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Characteristics of Flo11-dependent flocculation in *Saccharomyces cerevisiae*

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Abstract

The *FLO11*-encoded flocculin is required for a variety of important phenotypes in *Saccharomyces cerevisiae*, including flocculation, adhesion to agar and plastic, invasive growth, pseudohyphae formation and biofilm development. We present evidence that Flo11p belongs to the Flo1-type class of flocculins rather than to the NewFlo class. Both Flo1-type and NewFlo yeast flocculation are inhibited by mannose. NewFlo flocculation, however, is also inhibited by several other carbohydrates including glucose, maltose and sucrose. These differences have in at least one case been shown to reflect differences in the structure of the carbohydrate-binding site of the flocculins. We report that Flo11p-dependent flocculation is inhibited by mannose, but not by glucose, maltose or sucrose. Furthermore, Flo11p contains a peptide sequence highly similar to one that has been shown to characterise Flo1-type flocculins. Further characterisation of the properties of Flo11p-dependent flocculation revealed that it is dependent on calcium, occurs only at cell densities greater than 1×10^8 ml^{-1}, and only occurs at acidic pH.

Keywords: Biofilm; Flocculin; Flocculation; *FLO11*; *MUC1*; Pseudohyphae; *Saccharomyces cerevisiae*

1. Introduction

The flocculin genes of *Saccharomyces cerevisiae* encode a family of cell wall proteins that bring about asexual reversible aggregation of cells into flocs. Flocculation is an industrially-important phenomenon, particularly to the brewing industry [1,2]. The flocs, or large aggregates of cells, separate from the culture medium either by precipitating, in the case of lager yeasts, or by rising to the surface, in the case of ale yeasts.

Flocculation is frequently, though not always, inhibited by mannose. In some yeast strains, flocculation is also inhibited by glucose, maltose and sucrose. This latter phenotype is designated NewFlo-type flocculation [3]. Flo1-type flocculation, on the other hand, is inhibited only by mannose [3]. Brewing yeasts frequently exhibit the NewFlo phenotype, while most laboratory strains are of the Flo1-type [4].

The flocculin gene family comprises five genes: *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11* (also known as *MUC1*). The predicted protein products of *FLO1* and

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FLO5 are 96% similar. Flo9p is predicted to be 94% similar to Flo1p, and Flo10p is 58% similar to Flo1p [1]. Flo11p, the most recently discovered member of the flocculin family, is more distantly related to the other flocculins, being 37% similar to Flo1p [5,6]. Flo11p has a number of unusual properties that distinguish it from the rest of the flocculins. While all the flocculins produce cell–cell adhesion, Flo11p is also essential for the formation of pseudohyphae and biofilms, and for invasive growth, adhesion to agar and other substrates such as plastic [5–7]. The multiplicity of phenotypes associated with *FLO11* is reflected in its promoter, which is one of the largest in the yeast genome [8], and in its complex regulation. Expression of *FLO11* has been shown to be controlled by several major pathways, including the MAP kinase pathway and the protein kinaseA/cAMP pathway [8–21].

Studies of the pH dependence of flocculation have shown a wide range of pH at which flocculation can occur in various strains, from pH 1.5 to 9 [22–25]. The physical properties of flocculins have been most thoroughly studied in Flo1p, the prototype of Flo1-type flocculins. Recently the critical differences between Flo1-type and NewFlo-type flocculation were illuminated by the cloning of a flocculin gene, Lg-Flo1p, from a NewFlo strain of lager brewing yeast. Lg-Flo1 was shown to confer glucose inhibition of flocculation upon yeast strains that express it. Domain-swap and mutagenesis experiments were used to identify a key peptide sequence within Lg-Flo1p that confers glucose inhibition of flocculation upon Flo1p [26].

To date, these types of studies have not been done on the *FLO11*-encoded flocculin. Here we report that Flo11p contains a peptide sequence similar to that shown to be characteristic of Flo1-type flocculins, suggesting that it belongs to this class. In agreement with this suggestion, we show that Flo11p-dependent flocculation is of the Flo1-type because it is inhibited by mannose but not by glucose. Flo11p-dependent flocculation only occurs at a critical cell density, and only at acidic pH. These results might help explain why flocculation of the Flo1-type occurs primarily in late-exponential and stationary-phase cultures [27,28].

2. Materials and methods

2.1. Microbial strains

Escherichia coli strain DH5α was used for plasmid amplification (Gibco BRL/Life Technologies, Rockville, MD), whereas *S. cerevisiae* strain YIY345 (*MAT***a** *ura3 leu2-3,112 his4 sta*^{0}) [29] was used for all of the flocculation experiments. Strain YIY345 was derived from a *S. cerevisiae* var. *diastaticus* strain by crossing out the *STA1* glucoamylase gene. The *FLO11* knockout (Δ*flo*11) strain, YIY345flo11^{-}, was constructed as previously described [6].

2.2. Flocculation rate assay

Yeast cultures were grown overnight at 30 °C in YPD broth (1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose). Cells were harvested and washed twice in deflocculation buffer [20 mM citrate (pH 3.0), 5 mM EDTA]. After washing, cells were suspended in deflocculation buffer to an optical density (absorbance at 600 nm; A_{600}) of 2. An aliquot of 800 μl of this cell suspension was pipetted into a 1-ml cuvette, and 200 μl of 100 mM calcium chloride was added to initiate flocculation. The cuvette was vigorously shaken and the absorbance (A_{600}) was measured immediately and at 30-s intervals for 5 min using a spectrophotometer (Spectronic Model 601, Spectronic Instruments, Rochester, NY, USA). To investigate the effect of sugars on flocculation, the deflocculation buffer was supplemented with indicated concentrations of mannose or glucose. To determine the effect of pH on cell flocculence, the pH of the deflocculation buffer was changed by adjusting the initial pH of the citrate solution. Values of pH that are plotted in the graph represent the final pH of the cells in the deflocculation buffer, before calcium chloride was added. All of the assays were performed in triplicate.

3. Results

3.1. Flo11p-induced flocculation is calcium-dependent

S. cerevisiae var . *diastaticus* [29], which exhibits pronounced *FLO11*-dependent flocculation, crosses freely with all standard laboratory strains of *S. cerevisiae*. Strain YIY345 was derived from the *S. cerevisiae* var. *diastaticus* genetic background [29]. When *FLO11* was knocked out in strain YIY345, flocculation was abolished (Fig. 1), showing that flocculation is solely dependent on the *FLO11* gene.

To investigate the characteristics of *FLO11*-dependent flocculation, a spectrophotometric assay for flocculation was used. The level of the cell suspension in the cuvette was somewhat higher than the path of the light beam in the spectrometer. Thus, when calcium chloride was added to initiate flocculation, the light absorbance at 600 nm initially rose as the cells above the path of the light beam descended into the beam (see Fig. 1, YIY345 + CaCl2). After 30 s the A_{600} value decreased as the flocculating cells continued to settle out of solution. Flocculation is shown to be dependent on calcium (Fig. 1). Cell cultures that did not flocculate, either because of deletion of *FLO11* or absence of calcium, showed no decrease in light absorbance.

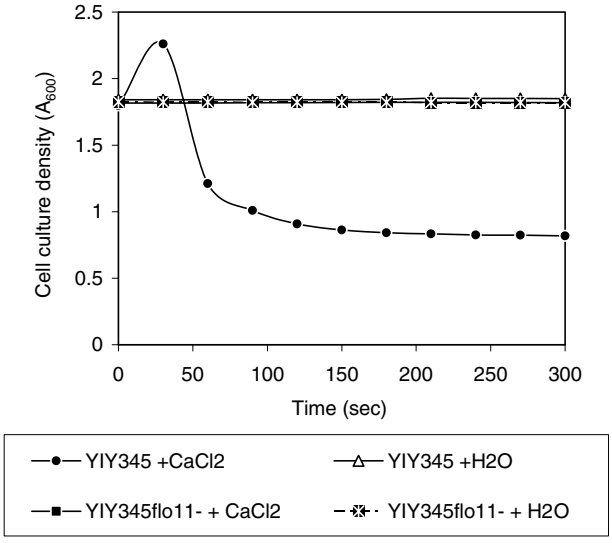

Fig. 1. Flo11/Muc1 flocculation is calcium dependent. Yeast expressing Flo11/Muc1 (YIY345) or yeast with a disruption of the Flo11/Muc1 gene (YIY345flo11-) were suspended in deflocculation buffer. Calcium chloride was added to 20 mM, or an equivalent amount of water was added. Flocculation was assayed by measuring the absorbance of light at 600 nm over time. Values plotted represent the average of 3 assays of the same culture.

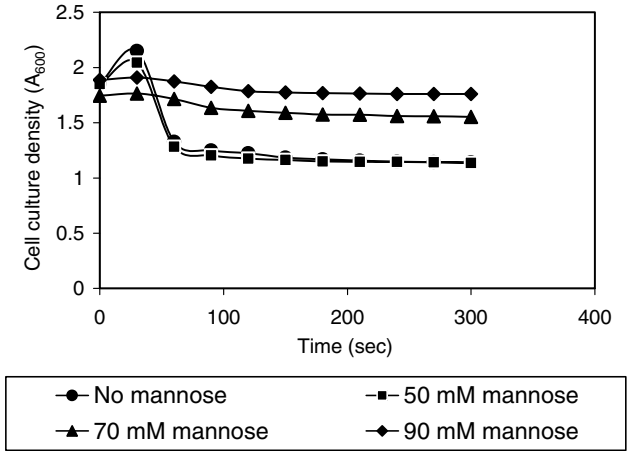

Fig. 2. Mannose inhibits Flo11/Muc1 flocculation. Yeast were suspended in deflocculation buffer containing the indicated concentrations of mannose for 15 minutes before being placed in a spectrophotometer cuvette. Calcium chloride was added to 20 mM to initiate flocculation. Flocculation was assayed by measuring the absorbance at 600 nm over time, as a measure of the decrease in cell density. An initial increase in cell density reflects the cells above the light path settling into the light path before settling to the bottom of the cuvette. A600 values represent the average of 3 assays of the same culture.

3.2. Mannose inhibits Flo11p-dependent flocculation

To investigate the targets of Flo11p cellular adhesion, we incubated the yeast cells with various carbohydrates for 15 min prior to initiation of flocculation. Flocculation was inhibited by concentrations of mannose greater than 50 mM when mannose was added to the deflocculation buffer before the addition of calcium (Fig. 2). This suggests that the adhesion receptor for Flo11p might be some component of the mannoprotein layer of the cell wall.

3.3. Flo11p-dependent flocculation is of the Flo1-type rather than the NewFlo-type

Yeast cells incubated in glucose remained flocculent, even in concentrations as high as 1 M glucose (Fig. 3). Similar results were obtained with sucrose and maltose (data not shown). The ability to be inhibited by mannose but not by glucose, sucrose or maltose is a defining characteristic of Flo1-type flocculation [3]. Therefore, Flo11p flocculation is of the Flo1-type, although Flo11p and Flo1p share little sequence homology [6].

To date, only one flocculin of the NewFlo-type has been cloned and characterised, Lg-Flo1. Kobayashi et al. [26] used domain swap experiments with Flo1p and site-specific mutagenesis to identify a peptide sequence responsible for the glucose inhibition that characterises NewFlo-type flocculation. As expected, the adhesion domain of the flocculin proved to be in the amino-terminal region of the protein, since the

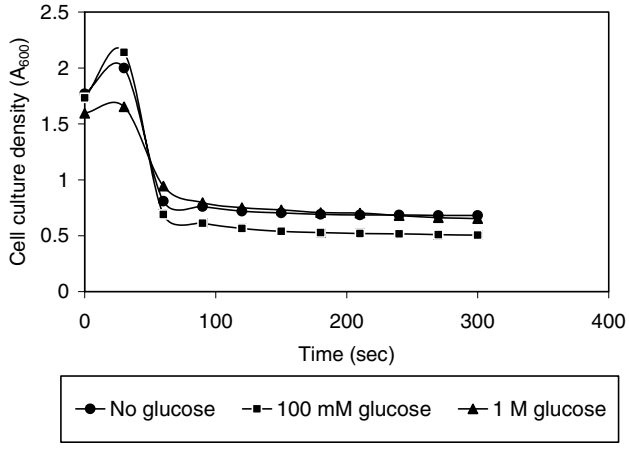

Fig. 3. Glucose does not inhibit Flo11/Muc1 flocculation. Yeast were suspended in deflocculation buffer containing the indicated concentrations of glucose. Calcium chloride was added to 20 mM to initiate flocculation. Flocculation was assayed by measuring the absorbance at 600 nm over time. A600 values represent the average of 3 assays of the same culture.

GPI anchor sequence is at the carboxyl-terminal end of the flocculins. One amino acid within this domain was identified as being particularly responsible for glucose inhibition, namely leucine 228. In order to confer glucose inhibition on a chimeric flocculin, it has been found that tryptophan 228 must be changed to a leucine [26]. In the present study, we investigated whether Flo11p contains a similar peptide sequence as Flo1p and, if so, whether it possesses a leucine or a trytophan residue at the homologous position. We used the lalign

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Flo11      162   YASSWQWGTTSFDLS   176
                 *     ***
                 **** **** ****
Flo1       222   YSNAVSWGTLPISVT   236
                 ****     ** **
                 ****   * ******
Lg-Flo1    222   YSNAKVLARLPVSVV   236
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Fig. 4. Alignment of Flo11/Muc1 sequences with the NewFlo-type flocculin Lg-Flo1 and the Flo1-type flocculin Flo1 illustrate that Flo11/Muc1 has sequences characteristic of a Flo1-type flocculin. Identity of 2 amino acid residues is symbolized by two stars between them; similarity is symbolized by one star. The critical tryptophan residue at position 228 of Flo1 (position 168 of Flo11/Muc1) that must be replaced by leucine in order to bring about glucose inhibition is shown in bold. Numbering and sequence of Flo1 and Lg-Flo1 are from Kobayashi et al. [26].

program [30] to explore Flo11p for sequences similar to the Flo1p sequence responsible for glucose inhibition. A region in the amino-terminal domain of Flo11p, from amino acids 162–176, was identified that exhibits 86.7% similarity to the Flo1p domain (amino acids 222–236) that governs substrate specificity (Fig. 4). Numbering of Flo1p and Lg-Flo1p residues are as in Kobayashi et al. [26]. The critical tryptophan/leucine residue is shown in boldface in Fig. 4. Flo11p contains a tryptophan at this position, which supports its assignment as a Flo1-type flocculin.

3.4. Flo11p flocculation is cell density-dependent

Fig. 5 shows that yeast did not flocculate when the density of the cell culture was less than 1.5 as measured by absorbance of light at 600 nm (approximately 1×10^8 cells per ml). In agreement with previous studies [31,32], mechanical agitation was also required for flocculation to occur.

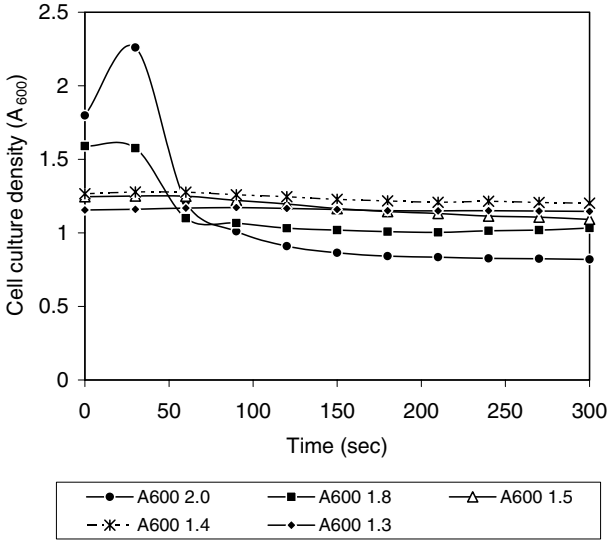

Fig. 5. Flo11/Muc1 flocculation is cell density dependent. Cell cultures with the indicated A600 values were suspended in deflocculation buffer. One-fifth volume of 100 mM calcium chloride was added to initiate flocculation. Flocculation was assayed by measuring the absorbance of light at 600 nm over time. Values plotted represent the average of 3 assays of the same culture.

3.5. Flo11p flocculation is pH-dependent

A study of the pH dependence of Flo11p flocculation is shown in Fig. 6. The usual deflocculation buffer is 20 mM citrate, pH 3. The pH of the flocculation reaction was adjusted immediately before the assay in order to avoid effects on synthesis of the flocculin RNA or protein. Flocculation did not occur at neutral pH, nor did it occur at the pH extremes of 2.5 and 10. Flocculation occurred at pH 3.9 and 5.5, which is a range typical of yeast growth media. The pH of YPD medium is approximately 5.5. By the end of exponential growth, the pH of the culture media dropped to below 3. Thus, flocculation occurred at physiological pH only.

4. Discussion

Most brewing strains of yeast exhibit NewFlo-type flocculation. We present evidence that Flo11p belongs to the class of Flo1-type flocculins. These flocculins are inhibited by mannose, but not by glucose. NewFlo-type flocculation, on the other hand, is inhibited by both mannose and glucose [3]. Flo1p and Flo5p belong to the class of Flo1-type flocculins [1]. The only NewFlo-type flocculin yet identified is Lg-Flo1 [26]. NewFlo flocculation might be completely inhibited by 160 mM glucose [26]. We show that Flo11p flocculation is resistant to glucose concentrations as high as 1 M, supporting its classification as a Flo1-type flocculin. However, Flo11 does share with the NewFlo-type

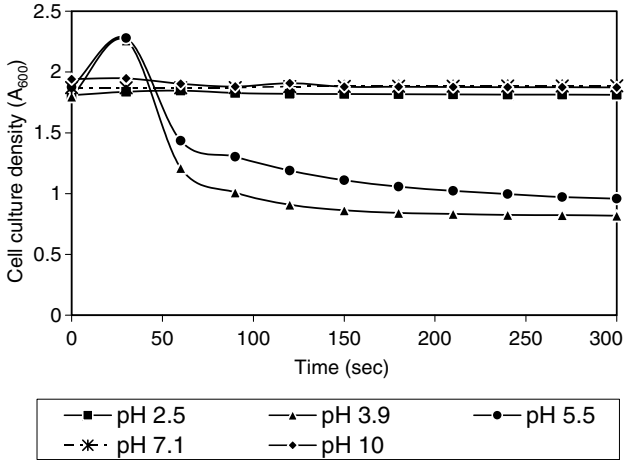

Fig. 6. Effect of pH on Flo11/Muc1-dependent flocculation. Cell cultures in deflocculation buffer were adjusted to the indicated pH levels. Calcium chloride was added to initiate flocculation, and the absorbance of light at 600 nm was measured as a function of time. Values plotted represent the average of 3 assays of the same culture.

flocculin Lg-Flo1 a great sensitivity to mannose inhibition. Lg-Flo1 is completely inhibited by 40 mM mannose, whereas Flo1 is still 22% active in 1 M mannose [26]. Flo11 is almost as sensitive to mannose inhibition as Lg-Flo1 and the transition to inhibition is as sharp as it is with Lg-Flo1. Flo11-dependent flocculation is not inhibited by 50 mM mannose, but inhibition is total at 70 mM. Lg-Flo1 also exhibits a sharp dependence on concentrations of mannose and glucose for inhibition of flocculation, unlike Flo1 where the effects of sugar inhibition are much more gradual [26].

Further evidence that supports the assignment of Flo11 to the Flo1-type class of flocculins is the tryptophan residue present at a key position corresponding to the sugar recognition domain of Flo1 and Lg-Flo1. This tryptophan residue is important for inhibition of flocculation by mannose and not by glucose. In contrast, when leucine is present at this position instead of tryptophan, flocculation shows the mannose and glucose inhibition characteristic of NewFlo-type flocculation [26].

Flocculation frequently occurs during late-logarithmic or stationary phases of growth upon depletion of the carbon source [28,33,34]. This might be partly due to increased transcription of flocculin genes at the post-diauxic shift [8,27]. Our work supports two other explanations for this phenomenon in the case of Flo11p, namely cell concentration dependence of flocculation and pH dependence. This is in agreement with previous work on other yeast strains that has clearly demonstrated the dependence of flocculation on cell concentration [31] and pH [35]. Flo11p-dependent flocculation only occurs under conditions of cell density and pH that are characteristic of late-exponential and stationary phase culture. Cultures flocculated only when cell density exceeded 10^8 cells ml^{-1} and when the pH fell to between 3.9 and 5.5 (Figs. 5 and 6). This effect of pH must be on activity of the flocculin rather than on its rate of synthesis, since in these experiments the pH was adjusted immediately prior to the flocculation assay.

FLO11 gene expression is regulated by glucose via the Gpr1 G protein-coupled receptor [15]. While glucose is an agonist of *GPR1*, mannose is an antagonist of the receptor [36]. Thus the effects of mannose and glucose on FLO11 gene expression and on activity of the protein are entirely congruent. Lack of inhibition of flocculation by glucose is a property that may have been selected for in Flo11, given the role of glucose in promoting pseudohyphal growth [15].

Our data on the calcium dependence and mannose inhibition of Flo11 stand in contrast to those published by Guo et al. [37] who expressed Flo11p from the *GAL1* promoter in strain Σ1278b, which does not ordinarily flocculate. They found that upon overexpression of *FLO11* in galactose, the Σ1278b cultures flocculated, but this flocculation was neither dependent on calcium nor inhibited by mannose or other sugars. The difference between their results and those presented here might be due to the different yeast strains used or to differing properties of Flo11p when overexpressed in the presence of galactose. These differences might indicate the existence of auxiliary factors to the flocculins that affect flocculation, perhaps in a strain-specific way.

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