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Flocculation of industrial and laboratory strains of *Saccharomyces cerevisiae*

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SUMMARY

A comparative study has been made of different laboratory and industrial wild-type strains of *Saccharomyces cerevisiae* in relation to their flocculation behavior. All strains were inhibited by mannose and only one by maltose. In regard to the stability of these characters in the presence of proteases and high salt concentrations, a relevant degree of variation was found among the strains. This was to such an extent that it did not allow their inclusion in the Flo1 or NewFlo phenotypes. Genetic characterization of one wild-type strain revealed that the flocculation-governing gene was allelic to *FLO1* found in genetic strains.

INTRODUCTION

Flocculation in yeasts is a process by which yeasts adhere to form clumps, usually developed during the late-exponential or stationary phases of growth. Reviews on yeast aggregation in general and flocculation in particular have been published by Calleja [2] and Stratford [20].

This phenomenon is of considerable interest for such industrial fermentations as brewing, wine making [19] and the biological production of ethanol [3] because it leads to an efficient separation of the yeast cells from the fermenting medium.

Two hypotheses have been advanced to explain the mechanism of flocculation; these differ essentially in the role of calcium ions. Thus, the calcium-bridging theory suggests that calcium ions form links between the surface proteins of flocculent cells [12]. In contrast, according to the lectin-like theory, surface proteins on flocculent cells (SPF) would bind to cell wall mannan, calcium being used only to maintain lectin-like proteins in an active conformation [11].

Protein denaturation [13] results in irreversible loss of flocculation even though flocculation receptor sites are insensitive to protease action [11]. Flocculation can also be reversibly inhibited by the presence of sugars. Sucrose, mannose, maltose and glucose are inhibitory [12]. Others [22] described specific inhibition by mannose and its derivatives, that is not shown by any other sugar. In this sense, Stratford and Assinder [21] defined two distinct 'lectin-like' mechanisms of flocculation by sugar, salt and low pH inhibitions, protease sensitivity and selective expression of flocculation. One group (Flo1 phenotype) was inhibited by mannopyranoses and contained all strains bearing genes known to affect flocculation. The other group (NewFlo phenotype) contains the majority of brewery strains and was inhibited by manno- and glucopyranoses.

Flocculation in yeasts is under genetic control. Several genes affecting flocculation have been reported. These comprise the dominant genes *FLO1* (allelic with *FLO2* and *FLO4*), now assigned to the *FLO1* locus, on chromosome I at 37 cM from the *ade1* marker [14], *FLO5* mitotically located on chromosome I [25] and *FLO8* linked to *arg4* on chromosome VIII [26]. Since the right ends of chromosomes I and VIII share homology [18], *FLO1* and *FL08* genes probably represent copies of the same gene. Different recessive genes *flo3* [5], *flo6* and *flo7* [6] have also been defined. These genes behave in a semi-dominant manner and are considered to be possible mutated alleles of *FLO1* more readily subject to suppression [17]. In addition, mutations giving rise to flocculation, many have pleiotropic phenotypes, have been reported. Among these are *tup1* (allelic with *flk1*, *umr7*, *cyc9*, *PD7*, *sfl2 amm1*, *aer2 aar1*), *cyc8* (*ssn6*) and *amy2* (*gam1*) [20]. Moreover, mutations in the *SFL1* and *CKA2* genes also give a flocculation phenotype [20]. Finally, flocculation identical to that of *FLO1* gene-containing strains has been reported in strains transformed with the HTLV-1 Tax transactivator [7].

MATERIALS AND METHODS

Organisms and media

The *S. cerevisiae* strains used are listed in Table 1. YEPD/YEPD-agar medium contained 1% yeast extract (Difco, USA), 2% peptone (Difco), 2% glucose and 2% agar. SD medium contained 0.7% Difco-yeast nitrogen base w/o aminoacids, 2% glucose and 2% agar (each w/v). Aminoacids were added to SD medium when necessary. Acetate agar was prepared as described in reference [16].

This paper is dedicated to Professor Herman Jan Phaff in honor of his 50 years of active research which still continues.

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TABLE 1

Strains of *Saccharomyces cerevisiae* used in this study

Strain	Genotype	Source/reference
IM1-8b	*MATa leu2-3,* 112 *his4 sta*^{0} *sta*^{10} *FLO1*	[17]
99R	*MATα SUC2 GAL CUP1 MAL mel ade1 can*^{r} *FLO1*	Y.G.S.C.[a]
STX347-1D	*MATa his2 ura3 gal1 FLO5*	Y.G.S.C.[a]
1389	wild-type	C.E.C.T.[b]
13	wild-type	[10]
V86	wild-type	Local winery
13A1	*MATa*	This study

[a]Y.G.S.C.: Yeast Genetic Stock Center, Berkeley, CA, USA.
[b]C.E.C.T.: Spanish Type Culture Collection.

Chemicals

Tunicamycin (Sigma, USA) was prepared in a stock solution containing 1 mg ml^{-1} of NaOH 0.01 M. The solution was filter-sterilized and appropriate amounts of the stock solution were added to autoclaved YEPD medium.

Genetic techniques

Mating, sporulation and ascus disection for tetrad analysis were carried out by standard procedures [16]. Genetic linkage and chromosome location studies were performed as described by Sherman et al. [16].

Measurement of flocculation

To disperse cells we washed them twice with 0.25 M EDTA and twice with sterile water. Flocculation was initiated by resuspending the cells in flocculation buffer (0.1 M citrate buffer, pH 4.0, containing 0.01 M $CaCl_2$) [21]. When flocculation was completed, samples of supernatant were withdrawn and dispersed in 0.25 M EDTA. Cell concentrations were then determined spectrophotometrically at 600 nm and expressed as percentages of total cell concentration. Alternatively, the degree of flocculation was expressed quantitatively by applying a subjective grading.

TABLE 2

Segregation of the flocculation genes

Cross	Segregation (F:NF)					
	2:2	3:1	1:3	4:0	0:4	Number of tetrads
13A1 × IM1-8b	0	8	0	23	0	31
13A1 × STX347-1D	0	4	0	29	0	33

F, flocculent; NF, non-flocculent. Segregation of the other markers was 2+:2−. Segregation 3F:1NF is attributed to gene conversion.

Experimental assays

Treatment with sugars. Cells were harvested, dispersed in EDTA 0.25 M and heat killed at 60 °C over 5 min. This treatment did not affect flocculation in the strains used in this study. Cells were then suspended in flocculation buffer containing mannose or maltose at different concentrations. When flocculation had been completed in controls, samples of supernatants were removed and dispersed in EDTA (0.25 M) and the degree of flocculation was calculated as described previously.

Treatment with proteases. Washed and dispersed cells were resuspended in 0.1 M sodium phosphate buffer (pH 7.5) containing different proteases. Suspensions were then incubated at 30 °C with gentle shaking and samples (1 ml) were removed at required intervals. Cells were washed, resuspended in flocculation buffer and flocculation expressed as described above. The proteinases used were pronase E (0.1 mg m^{-1}), proteinase K (1 mg ml^{-1}) and trypsin (1 mg ml^{-1}).

Treatment with salts. Cells were washed, dispersed and suspended in 0.1 M sodium citrate buffer (pH 4) with calcium chloride at different concentrations. The degree of flocculation was estimated as described previously.

Treatment with tunicamycin. In order to establish the MIC of tunicamycin for strains used in this study, they were grown in YEPD medium containing different amounts of this antibiotic. The cultures were then examined for growth or the absence of growth. The strains were grown in YEPD medium with tunicamycin at different subinhibitory concentrations. The degree of flocculation was calculated at the end of the stationary phase of growth.

Preparation of invertase extract and PAGE

Cells from a 5-ml mid-log-phase culture on YEPD with 5% glucose were collected, washed twice with sterile water and resuspended in YEPD containing glucose at 0.05% as for derepressing invertase for 2 h at 30 °C. Then 5×10^7 cells were centrifuged, washed with water and resuspended in 0.05 M Tris HCl (pH 7.5) with 400 U of zymolyase 20T (Seikagaku-Kogyo, Japan) and β-mercaptoethanol 40 mM. Under these conditions, samples were incubated at 37 °C for 30 min. Cell debris was removed by low speed centrifugation. Ten microliters of supernatant were used for polyacrylamide gel electrophoresis (PAGE). This was carried out according to Laemmli [8] in polyacrylamide gels at 4% w/o SDS that were run at 4 °C for 6–7 h at 80 V with a 0.005 M Tris-glycine buffer, pH 8.3 w/o SDS.

Activity stain for invertase

Polyacrylamide gels were immersed in 50 ml of cold (4 °C) sucrose solution (0.1 M) in 0.1 M sodium acetate, pH 5.1) and incubated at 37 °C for 30 min. The gel was rinsed twice with water and then transferred to a petri dish containing 50 ml of TTC solution (1% 2,3,5-triphenyltetrazolium chloride in 0.5 M NaOH) and heated to boiling under the color developed [4].

RESULTS

Onset of flocculation

The ability to flocculate in YEPD medium was studied in three genetic strains of *S. cerevisiae*, 99R (*FLO1*), IM1-8b (*FLO1*) and STX347-1D (*FLO5*) as well as in three wild-type strains; namely, 1389, 13 and V86. As shown in Fig. 1, the three wild-type strains grew as free cells and only in the late exponential and particularly the stationary phase was flocculation apparent. Conversely, the three genetic strains exhibited their clumpy phenotypes from the very beginning of growth (i.e. constitutive flocculation). The constitutive flocculation appeared both in haploid strains obtained from wild-type homothalic diploids and heterothalic diploids obtained by conventional crosses of wild-type derived haploids (Fig. 1).

Effect of mannose, maltose and calcium salts

Flocculation capacity in the presence of 1 M mannose and 1 M maltose was also investigated. As shown in Fig. 2(A), flocculation was completely inhibited in all strains when the first sugar was present but was unaffected by the second one (Fig. 2(B)) except for strain V86 which showed 75% free cells. The effect of low sugar concentrations was also studied (Fig. 2(A,B)). Strain V86 was the only one whose flocculating phenotype was inhibited by 0.2 M maltose. It was more affected by mannose than the other strains (i.e. 0.2 M of mannose induced 75% of free cells, in contrast with 0.8 M mannose for the other strains). As depicted in Fig. 3, flocculation was less affected by high $CaCl_2$ concentrations in genetic strains than in the wild-types.

Effect of different proteases

The effect of different proteases on the flocculating phenotype of genetic and wild-type strains was studied (Fig. 4). Pronase E, proteinase K and trypsin irreversibly inhibited flocculation in all strains so far tested. With respect to the first protease, it (Fig. 4(A)) had a very rapid effect on deflocculating cell strains even though strain STX347-1D was less susceptible to the treatment. The same applied for the second protease (Fig. 4(B)). Trypsin treatment, however, revealed a clear-cut difference between genetic and industrial strains (Fig. 4(C)). Accordingly, the former were by far less susceptible to deflocculation (up to 120 min) than the second (strain V86 was totally deflocculated in 25 min).

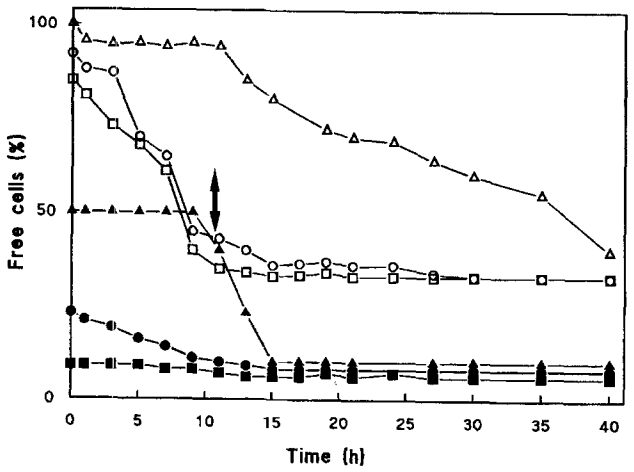

Fig. 1. Appearance of flocculation phenotype in strains: IM1-8b (■), 13 (○), 1389 (□), V86 (△), 13A1 (▲) and 13A1 × IM1-8b (●). Arrow indicates onset of stationary phase.

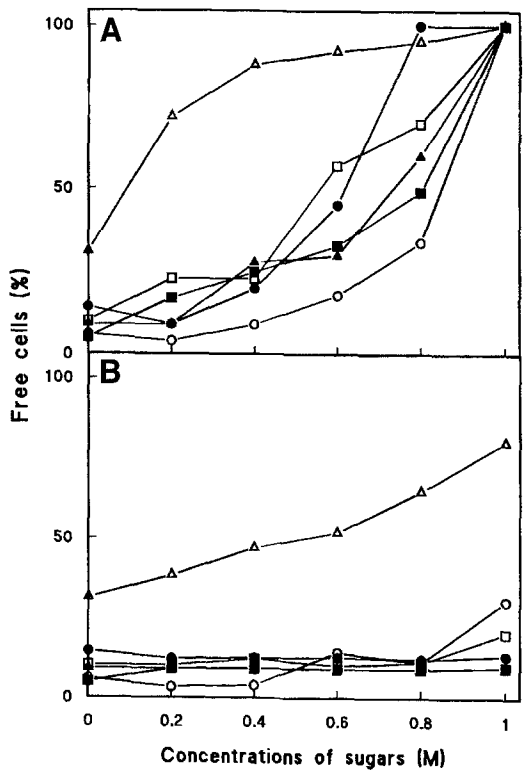

Fig. 2. Effect of different mannose (A) and maltose (B) concentrations on the flocculation of strains IM1-8b (■), 99R (●), STX347-1D (▲), 13 (○), 1389 (□) and V86 (△).

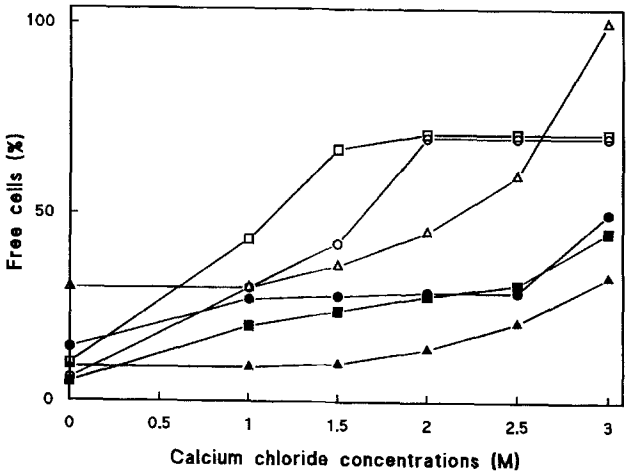

Fig. 3. Effect of calcium chloride concentrations on flocculation of strains IM1-8b (■), 99R (●), STX347-1D (▲), 13 (○), 1389 (□) and V86 (△).

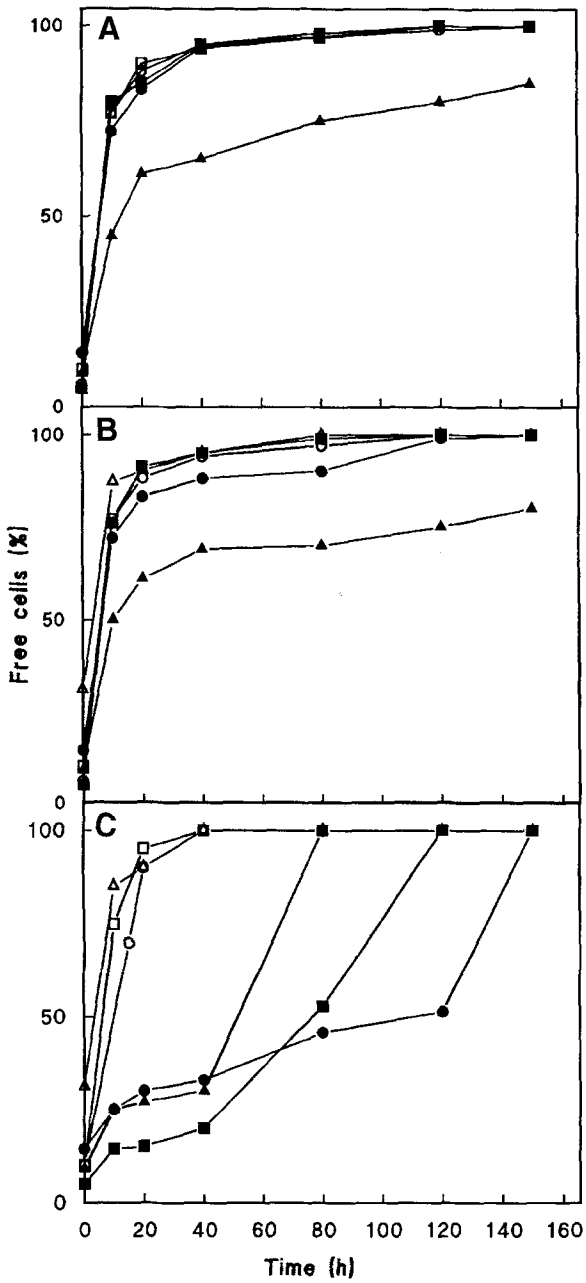

Fig. 4. Effect of pronase E (A), proteinase K (B) and trypsin (C) on the flocculation of strains IM1-8b (■), 99R (●), STX347-1D (▲), 13 (○), 1389 (□) and V86 (△).

Effect of tunicamycin

Yeast flocculation is the result of the binding of lectin-like glycoproteins present on the surface of flocculating strains to receptors thought to be made up of cell wall mannan [11]. The effect of tunicamycin was studied in the genetic strains IM1-8b and wild-types 1389 and 13 as shown in Fig. 5. The MICs for the strains were, respectively, 10, 9 and 8.5 $\mu g\ ml^{-1}$. As shown, flocculation occurred in all strains regardless of the antibiotic used. Invertase, used in these experiments as a control because tunicamycin was causing subglycosylation, could be detected in sub/non-glycosylated form. Thus glycosylation is not necessary for flocculation to occur.

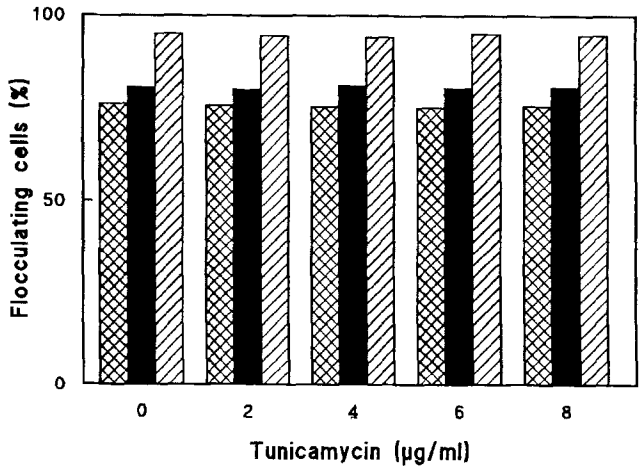

Fig. 5. Effect of different concentrations of tunicamycin on flocculation of strains 13 (▨), 1389 (■) and IM1-8b (▨).

Genetic characterization of a wild-type strain

In order to determine whether or not the flocculation phenotype in wild-type strains was also under control of locus *FLO1*, it was first necessary to obtain flocculation haploids derived from the homothalic wild-type diploids 13 and V86. Strain 13 was converted to a heterothalic state by specific gene interruption at the HO locus. Among the segregants of this diploid, the flocculating one 13A1 was selected. This later strain was crossed with IM1-8b (*FLO1*) and with STX347-1D (*FLO5*) and the tetrads were analyzed. Analysis of 31 tetrads, derived from the first cross, showed segregation 4F:0NF in 23 cases whereas in the second cross this segregation increased to 29 for 31 asci. It thus follows that the flocculation gene of strain 13A1 is allelic with the one present in strains IM1-8b and STX347-1D.

DISCUSSION

It is currently accepted that yeast flocculation is the result of a network formed among lectin-like glycoproteins present on the cell surface and mannan [11]. Based on this theory, two distinct flocculating phenotypes have been described [21]. The first Flo1 (including all those strains harboring any of the genes *FLO1*, *FLO5*, *FLO8*, *tup1* and *cyc8*) shows constitutive flocculation and is rapidly inhibited by mannopyranosides and it is quite sensitive to pronase E as well, although to a much lower extent than proteinase K and trypsin; calcium chloride exerts almost no action on this kind of flocculation. The second phenotype, known as the NewFlo phenotype (widespread among brewery strains), is sensitive to both gluco- and mannopyranosides, many proteases, and to calcium chloride. This is only apparent at the end of the log-phase of growth.

The three genetic strains studied here, 99R (*FLO1*), IM1-8b (*FLO1*) and STX347-1D (*FLO5*), exhibited constitutive flocculation, thus they belong to the Flo1 phenotype. Wild type strains, however, exhibited flocculation either at the end of the log-phase (strains 13 and 1389) or when the stationary phase was quite advanced (strain V86). They are therefore ascribed

to the second phenotype. This simple scheme worked apparently well for many genetic or wild-type strains. It did not, however, work so well when the latter began to be manipulated by means of genetic techniques. In this sense, the haploid strains derived from the wild homothalic diploids by HO gene disruption showed constitutive flocculation as well as their respective heterothalic diploids. This would imply that homo/heterothalim is somehow involved in the flocculation phenotype.

As far as the response to different sugars is concerned, all the strains studied, with the exception of strain V86, would have to be included in the Flo1 phenotype since they are inhibited by mannose. With respect to the other afore-mentioned criteria (i.e. effect of proteases and calcium chloride), it was found that the genetic strains studied here could be either ascribed to the Flo1 phenotype (response to high calcium chloride concentrations and trypsin) or to the Newflo phenotype (if the response to proteinase K is also taken into account). The wild-type strains could be (again following these criteria) unambiguously classified in the last phenotype, specially strain V86 in the sense that it was the only one inhibited by maltose.

The difference between both flocculation phenotypes has been explained by assuming the existence of at least two differentiated lectins [21]. As reported here, the existence of a certain variability in the flocculation process (possibly due to small differences in the respective flocculation genes) among genetic and wild-type strains can be concluded. This, in turn, would make it difficult to assign them to either of the phenotypes; the acceptance of such variability in applying the different criteria would question the very existence of different flocculation mechanisms in *S. cerevisiae*.

The role of *N*-linked carbohydrate in surface glycoproteins was investigated by following the effect of tunicamycin on flocculation. This antibiotic specifically inhibits the synthesis of dolichol-pp-*N*-acetylglucosamine, the first step in *N*-glycosylation of proteases [9]. This antibiotic did not inhibit flocculation even at a concentration close to the inhibitory one, in conflict with the results of other authors [1]. The antibiotic readily affected glycosylation of other glycoproteins, such as 1,3-β-D-glucanase [15], being then more susceptible to degradation by proteases. This again raises the issue of the different effect of proteases on both flocculation phenotypes which could be due, in turn, to different degrees of glycosylation rather than to different surface lectins and therefore different genes.

According to the results reported by Russel et al. [14] and Teunissen et al. [23,24] in *S. cerevisiae*, the flocculation mechanism is primarily governed by the *FLO1* locus on the right arm of chromosome I at 37 cM from *ade1* [14] and 4.7 cM from *pho11* [17]. In this sense, the *FLO* locus from the wild-type strain 13 was genetically characterized employing haploid-derived strains. It was therefore possible to establish that the respective gene was allelic with *FLO1* and *FLO5* from strains IM1-8b and STX347-1D, respectively (*FLO1* and *FLO5* are allelic themselves; unpublished results). Moreover, the *FLO8* gene [26] is probably a copy of *FLO1* because 28 kbp of the right arm on chromosomes I and VIII are homologous as reported by Steensma et al. [18]. Most probably the mechanism of *S. cerevisiae* flocculation is always the same and governed by the same genes. The differences found may be related to the homo/heterothalic strains and, of course, to differences in the glycosylation of SPF and mannan.

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