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Cloning and analysis of a *FLO5* flocculation gene from *S. cerevisiae*

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Abstract. A yeast flocculation gene was isolated from a genomic library of an FLO5 strain of *S. cerevisiae* on the basis of its ability to trigger flocculation in a non-flocculent strain. Characterization of the cloned gene by restriction mapping, Southern analysis, and chromosome mapping have shown that it corresponds to a *FLO5* gene previously located on chromosome I and that this gene is related to the already described *FLO1* gene. A study of gene expression in different yeast strains has indicated that, while this gene is dominant, its expression can be suppressed in some genetic backgrounds. A Northern-blot analysis has demonstrated that the same 5000-nt transcript was present in an *FLO5* and an *FLO1* strain. A gene disruption experiment has led to the conclusion that another flocculation gene is present and can be active in the FLO5 strain we used.

Key words: Yeast – Flocculation – Cloning – Expression

Introduction

Yeast flocculation describes a non-sexual aggregation of the cells which is calcium dependent and reversible. This phenomenon is of considerable interest for some industrial fermentations because it leads to an efficient separation of the yeast cells from the fermenting medium. The generally accepted mechanism of flocculation involves a protein-sugar interaction between a specific cell-surface protein and mannan of the cell wall (Miki et al. 1982; Stratford 1992). Several genes, *FLO1*, *FLO5* (Johnston and Reader 1983) and *FLO8* (Yamashita and Fukui 1984), have been described as dominant flocculation genes, while some pleiotropic mutations like *TUP1* or *CYC8* (Lipke and Hull-Pillsbury 1984) are also known to give rise to flocculence. The dominant flocculation gene *FLO1* is the best characterized and has been cloned by Watari et al. (1989) and Teunissen and Steensma (1990). FLO5 flocculation has received less attention, the corresponding gene has resisted several attempts at mapping by classical approaches but an *FLO5* gene has recently been located on chromosome I by cytoduction (Vezinhet et al. 1991). FLO5 and FLO1 types of flocculation have been distinguished on the base of differences in flocculation intensity or resistance to various treatments (Hodgson et al. 1985) but the genetic basis of these differences has not been established. Cloning the different genes which confer flocculation will allow one to determine the relationships between these genes and will provide new tools to investigate the flocculation mechanism. This paper reports the cloning and analysis of an *FLO5* gene which is related to *FLO1* and is implicated in the flocculation of a FLO5 strain.

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Materials and methods

Strains and media. The *Saccharomyces cerevisiae* strains used in this study are presented in Table 1. The flocculent strain 17-13D is derived from STX 347-1D (YGSC). The V5 strain was used as the standard strain for transformations. Yeast were cultivated as described in Sherman (1991). *E. coli* strain DH5α was used for cloning experiments and plasmid preparation. *E. coli* was grown in LB medium supplemented when necessary with ampicillin (50 μg/ml).

Transformation methods. E. coli transformation was carried out by the $CaCl_2/RbCl_2$ method. Transformation of yeast was performed using either the LiCl procedure (Ito et al. 1983) or the protoplast method (Burgers and Percival 1987).

DNA-RNA manipulation and cloning techniques. Restriction enzymes and modifying enzymes were used according to the manufacturer's instructions. Chromosome block preparations and pulsed-field gel electrophoresis were carried out as described by Vezinhet et al. (1990). Yeast DNA was prepared by the method of Cryer et al. (1975) with minor modifications. *E. coli* plasmid DNA was prepared using standard protocols (Sambrook et al. 1989). RNA was extracted and enriched in poly $(A)^+$ following the procedure described by Köhrer and Domdey (1991). Southern and Northern blots and DNA labelling were performed according to the supplier's instructions.

Table 1. Yeast and *E. coli* strains used in this study

S. cerevisia strains	Genotype	Origin or reference
17-13D	*Matα leu2 ade1 FLO5*	IPV-INRA
ABXL-1D	*Mata FLO1*	YGSC
BX24-2B	*Matα FLO1*	YGSC
V5	*Mata ura3*	IPV-INRA
YPH250	*Mata ura3 lys2 ade2 leu2 trp1 his3*	Sikorski and Hieter (1989)
YPH252	*Matα ura3 lys2 ade2 leu2 trp1 his3*	Sikorski and Hieter (1989)
YPH274	*Mata/α ura3 lys2 ade2 leu2 trp1 his3*	Sikorski and Hieter (1989)
OL1	*Mataα leu2 his3 ura3*	Laboratoire de Génétique Université Paris Sud
E. coli strain		
DH5α	F⁻ (Y80 *lac Z* MIS), *Δ(lac ZYA-argF)* B169, *recA1, end A1, nsd R17, sup E44, λthi-1, gyr A, rel A1*	Gibco BRL

Genomic library constitution. A genomic library of the flocculent strain 17-13D was constituted in the yeast-*E. coli* shuttle plasmid YCp50. The DNA was partially digested by *Sau*3A, fractionated on a 10–40% sucrose gradient and ligated to the dephosphorylated plasmid. The library was amplified by transformation of *E. coli* and extraction of the plasmid pool.

Plasmid constructions and subcloning. The plasmid pou27 isolated from the YCp50 library of the strain 17-13D is displayed in Fig. 1. The pouBis plasmid was obtained by a *Hin*dIII digestion of the pou27 plasmid and religation. The 5.5-kb *Bam*HI-*Hin*dIII fragment was then subcloned in pRS316 (Sikorski and Hieter 1989) to give pRSFLO5 and in YEp352 to give YEpFLO5.

Gene disruption procedure The one-step gene disruption technique was used (Rothstein 1983). The *Sal*I-*Bgl*II fragment of the *LEU2* gene was inserted in place of the 1.5-kb *Pvu*II-*Bgl*II *FLO5* fragment of YEpFLO5. An *Eco*RI site in association with the multiple cloning sites is present at the left junction of *FLO5-LEU2*. The 6.5-kb *Bam*HI–*Hin*dIII fragment of the plasmid pFLO5ΔLEU2 was purified and used to transform the strain 17-13D.

Flocculation assays. To screen the library for the acquisition of flocculation the clones were cultivated in 250 µl of YEPD medium in microtitration plates. After 2 days at 28°C a 2-mm glass bead was added into each microcuvette, plates were agitated for 10 s and then examined. Tests of sensitivity of flocculation towards heat and chymotrypsin treatments were performed as described by Hodgson et al. (1985).

Results

Cloning of an FLO5 gene

The genomic library of the FLO5 strain 17-13D constructed in YCp50, as described in Materials and methods, was used to transform the non-flocculent strain V5. The latter strain was chosen because previous cytoduction experiments had demonstrated that it could efficiently express flocculation after the transfer of a single chromosome (Vezinhet et al. 1990). Five-thousand Ura + transformants were screened for flocculation acquisition and one flocculent clone was isolated. The recombinant plasmid pou27 responsible for the flocculent character carried a 12-kb insert whose partial restriction map is displayed in Fig. 1. The cloned fragment was able to trig-

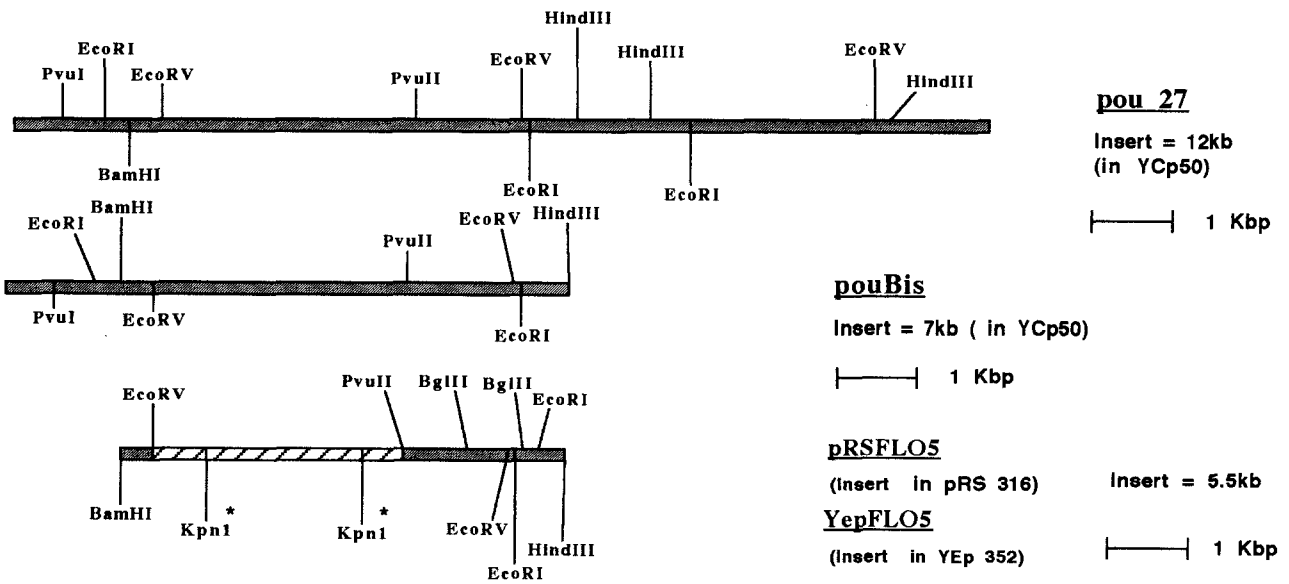


Fig. 1. Restriction maps of the constructions used in this study. * denotes that other *Kpn*I sites are present between the two corresponding sites but have not been mapped. The hatched region defined for the pRSFLO5 insert differs in size from the *FLO1* gene map reported by Watari et al. (1989)

ger a strong flocculation in the tester strain V5 as shown in Fig. 2. The ability to induce an efficient flocculation was conserved in the three different constructions presented, pouBis, pRSFLO5 and YEpFLO5. The flocculation induced was intense with any of the centromere-based plasmids used but was increased with the multicopy plasmid YEpFLO5. It was confirmed that the induced flocculation was inhibited by EDTA, and restored by adding Ca^{++} ions. The restriction map of the *Bam*HI-*Hin*dIII fragment of pRSFLO5 displays striking similarities with the *FLO1* gene restriction maps published by Watari et al. (1989) and Teunissen et al. (1990). The main difference is found in the region defined by the *Eco*RV and *Pvu*II restriction sites, which is 1.7 kb larger in the present case.

Structural relationships between FLO5 and FLO1

We first checked, by Southern analysis, that the cloned gene had not been modified during the cloning procedure. An *FLO5* probe was used to hybridize simultaneously the digested genomic DNA of the FLO5 strain 17-13D and the digested plasmid pou27. Fig. 3 A shows that one of the signals (*Bam*HI-*Eco*RI = 4.9 kb) observed with the FLO5 strain is identical to that signal displayed by the plasmid pou27. Thus, the cloned form of the *FLO5* gene seems to be identical to the naturally occurring one in the FLO5 strain.

In order to gain more information about the structural relationships between the *FLO5* and *FLO1* genes we have performed a Southern analysis with different strains. As shown in Fig. 3 B the strong hybridization signal observed at 4.9 kb on the *Bam*HI-*Eco*RI digest of the FLO5 strain is also present in the two FLO1 strains analysed. No signal which could correlate with the restriction map of *FLO1*, previously reported by Watari et al. (1989), is observed with the FLO1 strains. Recently, Teunissen et al. (1993 a) have shown that their cloned *FLO1* gene was incomplete and deleted for a fragment of 1.8 kb. Thus it is probable that the observed signal corresponds to the *FLO1* gene. Therefore, the FLO5 and FLO1 strains bear

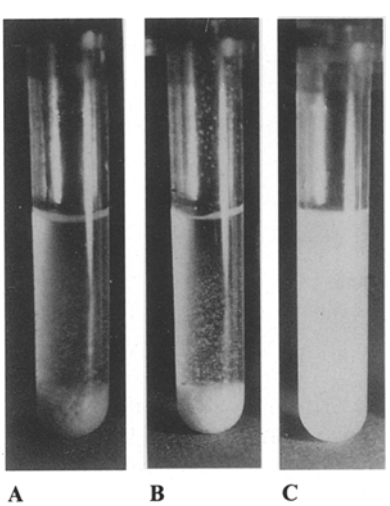


Fig. 2. Flocculation of the strains 17-13D (*A*), V5 transformed with pou27 (*B*), V5 non-transformed (*C*)

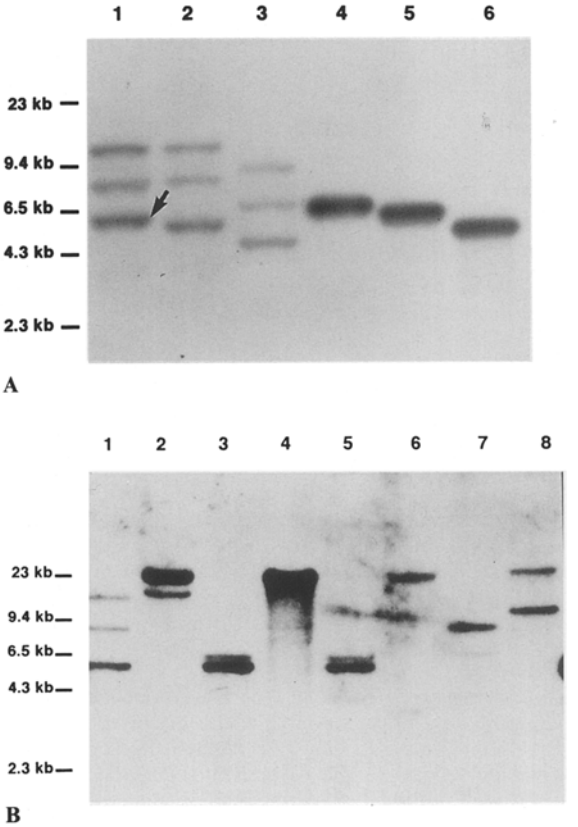


Fig. 3 A, B. Southern-blot analysis. **A** DNA from the strain 17-13D (*lanes 1, 2, 3*) and the plasmid pou27 (*lanes 4, 5, 6*) was digested by *Eco*RI (*lanes 1, 4*), *Bam*HI + *Eco*RI (*lanes 2, 5*), or *Bam*HI + *Bgl*II (*lanes 3, 6*) and hybridized with the 4.9-kb fragment of the pouBis plasmid. **B** DNA from the strains 17-13D (*lanes 1, 2*), ABXL-1D (*lanes 3, 4*), BX24-2B (*lanes 5, 6*) and OL1 (*lanes 7, 8*) was digested with *Bam*HI (*lanes 2, 4, 6, 8*) or *Bam*HI + *Eco*RI (*lanes 1, 3, 5, 7*). The probe used was the 5.3-kb *Eco*RI fragment of the pouBis plasmid

genes which are structurally related and we assume that we have cloned the *FLO5* gene intact. We find that flocculent strains possess other sequences sharing homology with the *FLO5* gene and that related sequences are also present in a non-flocculent strain.

Mapping of the FLO5 gene

Mapping of the cloned *FLO5* gene was performed by hybridization to a blot of pulsed-field-gel-electrophoresis-fractionated chromosomes. Figure 4 shows that the *FLO5* gene is located on chromosome I as reported for *FLO1*. This mapping is in good agreement with the previous mapping of the *FLO5* gene on this chromosome by cytoduction (Vezinhet et al. 1991). It can be observed that sequences homologous to the *FLO5* gene exist on chromosome VIII (the doublet V-VIII is resolved in the strain A364A). This may be correlated with the existence of an *FLO8* gene mapped on this chromosome by Yamashita and Fukui (1984) in a strain of *S. diastaticus*. It must be mentioned that the same distribution of signals on chromosomes I and VIII is observed in all the strains tested independent of their type or their ability to flocculate.

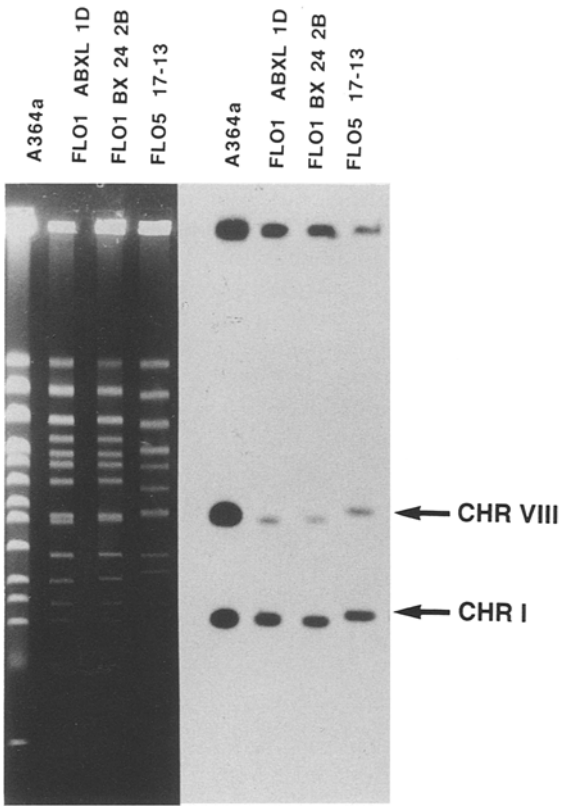


Fig. 4. Chromosome mapping of the *FLO5* gene. The probe used was the 5.3-kb *Eco*RI fragment of the poubis plasmid

Table 2. Phenotypic analysis of the flocculation induced by the pou27 plasmid

Strain	Initial flocculation grade	Flocculation grade after treatment	
		Heat	α-chymotrypsin
17 13D (FLO5, STX 347-1D derived)	4	1	4
ABXL1-D (FLO1)	3	3	0
V5 transformed with pou27	3	2	1

Table 3. Induction of flocculation with different constructions in several yeast strains (+, flocculation; –, no flocculation)

Strain	Plasmid		
	pou27	pouBis	pRSFLO5
V5	+	+	+
YPH250	–	–	+
YPH252	–	–	+
YPH274	–	–	+

Phenotype characterization

We have attempted to characterize the flocculation phenotype according to the method of Hodgson et al. (1985). They reported that the FLO5 and FLO1 phenotypes could be distinguished on the basis of their behaviour towards chymotrypsin treatment and heat treatment. FLO1 cells are supposed to be heat resistant and chymotrypsin sensitive while FLO5 cells are chymotrypsin resistant and heat sensitive. This assay was performed on the V5 strain bearing the pou27 plasmid. As shown in Table 2 the transformed yeast does not exhibit a clear FLO5 or FLO1 phenotype but rather an intermediate one more similar to FLO1. It is heat resistant and partially chymotrypsin resistant. The significance of this assay is doubtful and Watari et al. (1991) have observed that results can vary with strains when transformed with the *FLO1* gene. The genetic background is assumed to influence the behaviour of the yeast towards these treatments.

Regulation of FLO5 expression

We have checked some laboratory strains for their ability to express flocculation after the introduction of the *FLO5* gene. The different constructions presented in Fig. 1 were used to transform the strains YPH250, YPH252, YPH274 and V5. Results summarised in Table 3 show that strains of the YPH series do not express flocculation with two of the plasmids we used, while the V5 control strain expresses it with all three constructions. This demonstrates that a repression of *FLO5* gene expression occurs in YPH strains. The regulation is abolished in the pRSFLO5 construction because the target sequence for this regulation has been eliminated. Preliminary results of sequence analysis (Bidard et al., unpublished) suggest that the *Bam*HI site lies within the promoter region. Thus a target site for regulation is located in the 5′ region of the *FLO5* gene. These results demonstrate that regulation of *FLO5* gene expression can vary with the genetic background of the yeast and that strains carry a regulator of flocculation acting probably at the transcriptional level.

Northern analysis

A Northern analysis was performed on mRNA extracted from stationary-phase grown FLO5 and FLO1 strains. Results presented in Fig. 5 show that both strains display the same transcript estimated at 5000 nt when probed with an *FLO5* fragment. No transcript is detected in the mRNA of the non-flocculent strain V5 when analysed with the same *FLO5* probe. *LEU2* being a moderately expressed gene, the ratio of intensity of the *FLO5* signal versus that of *LEU2* indicates that the *FLO5* transcript is relatively abundant. It is likely that the transcript observed in the FLO1 strain corresponds to the *FLO1* gene transcript which Teunissen et al. (1992) have estimated at a slightly lower size (4500 nt).

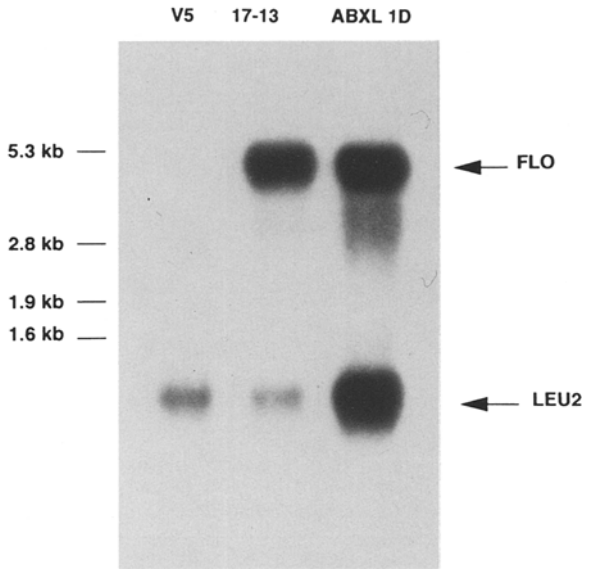


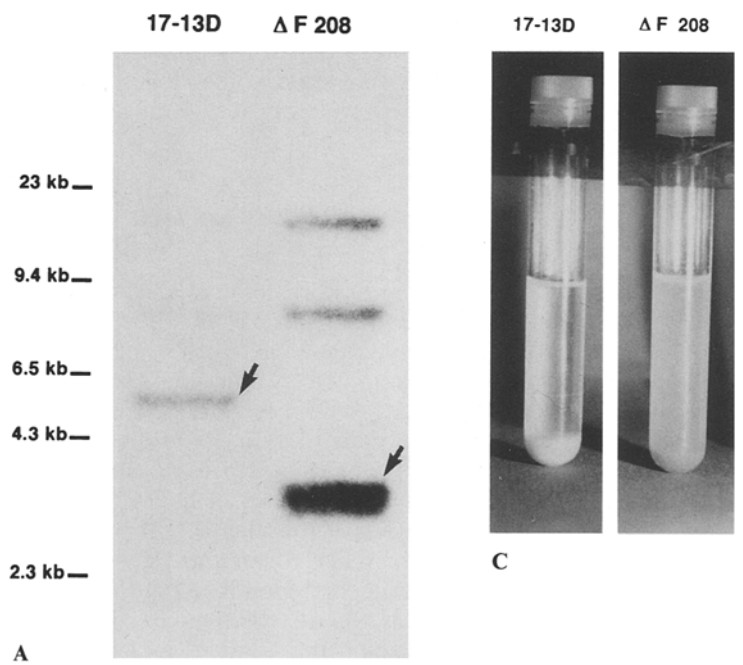
Fig. 5. Northern-blot analysis of the transcripts in the strains 17-13D, ABXL-1D and V5. The *FLO5* probe was the 2.1-kb *Kpn*I-*Eco*RI fragment from the pRSFLO5 plasmid. The *LEU2* probe was the 2.2-kb *Xho*I-*Sal*I fragment of the *LEU2* gene. Transcript size was estimated with the RNA molecular weight marker II from Boehringer

Gene disruption

In order to assess the role of the cloned gene in the flocculation of the FLO5 strain, a gene disruption experiment was performed. The *Bam*HI-*Hin*dIII fragment of the pFLO5ΔLEU2 construction was used to disrupt the *FLO5* gene as described in Materials and methods. Correct replacement of the chromosomal copy by the disrupted one was checked by Southern analysis and is presented in Fig. 6. Surprisingly, the clones disrupted for the *FLO5* gene, like ΔF208, display only a slight decrease in flocculation intensity as shown in Fig. 6C. It was verified that the disrupted construction pFLOΔ5LEU2 (placed on the multicopy plasmid YEp352) could not induce any flocculation in the V5 strain. Therefore, the remaining high level of flocculence must be trigged by another flocculation gene. To test this hypothesis we crossed the flocculent strain (17-13D) and the disrupted clone ΔF208 with the tester strain V5 and analysed the segregation of flocculation in the progeny. Results presented in Table 4 show that the segregants from the cross (17-13D × V5) exhibit an excess in flocculent clones (63%). A lower level of flocculent clones is found in the progeny of the cross (ΔF208 × V5) which reaches 45%. These results are compatible with the presence of two flocculation genes in strain 17-13D and one flocculation gene in ΔF208. The lack of recombined ditype (4+ : 0−) in the progeny of the cross 17-13D × V5 suggests that the two flocculation genes are linked. Thus the second flocculation gene may also map on chromosome I.

Discussion

Several lines of evidence indicate that the flocculation gene we cloned, while isolated from an FLO5 strain, is structurally similar to the *FLO1* gene. These two genes map on chromosome I, display the same transcript, and their restriction maps share obvious similarities. As indicated by Southern analysis, it is likely that the differences in the restriction maps of these two genes result mainly from a cloning artefact of the *FLO1* gene. From this point of view, the present paper reports the first cloning of a complete flocculation gene from *S. cerevisiae*. Recently Teunissen et al. (1993b) have obtained the partial nucleotide sequence of the *FLO1* gene and have con-



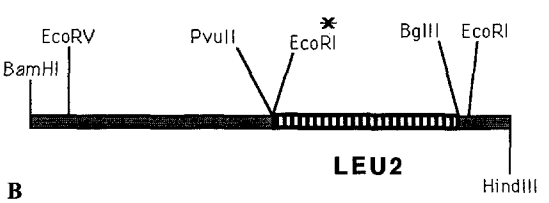


Fig. 6. Gene disruption of the *FLO5* gene. **A** Southern analysis of the wild-type and a *FLO5* disruptant. Total DNA was digested with the *Eco*RI restriction enzyme and probed with the 2.1-kb *Kpn*I-*Eco*RI fragment. **B** the pFLO5ΔLEU2 construction used to perform the one-step gene disruption.* denotes a created site. **C** flocculation of the wild-type FLO5 strain 17-13D and the disruptant ΔF208

Table 4. Tetrad analysis of crosses 17-13D × V5 and ΔF208 × V5

Cross	Number of tetrads analysed	Number of tetrads with ratio flocculent (+): :non flocculent (−)					Total + segregants	% Flocculent segregants
		4+:0−	3+:1−	2+:2−	1+:3−	0+:4−		
17-13D × V5	13	0	7	6	0	0	33	63
ΔF208 × V5	11	0	1	7	3	0	20	45

firmed that an internal part of the gene was missing in their isolate. Preliminary results in the nucleotide-sequence analysis of the *FLO5* gene (Bidard et al., unpublished) indicate that the *FLO5* and *FLO1* genes are very similar. Complete sequence analysis of the *FLO5* and *FLO1* genes will reveal the exact extent of homology between them.

Results obtained on the regulation of *FLO5* expression emphasize the role of the genetic background of the yeast in respect of flocculation behaviour. The observed regulation obviously differs from the mating-type control of flocculation reported by Watari et al. (1990). The existence of suppressors of flocculation is well-known (Johnston and Reader 1982) but their mode of action is still not understood. The availability of a cloned gene will allow the identification of the target sequences and will aid in resolving the mechanism of this regulation.

Gene disruption experiments have revealed that probably two flocculation genes are present in the FLO5 strain we used. This does not seem to be the case for the FLO1 strains since gene disruption of the *FLO1* gene led to a complete loss of flocculation (Teunissen et al. 1990). The fact that the disruptant clones display a high level of flocculence, does not allow one to conclude that the remaining gene plays a major role in FLO5 flocculation since the regulation of this gene may have been modified by the disruption of the first *FLO5* gene. Other investigations will be necessary to assess the exact contribution of each gene to overall FLO5 flocculation. The presence of two genes, perhaps subject to different regulation, may explain the difficulties encountered in mapping the *FLO5* gene by a classical genetic approach. The view we have of FLO5 flocculation is that it results from the expression of two genes and that one of them is highly similar to *FLO1*. In this respect, it would be interesting to check whether this situation is common to all FLO5 strains.

An unresolved question in the understanding of flocculation genetics is whether the *FLO* genes are structural or regulatory (Stratford 1992). Present data do not provide an answer but we can assume that the *FLO5* gene acts rather directly because flocculation intensity is dependent on the *FLO5* gene copy number. Moreover, information obtained elsewhere in a sequence analysis of these genes (Bidard et al., unpublished) and of *FLO1* (Teunissen et al. 1993 b) suggests that *FLO* genes encode for cell-surface proteins. Thus *FLO1/5* genes are probably the structural genes for flocculation. As indicated by Southern analysis, sequences related to *FLO5* are relatively widespread in the yeast genome and are present even in non-flocculent strains. In this case we can hypothesize that they correspond to silent copies of *FLO1/5* genes which are switched on under certain circumstances, such as mutation of *TUP1* or *CYC8*, which induce flocculation.

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