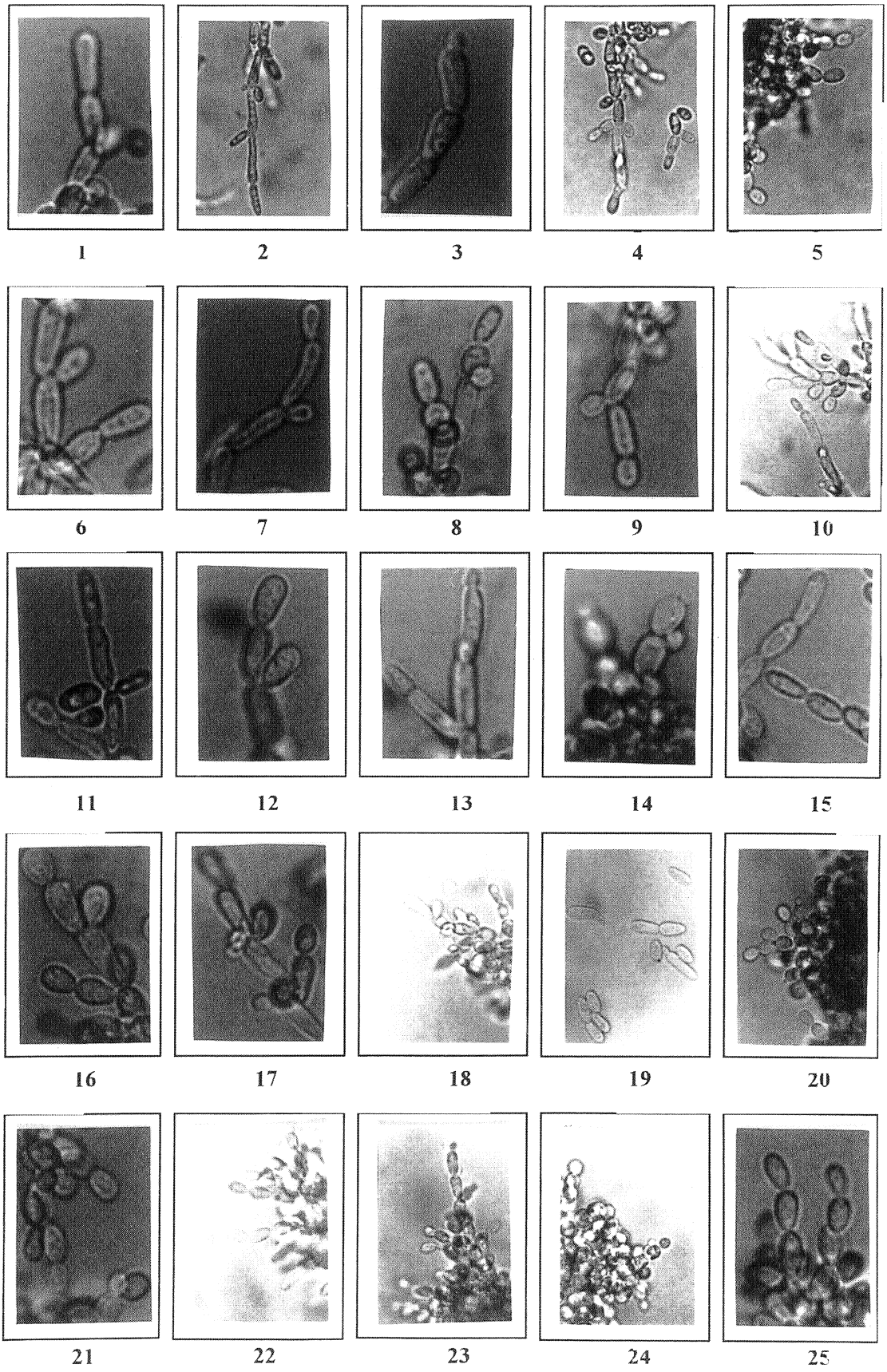

FIGURE 1
Pseudohyphal growth of 25 commercial wine yeast strains (Table 1). Cells were precultured in YPD medium for 48 h before spotted onto SLAD agar plates and incubated for 7 days at 30°C. Cells that penetrated the agar medium were photographed on the agar medium under the 40x objective. Cell lengths were measured using different cultures, but under similar culture conditions.

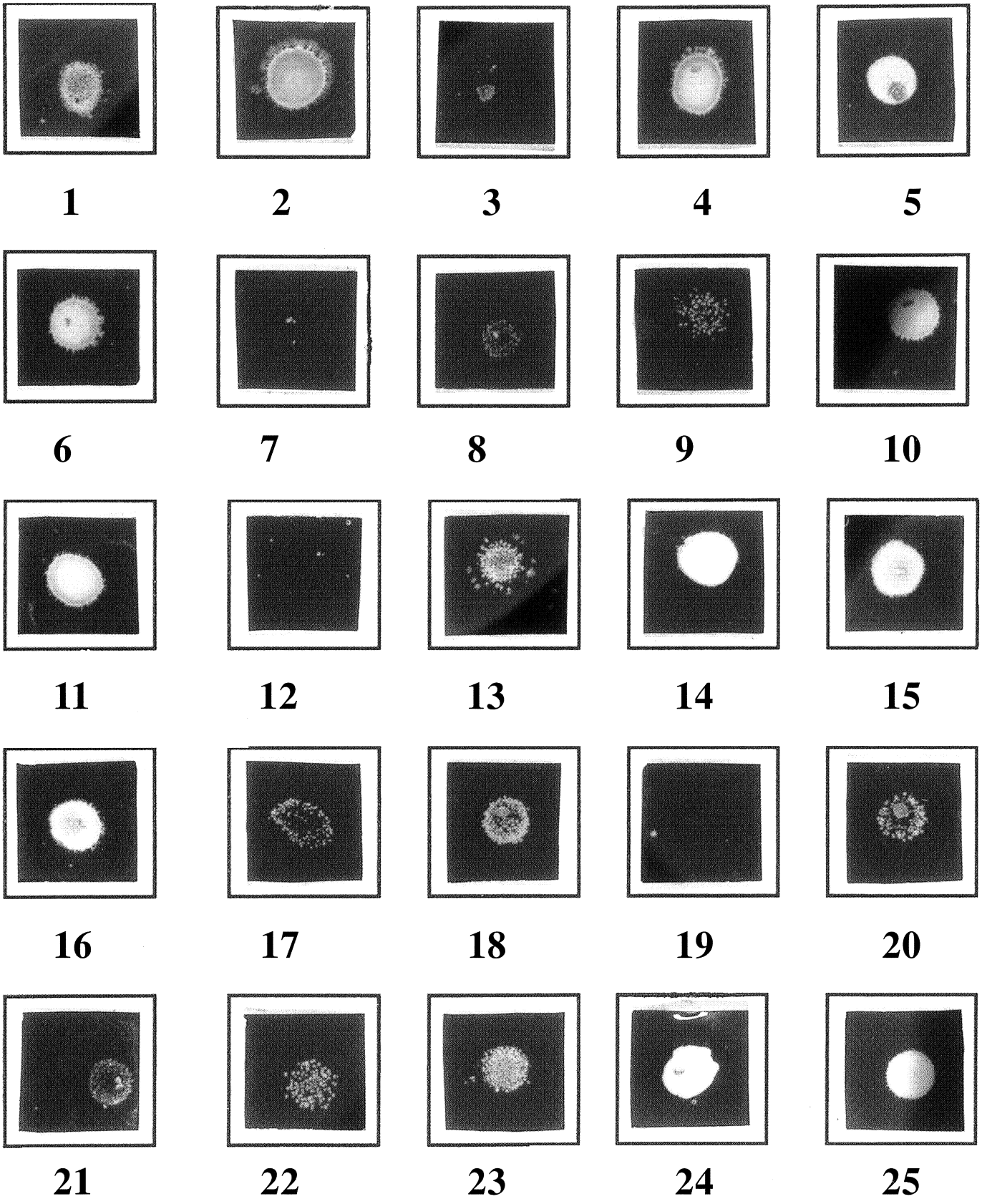

FIGURE 2

Invasive growth of 25 commercial wine yeast strains (Table 1) after 7 days of growth on a nitrogen-depleted medium (SLAD). Cells were precultured in YPD media for 48 hours before being spotted onto the SLAD agar plates. Invasive growth was assayed after 7 days by washing the cells off the surface of the agar. The remaining cells had penetrated the agar, since they could only be removed by piercing the agar with a micro-dissection needle.

formed elongated cells under these conditions were investigated. It seems that only the cells growing closest to the surface of the medium show strong cell elongation and chain formation. The cells growing at the upper surface of the colony are ellipsoidal without pseudohyphae.

Flocculation assay: To examine the ability of the wine yeasts to flocculate, cultures of each were grown in grape juice, SLAD, SCGE, SC, YPD or YPGE media containing either 0% or 1% calcium chloride. Cell aggregation was found in five of the 25 strains tested whereas only three strains flocculated when grown in YPGE medium after preculturing in a calcium-containing medium (Table 1). EDTA (250 mM) was added to the medium to determine whether the observed cell aggregation was due to true flocculation. Three of these strains, namely Uvaferm ALB (no. 14), Lalvin 016 (no. 10) and VIN13 (no. 20) showed reversible aggregation, that is true flocculation, while the other two strains, namely WE228 (no. 24) and WE14 (no. 28), did not undergo reversible aggregation in any of the media tested.

Southern blot analyses:

Flocculation Genes: Most of the commercial wine yeast strains used in this study did not flocculate in any of the conditions tested (Table 1). Therefore, these strains were investigated for the presence of the structural *FLO* genes (Fig. 3). *FLO1* was chosen as the probe, as it is the gene predominantly responsible for flocculation. The other dominant flocculation genes, namely *FLO5* and *FLO9,* show homology with the *FLO1* sequence (Teunissen *et al.,* 1995b; Teunissen & Steensma, 1995) and should also hybridize to *FLO1* under conditions of high stringency. Both the *FLO1* (Fig. 3) and *FLO8* (Fig. 4) genes are present in all of the strains tested. The *FLO1* sequence was homologous to more than one locus in the genomes of all these strains. All these different loci correlated to chromosomes known to carry *FLO* loci (Teunissen & Steensma, 1995), except in one case. This locus was present in 13 out of the 29 strains tested (the uppermost band, Fig. 3). It is noticeably absent from the four laboratory strains (strains no. 26 - 29). Using the *DAL80* gene as a chromosomal marker for chromosome XI, it was established that this additional locus is not located on chromosome XI (since *FLO10* is located on chr. XI) as was first thought (data not shown). It is therefore tempting to speculate that this might be a new locus of a gene homologous to *FLO1*. Comparing the positions of this additional locus to that of *MSS11* located on chromosome XIII, we believe this new locus is also located on chromosome XIII. The other loci visible on the blots in Fig. 3 correspond to chromosomes I, IX and VIII.

Genes involved in pseudohyphal and invasive growth: The sequences of *MUC1*, the gene involved in pseudohyphal and invasive growth and flocculation, and *MSS10* and *MSS11* (encoding two putative transcriptional activators of *MUC1*), are present in all of the strains tested (Fig. 5). In strains WE14 (no. 23) and ISP20 (no. 29) the *MSS11* gene hybridized to two loci. This was not investigated further.

Northern blot analysis: Transcription of the structural *FLO* genes and *MUC1* was investigated during growth in YPGE medium, since growth in this medium resulted in flocculation in 5 of the 25 strains tested. The *MUC1* gene was transcribed in 18 of the 25 strains when grown for 3 days in YPGE liquid medium (Fig. 6). A variation in the size of the mRNA was found. No *FLO1* or homologous (*FLO5, FLO9*) mRNA transcripts were found in any of the strains, except in strain LL14 (data not shown).

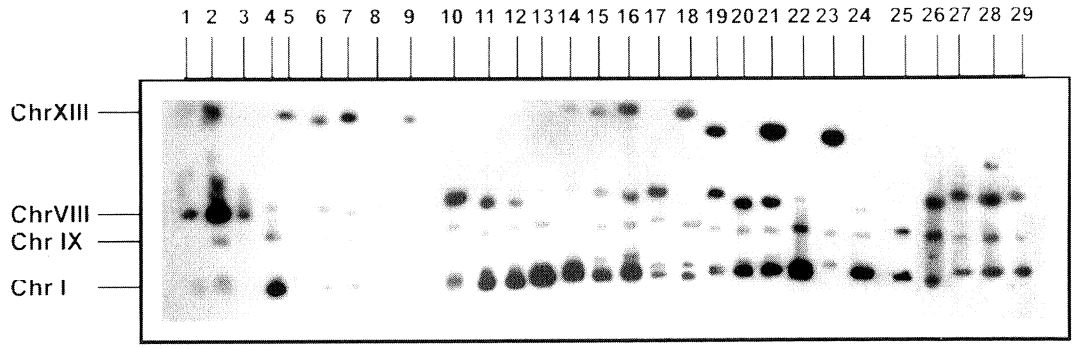

FIGURE 3

Chromoblot analysis of 25 commercial wine yeast strains and 4 laboratory strains (Table 1). The DNA of the wine yeast strains were transferred to nylon membranes and probed with a 2,7 kb *Eco*RI-*Eco*RV fragment containing a partial sequence of the *FLO1* (Chr. 1) gene, which is homologous to the other dominant flocculation genes, *FLO5* (Chr. VIII), *FLO9* (Chr. I) and *FLO10* (Chr. XI).

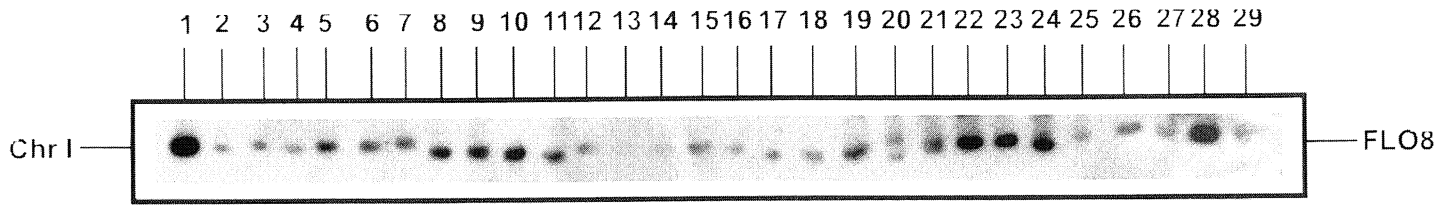

FIGURE 4

Chromoblot analysis of *FLO8* in 25 commercial wine yeast strains and 4 laboratory strains (Table 1). The DNA of these strains was transferred to nylon membranes and probed with a 1,6 kb HindIII fragment of the *FLO8* gene.

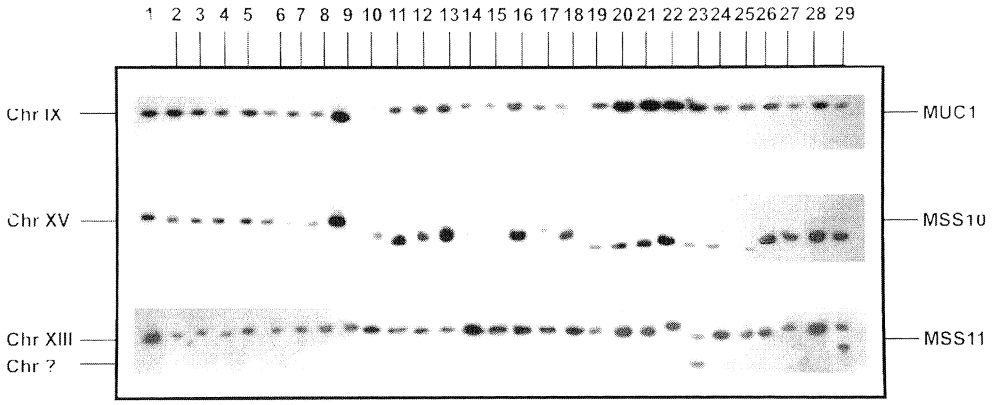

FIGURE 5

Chromoblot analysis of *MUC1*, *MSS10* and *MSS11* in 25 commercial wine yeast strains and 4 laboratory strains (Table 1). The DNA of these strains was transferred to nylon membranes and probed with *MUC1* (*Bam*HI-HindIII, 2,6 kb), *MSS10* (*Eco*RV-*Bam*HI, 1,9 kb) and *MSS11*(*Pst*1, 0,769 kb).

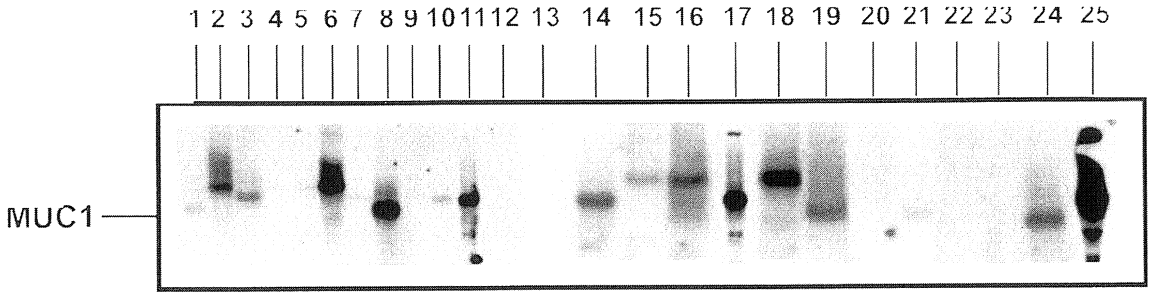

FIGURE 6

Northern blot analysis of *MUC1* in 25 commercial wine yeast strains (Table 1). Total RNA of these strains was isolated after growth in YPGE medium for 3 days. The RNA (10 µg) were separated on a formaldehyde gel and transferred onto a nylon membrane. These were probed with a 2,6 kb *Bam*HI-HindIII fragment of *MUC1*.

DISCUSSION

Flocculation, pseudohyphal development and invasive growth are genetically determined cellular responses to nutrient-limitation. The signal transduction pathways involved in these phenomena are still unclear, although several genes have been shown to be involved in these phenomena. Since it has been shown that the *MUC1*, *FLO8* and *TUP1* genes are mutual for these processes, flocculation, pseudohyphal development and invasive growth might possibly be co-regulated.

Pseudohyphal and invasive growth: Pseudohyphal and invasive growth are frequently observed under identical conditions of nutrient limitation. The SLAD medium used in this study seems to be optimal to induce these characteristics (Gimeno *et al.*, 1992). Even under these optimal conditions both these phenotypes are not expressed simultaneously in all wine yeast strains. In strains that do not show these characteristics simultaneously, at least, either pseudohyphal growth or invasive growth was observed. In all the strains tested pseudohyphae were formed under the abovementioned conditions except for VIN7 (no. 19), which formed only elongated cells. The degree of pseudohyphal formation, with regard to the length of the cells and the length of the pseudohyphae themselves, differed among the strains (Table 1) and was not dependent on the ploidy of the cells. This contradicts findings by Roberts & Fink (1994) that diploids generally form longer cells than haploids during pseudohyphal growth. According to our results, the poidy of the cells does not influence the degree of invasive growth, the length of the pseudohyphal cells or the length of the pseudohyphae (strains no. 5, 24, 16; Figs. 1 and 2). In three of these strains no invasive growth occurred, although the degree of cell elongation in these strains was high. There seems to be no direct correlation between the extent of pseudohyphal formation, the length of the pseudohypahe and the extent of invasive growth. Our results confirm those of Roberts & Fink (1994) that pseudohyphal formation and invasive growth are two different processes.

Pseudohyphal development and invasive growth were shown to be dependent on *MUC1* and its activators, *MSS10* and *MSS11* (Lambrechts *et al.*, 1996; Gagiano *et al.*, 1998). *FLO8* were previously shown to be involved in pseudohyphal development and invasive growth. *MUC1* and *FLO8* were also shown to be involved in flocculation (Liu *et al.*, 1996; Lo & Dranginis, 1996). Southern blot analyses revealed homologous sequences to these genes in all the strains tested.

Flocculation: Cells of strains aggregating in a liquid culture are not necessarily of the flocculant phenotype, since those showing pseudohyphal formation may also form clumps during growth in a liquid culture (Roberts & Fink, 1994). Agglomeration, or cluster formation, resembles true flocculation only with regard to the macroscopic structure and sedimentation

ability of the yeasts, although the settling profiles differ completely (Amory *et al.*, 1998). This was also found in the present study (data not shown). These two processes are distinguished by means of the addition of a chelating agent which removes calcium ions, imperative for flocculation but not involved in cell attachment during pseudohyphal growth, from the medium. The same strain may have the ability to choose between both these responses (Lo & Dranginis, 1996). Our results confirm these findings in strains Uvaferm ALB (no. 14), Lalvin 016 (no.10) and VIN13 (no. 20). These strains have the ability to either flocculate or form pseudohyphae under specific growth conditions.

Flocculation is influenced by the genetic background of the host as well as environmental factors, e.g. pH, the sugar content, agitation, aeration and Ca^{2+}. The absence of flocculation could be due to the absence of a flocculation gene(s), by inhibition of transcription and translation, or by post translational modification (Teunissen *et al.*, 1995a). In a natural fermentation, environmental factors are not always controllable, e.g. the decrease in pH during fermentation due to sugar metabolism may decrease the flocculation ability of the strains used. Such naturally occurring phenomena are major determinants for the appearance of flocculation. Therefore, although the genetic composition of yeast strains may indicate a flocculent phenotype, not all such strains flocculate.

Structural genes of the *FLO1* phenotype are ubiquitous in *S. cerevisiae* strains, but are only expressed under certain environmental conditions (Stratford, 1992a) and are not transcribed in non-flocculent strains (Teunissen *et al.*, 1995a). Except for three strains [Uvaferm ALB (no. 14), Lalvin 016 (no. 10) and VIN13 (no. 20)], none of the strains examined in this study is able to flocculate under the conditions tested. It was possible to induce five of the strains [Uvaferm ALB (no. 14), VIN13 (no. 20), Lalvin 016 (no. 10), WE14 (no. 23), WE228 (no. 24)] to aggregate to a limited degree after five days of growth in YPGE. Flocculation of strains Uvaferm ALB (no. 14), VIN13 (no. 20) and Lalvin 016 (no. 10) was reversed by the addition of 0,25 M EDTA, but not in the other strains. These three strains therefore show true flocculation. Aggregation of the other two strains [WE14 (no. 23) and WE228 (no. 24)] might be due to clumpy growth found with some pseudohyphal strains (Stewart & Russell, 1981).

Strain VIN13 (no. 20) could be induced to flocculate in YPGE medium to a limited degree only after being precultured in YPD containing 0.1% calcium chloride. The inability of this strain to flocculate in different growth media and wine might be ascribed to environmental conditions which may influence the expression of the specific genes, rather than the presence of possible suppressor genes. In the other strains where flocculence was not inducible in any medium, the phynotype might be ascribed to the presence of suppressor genes, such as *TUP1* and *CYC8*, that have previously been shown to repress transcription of the flocculation genes.

All the strains tested possess at least one flocculation gene. No correlation exists between the number of loci homologous to the *FLO1* gene present in the genome of a strain and the expression of the flocculent characteristic. Although the *FLO1* gene is present in all of the strains tested, only two strains, namely Uvaferm ALB (no. 14) and Lalvin 016 (no. 10), flocculated spontaneously in a variety of media. It is unknown whether the genes identified by Southern blot hybridization are intact or not. A possible chromosomal polymorphism exists on chromosome I (strains 15 to 18, Fig. 3), because of the double band present in these strains.

Northern blot analyses revealed *FLO1* mRNA in only strain Uvaferm ALB (no. 14), a constitutive flocculating strain. A possible explanation could be that one of the less homologous genes, *FLO10* or pseudogenes could be responsible for flocculation. We would therefore be unable to detect such genes with our probe or the conditions used in this study for probing.

In this study a liquid culture was used to investigate the expression of *MUC1*. Although this gene was said to be critical for flocculation (Lo & Dranginis, 1996), strains in which this gene was expressed did not flocculate under conditions used in this study. This might be due to the absence of transcription of a *FLO* gene in these strains (data not shown). Expression of *MUC1* alone can therefore not induce flocculation. We therefore suggest that *MUC1*, as opposed to *FLO11*, be retained as the most appropriate designation of this gene encoding the *S. cerevisiae* mucin-like protein.

It has been shown by Lambrechts *et al.* (1996) that the *MUC1* gene contains numerous repeats encoding sequences rich in serine and threonine residues. Possible differences in the number of repeat sequences might explain the variation in the size of the mRNA transcripts seen in the Northern blots.

Another gene found to be involved in flocculation, pseudohyphal development and invasive growth is *FLO8* (Liu *et al.*, 1996), the transcriptional activator of the *FLO1* gene (Kobayashi *et al.*, 1996). Our results do not clarify the possible link between flocculation, pseudohyphal development and invasive growth, although the genes *MUC1*, *FLO8* and *TUP1* are involved in these processes. Investigation of the effect of *FLO8*, *MSS10* and *MSS11* on genes involved in flocculation, pseudohyphal differentiation and invasive growth is therefore essential. It is also not yet clear what the influence of the expression of these genes might be on the performance of the yeasts during fermentation and on wine quality.

In conclusion, in order to construct wine yeast strains that are able to flocculate at the appropriate time during wine fermentations, the structural genes involved in flocculation will have to be deregulated, for example, exchanging the promoters of the structural genes (*MUC1*, *FLO1*, etc.) with a promoter that is switched on during the late stationary phase of growth.

LITERATURE CITED

AMORY, D.E., ROUXHET, P.G. & DUFOUR, J.P., 1988. Flocculence of brewery yeasts and their surface properties: chemical composition, electrostatic charge and hydrophobicity. *J. Inst. Brew.* **94,** 79-84.

BANUETT, F., 1998. Signalling in the yeasts: An informational cascade with links to the filamentous fungi. *Microbiol. Mol. Biol. Rev.* **62,** 249-274.

BRAUN, B.R. & JOHNSON, A.D., 1997. Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1. *Science* **277,** 105-109.

BLACKETER, M.J., MADUALE, P. & MYERS, A.M., 1995. Mutational analysis of morphologic differentiation in *Saccharomyces serevisiae*. *Genetics* **140,** 1259-1275.

BOWEN, W.R. & VENTHAM, T.J., 1994. Aspects of yeast flocculation. Size distribution and zeta-potential. *J. Inst. Brew.* **100,** 167-172.

CARLE, G.F. & OLSON, M.V., 1985. An electrophoretic karyotype for yeast. *Proc. Natl. Acad. Sci. USA* **82,** 3756-3760.

GAGIANO, M.,VAN DYK, D., BAUER, F.F., LAMBRECHTS, M.G. & PRETORIUS, I.S., 1998. Msn1p/Mss10p, Mss11p and Muc1p/Flo1p are part of a signal transduction pathway downstream of Mep2p regulating invasive growth and pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Mol. Microbiol* (In press).

GIMENO, C.J. & FINK, G.R., 1992. The logic of cell division in the life cycle of yeast, *Science* **257,** 626.

GIMENO, C.J., LJUNGDAHL, P.O., STYLES, C.A. & FINK, G.R., 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: Regulation by starvation and RAS. *Cell* **68,** 1077-1090.

GIMENO, C.J., LJUNGDAHL, P.O., STYLES, C.A. & FINK, G.R., 1993. Characterization of *Saccharomyces cerevisiae* pseudohyphal growth. In: Van den Bossche, H., Odds, F.C. and Kerridge, D. (eds) Dimorphic fungi in bilogy and medicine, Plenum Press, New York and London, pp. 83-103.

JOHNSTON, J.R. & READER, H.P., 1983. Genetic control of flocculation. In: Spencer. J.F.T., Spencer, D.M. & Smith, A.R.W. (eds) Yest Genetics: Fundamental and applied aspects. Springer-Verlag, new York Inc. pp. 205-224.

KOBAYASHI, O., SUDA, H., OHTANI, T. & SONE. H., 1996. Molecular cloning and analysis fo the dominant flocculation gene *FLO8* from *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **251,** 707-715.

LAMBRECHTS, M.G., BAUER, F.F., MARMUR, J. & PRETORIUS, I.S., 1996. Mucl, a mucin-like protein that is regulated by Mss10, is critical for pseudohyphal differentiation in yeast. *Proc. Natl. Acad. Sci. USA* **93,** 8419-8424.

LIU, H., STYLES, C.A. & FINK, G.R., 1996. *Saccahromyces cerevisiae* S288C has a mutation in *FLO8*, a gene required for filamentous growth. *Genetics* **144,** 967-978.

LO, W.-S. & DRANGINIS, A.M., 1996. *FLO11*, a yeast gene related to the *STA* genes, encodes a novel cell surface flocculin. *J. Bacteriol.* **178,** 7144-7151.

LORENZ, M.C. & HEITMAN, J., 1997. Yeast pseudohyphal growth is regulated by GPA2, a G protein x homolog. *EMBO J* **16,** 7008-7018.

MADHANI, H.D. & FINK, G.R., 1997. Combinatorial control required for the specificity of yeast MAPK signaling. *Science* **275,** 1314-1317.

MÖSCH, H-U. & FINK, G.R., 1997. Dissection of filamentous growth by transposon mutagenesis in *Saccharomyces cerevisiae*. *Genetics* **145,** 671-684.

ROBERTS, R.L. & FINK, G.R., 1994. Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genes Dev.* **8,** 2974-2985.

RUSSEL, I., STEWART, G.G., READER, H.P., JOHNSTON, J.R. & MARTIN, P.A., 1980. Revised nomenclature of genes that control yeast flocculation. *J. Inst. Brew.* **86,** 120-121.

SAMBROOK, J., FRITSCH, E.F., MANIATIS, T., 1989. Molecular Cloning. A laboratory manual, 2nd edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

SIEIRO, C., LONGO, E., CANSADO, J., VELáZQUEZ, J.B., CALO, P., BLANCO, P. & VILLA, T.G., 1993. Genetic evidence of a new flocculation suppressor gene in *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **112,** 25-30.

SMIT, G., STRAVER, M.H., LUGTENBURG, B.J.J. & KIJNE, J.W., 1992. Flocculence of *Saccharomyces cerevisiae* cells is induced by nutrient limitation, with cell surface hydrophobicity as a major determinant. *Appl. Enrivon, Microbiol.* **58,** 3709-3714.

STEWART, G.G. & RUSSELL, I., 1981. Yeast flocculation. In: POLLOCK, J.R.A. (ed.) Brewing Science, vol. 2 Academic Press, London. pp. 61-89.

STRATFORD, M., 1992a. Yeast flocculation: Reconciliation of physiological and genetic viewpoints. *Yeast* **8,** 25-38.

STRATFORD, M., 1992b. Yeast flocculation: A new perspective. *Adv. Microbiol Physiol.* **33,** 1-71.

STRAVER. M.H., VAN DEN AAR, P.C., SMIT, G. & KIJNE, J.W., 1993. Determinants of flocculence of brewer's yeast during fermentation in wort. *Yeast* **9,** 527-532.

TEUNISSEN, A.W.R.H. & STEENSMA, H.Y., 1995. Review: The dominant flocculation genes of *Saccharomyces cerevisiae* constitute a new subtlelomeric gene family. *Yeast* **11,** 1001-1013.

TEUNISSEN, A.W.R.H., VAN DEN BERG, J.A. & STEENSMA, H.Y., 1995a. Transcriptional regulation of flocculation genes in *Saccharomyces cerevisiae*. *Yeast* **11,** 435-446.

TEUNISSEN, A.W.R.H., VAN DEN BERG, J.A. & STEENSMA, H.Y., 1995b. Localization of the dominant flocculation genes *FLO5* and *FLO8* of *Saccharomyces cerevisiae*. *Yeast* **11,** 735-745.

VEZINHET, F., BLONDIN, B. & BARRE, P., 1991. Mapping of the *FLO5* gene of *S. cerevisiae* by transfer of a chromosome during cytoduction. *Biotehcnol. Lett.* **13,** 47-52.

VIVIER, M.A., LAMBRECHTS, M.G. & PRETORIUS, I.S., 1997. Co-regulation of starch degradation and dimorphism in the yeast *Saccharomyces cerevisiae*. *Crit. Rev. Biochem, Mol. Biol.* **32,** 405-435.

WEBBER, A.L., LAMBRECHTS, M.G. & PRETORIUS, I.S., 1997. *MSS11,* a novel yeast gene involved in the regulation of starch metabolism. *Curr. Genet.* **32**, 260-266.

WILCOCKS, K.L. & SMART, K.A., 1995. The importance of surface charge and hydrophobocity for the flocculation of chain-forming brewing yeast strains and resistance of these parameters to acid washing. *FEMS Microbiol. Lett.* **134,** 293-297.