MIG1 overexpression causes flocculation in *Saccharomyces cerevisiae*

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MIG1, encoding a C₂H₂ zinc-finger repressor protein involved in carbon catabolite repression, was found to play a role in non-sexual flocculation of *Saccharomyces cerevisiae*. Disruption of MIG1 in a flocculent mutant strain of NCYC 227, resulted in a non-flocculent phenotype. Expression of MIG1 on a 2 µ pRS426 vector in a non-flocculent strain, YM 4134, caused flocculation; MIG1 on a high-copy-number LEU2-d plasmid caused intense flocculation in the same strain. Mutations in the SSN6 and TUP1 genes confer a flocculent phenotype in non-flocculent strains of *S. cerevisiae*, and it has been shown that Mig1 can tether the Ssn6p–Tup1p complex to the regulatory regions of glucose-repressible genes. Mutations in *tup1* in a MIG1 background caused flocculation while double mutants of TUP1 and MIG1 did not flocculate. Based on these results, a model for the role of MIG1 in flocculation gene regulation is proposed.

Keywords: flocculation, regulation, MIG1, SSN6–TUP1

INTRODUCTION

Yeast flocculation and cell sedimentation to the bottom of the fermenter are desirable properties of *S. cerevisiae* strains used in industrial fermentations (Stratford, 1992). Flocculation might serve as a useful tool in modern biotechnological processes such as the production of heterologous proteins from yeast, since it is a cheap and easy way to separate the cells from culture fluids, thus facilitating further downstream processing. Flocculation in all yeast genera appears to conform to the Lectin-like Hypothesis (Miki *et al.*, 1982), and a mannose-specific flocculation lectin has recently been identified (Shankar & Umesh-Kumar, 1994).

Flocculation is under genetic control and may be transferred by mating, cytoduction or electrofusion (Stratford, 1994). Much interest has centred on *FLO1*, a dominant gene sufficient for flocculation (Teunissen *et al.*, 1993a). It is located on chromosome I and a full sequence has been published (Watari *et al.*, 1994). Another gene, *FLO5*, is also known to be dominant and is located on chromosome VIII (Teunissen *et al.*, 1995b). Some non-flocculent strains if mutated in certain genes become flocculent. These genes include *TUP1* (Lipke & Hull-Pillsbury, 1984) which is allelic to *SFL2* (Fujita *et al.*, 1990), *CYC9* (Rothstein & Sherman, 1980) and *SSN6* allelic to *CYC8* (Trumbly, 1988). Both *TUP1* and *SSN6* encode proteins which form a repressor complex in a regulatory cascade (Keleher *et al.*, 1992). Many of these non-flocculent strains contain intact *FLO1* sequences (Teunissen *et al.*, 1995a).

Thus, the induction of flocculation in these strains suggested the presence of one or more flocculation genes that were silent in the corresponding wild-type strains. In contrast, some strains with *FLO1* flocculate in the presence of intact *TUP1* and *SSN6* genes, and it has been shown that in these strains flocculation is insensitive to the *TUP1/SSN6* regulatory cascade (Teunissen *et al.*, 1995a). Thus, much remains unknown about the regulation of flocculation at the molecular level. In this paper, we present evidence to indicate constitutive overexpression of *MIG1* in flocculating strains as a cause for flocculation in *S. cerevisiae*.

METHODS

Strains and growth media. YM 4134 (MATa *MIG1 TUP1 ura3 his3 leu2 ade2 lys2 trp1*) and YM 4230 (MATa *mig1::LEU2 TUP1 ura3 his3 leu2 ade2 lys2 trp1*) are two strains of *S. cerevisiae* obtained from the collection of Dr M. Johnston, Department of Genetics, Washington University School of Medicine, St Louis, MO, USA. Flocculent strains of *S. cerevisiae* NCYC 227 and NCYC 1119 and non-flocculent strains NCYC 431 and SD 164 are cultures maintained in this laboratory. The original cultures were obtained from the National Collection of Yeast Cultures (NCYC), UK, and Labatt Breweries, Canada. The cultures were routinely grown in yeast extract/peptone/dextrose (YPD) broth or agar. For some of the experiments, dextrose was replaced with raffinose or glycerol in the medium. Yeast minimal medium with necessary amino acid selection was used for genetic studies.

Measurement of flocculation. Flocculation was determined by an assay method described by Nagarajan & Umesh-Kumar

(1990) with the following modifications. Cells washed with 250 mM EDTA and resuspended in deionized water to 2.0×10^8 cells ml^{-1} were distributed in tubes. Flocculation was initiated by the addition of 1 ml 250 mM CaCl_2 solution and OD_{660} was determined after 5 min of settling. The percentage of cells in the supernatant was proportional to the difference in the mean optical density between the experimental tubes and the control tubes without added CaCl_2 .

For studies on the effect of inhibitors on flocculation, the assay was carried out with cells suspended in 100 mM Tris/HCl buffer, pH 8.0. Heat-killed cells were used for sugar-inhibition tests (Stratford & Assinder, 1991). The effect of Pronase E on flocculation was studied after incubating the cells for 15 min in Tris/HCl buffer at 37 °C.

Cloning. The *MIG1* gene on plasmid pBM2220 (*pMIG1*; Nehlin & Ronne, 1990) was used for overexpression studies. This plasmid contained a *LEU2* defective gene for high-copy-number and *URA3* for selection. Yeast strain YM 4134 was transformed with this plasmid and the transformants were selected on uracil-less glucose plates. They were then replicated on leucine-less plates or broth for high-copy plasmids. For normal-copy expression of *MIG1*, the ~3.2 kb *XbaI*–*HindIII* fragment of *MIG1* (for restriction map see Nehlin & Ronne, 1990) was cloned into the yeast centromere vector, pRS316, linearized with the same enzymes. *Escherichia coli* strain DH5 α was transformed with the cloned plasmid by electroporation. The plasmid preparation obtained from the transformed bacteria was confirmed for the presence of this construct by restriction digestion and electrophoresis. A portion of this plasmid stock was used to transform the yeast strain. The *MIG1* gene located within the *NotI*–*HindIII* fragment of the above construct was subcloned into the yeast 2 μ plasmid (pRS426) and used as the plasmid for moderate level expression of *MIG1*.

Yeast strains were transformed with 10 μg plasmid DNA using the lithium acetate procedure (Ito *et al.*, 1983). For quantification of flocculation phenotype, the transformed yeast strains were grown in yeast minimal broth with appropriate amino acid selection, and intensity of flocculation was assayed as described above. In all these experiments, controls consisted of yeast strains transformed with plasmids containing no *MIG1* gene.

mRNA isolation and Northern analysis. Total mRNA was isolated from late exponential phase cultures (Kohren & Domdey, 1993) by passing through an oligo(dT) cellulose spun column (Pharmacia) previously equilibrated with high-salt buffer [10 mM Tris/HCl (pH 7.5), 0.2% SDS, 5 mM EDTA; 0.5 M NaCl]. The mRNA in the preparation was determined by measuring OD_{260} using a Shimadzu spectrophotometer for Northern dot blot analysis. Identical concentrations of mRNA from strains of *S. cerevisiae* were applied as dots on to the GeneScreen nylon membrane. The bound *MIG1* mRNA on the membrane was detected using ~1.5 kb *MIG1* DNA probe labelled with digoxigenin 11-UTP. The DIG DNA labelling and detection kit of Boehringer Mannheim was used according to the manufacturer's instructions. The probe was hybridized at 68 °C for 12 h with gentle shaking. The probe used for Northern analysis was the *MIG1* gene amplified by PCR. The two primers synthesized for *MIG1* amplification, 5'-AGTGTCTAACGT-TGATGA-3' and 5'-GTCCATGTGTGGGAAGGGC-3', were based on the published *MIG1* gene sequence (Nehlin & Ronne, 1990).

Genetic studies. A *leu2* mutant of NCYC 227, obtained by UV irradiation and confirmed for the mutation by complementation with a *LEU2* centromere plasmid, was used for *MIG1*

disruption. *MIG1* was disrupted in this strain (*mig1::LEU2*) by transformation with the *XbaI*–*HindIII* fragment of the *MIG1* disruptor plasmid pBM2169 (Nehlin & Ronne, 1990). *TUP1* was disrupted in the non-flocculent *mig1* strain of *S. cerevisiae* (YM 4230) using the *HindIII* fragment of the *TUP1* disruptor plasmid pBM2004 (*tup1::URA3*). The desired strains were identified by 2-deoxyglucose resistance and clumpy growth (*mig1* and *tup1* phenotypes, respectively).

RESULTS AND DISCUSSION

The strain of *S. cerevisiae* YM 4134 used in this study was a non-flocculent haploid. Disruption of *TUP1* in this strain (*tup1::URA3*) resulted in mutants that were flocculent. This result was similar to that previously reported (Lipke & Hull-Pillsbury, 1984). Table 1 shows a comparison on the effect of inhibitors of flocculation in a *tup1* strain in relation to the brewery strains NCYC 1119 and NCYC 227. The inhibitory effects were similar in all strains and specific inhibition of flocculation by mannose and α -methyl D-mannoside suggested the strains to conform to *FLO1*, *FLO5* or *FLO8* phenotypes (Stratford & Assinder, 1991; Stratford, 1992). At 10 μg Pronase E ml^{-1} , a concentration that almost completely inhibited flocculation in NCYC 227, flocculation of NCYC 1119 and *tup1* mutants was 26 and 84% of the control values, respectively. This reflected the flocculation intensity of the strains: growth experiments showed higher flocculation intensity with the *tup1* strain compared with NCYC 1119 which was more flocculent compared with NCYC 227 (Shankar & Umesh-Kumar, 1994). Increased flocculation intensity appeared to reflect the synthesis of more flocculation proteins as indirectly shown by the inhibitory effects of mannose and α -methyl D-mannoside. Mannose and α -methyl D-mannoside at 500 mM completely inhibited flocculation in NCYC 227 and 1119, but caused only about 60% flocculation inhibition in the *tup1* strain. The antibody raised against the flocculation protein of NCYC 227 reacted with NCYC 1119 (Shankar & Umesh-Kumar, 1994), and also cross-reacted with the *tup1* mutant (data not shown). Thus, it appeared that the flocculation gene in the strain YM 4134 was silent due to its sensitivity to the *TUP1* regulatory cascade. However, some dispersion of flocculation in the *tup1* strain due to 200 mM NaCl differentiated it from the other strains used in this study.

TUP1, together with *SSN6*, is known to be involved in the repression of many important yeast genes. Keleher *et al.* (1992) suggested that Tup1p–Ssn6p locates the wide variety of genes that it represses by recognizing the specific DNA-bound proteins present at each promoter. A strong candidate for such a protein was Mig1p (Multicopy Inhibitor of *GAL* genes), which has been shown to bind to regions of the promoter required for glucose-dependent repression of *SUC2* and *GAL4*, and is required in addition to Ssn6p and Tup1p for complete repression of both genes (Nehlin & Ronne, 1990; Nehlin *et al.*, 1991; Griggs & Johnston, 1992). An earlier report had shown flocculation identical to that of *FLO1* in *S. cerevisiae* when C_2H_2 zinc-finger HTLV-1 Tax-transactivator protein was overexpressed (Kramer *et al.*, 1990). Since Mig1p is also a C_2H_2 zinc-finger DNA-binding

Table 1. Effect of inhibitors on flocculation

Values are means of triplicate determinations. The maximum variation between tubes was 10%, with mean standard errors of 3%. Control values ranged from 84 to 91% aggregated cells.

Inhibitor	Percentage of control flocculation		
	<i>tup1</i>	NCYC 1119	NCYC 227
None	100	100	100
100 mM Tris/HCl buffer (pH 8.0)	100	100	100
200 mM NaCl	86	100	100
250 mM EDTA	0	0	0
10 µg Pronase E ml ⁻¹	84	26	10
100 µg Pronase E ml ⁻¹	30	2	0
1 mg Pronase E ml ⁻¹	0	0	0
500 mM Glucose	96	98	98
500 mM Galactose	96	100	100
500 mM Mannose	45	0	0
500 mM α -Methyl D-mannoside	40	0	0

Table 2. Effect of *MIG1* overexpression on flocculation

Strain	<i>MIG1</i> on plasmid	Plasmid copy no.	Flocculation intensity*
YM 4230	pRS316	1–2	—
YM 4134	pRS426	10–40	++
YM 4134	pBM2220	100–200	++++

* —, Non-flocculent; + + + +, highly flocculent.

protein with domains for Tup1p and Ssn6p (Treitel & Carlson, 1995), we proceeded to test the importance of this protein in flocculation. When *TUP1* was disrupted (*tup1::URA3*) in the non-flocculent *mig1* strain (YM 4230) the double mutants were non-flocculent. Disruption of *MIG1* in a flocculent *leu2* mutant of NCYC 227 also resulted in a non-flocculent phenotype. These results showed an important role for *MIG1* in flocculation.

In another experiment, the role of *MIG1* gene dosage on yeast flocculation was determined. *MIG1* was overexpressed at two levels in strain YM 4134. High-copy-expression of *MIG1* was obtained using the gene cloned in a *LEU2-d* plasmid (pBM2220). *MIG1* cloned in a 2 µ multicopy vector pRS426 when expressed in this yeast strain (YM 4134) resulted in moderate levels of the gene. As a control, *MIG1* was cloned in a centromere vector, pRS316, and expressed in a *mig1* strain (YM 4230). While *MIG1* in normal copy-numbers did not influence flocculation, increasing the *MIG1* gene dosage caused an increase in the intensity of flocculation in YM 4134 (Table 2). Heavy flocculation in YM 4134 due to overexpression of *MIG1* on a *LEU2-d* plasmid affected the growth of the yeast in glucose broth. It could be argued that titration of Ssn6p/Tup1p repressors due to overexpression of *MIG1* caused flocculation similar to the *TUP1* and *SSN6* mutations. However, it is unlikely that this is the cause for flocculation because increasing the gene dosage of *MIG1*

Table 3. Effect of inhibitors on flocculation in relation to *MIG1* gene dosage

Values are means of triplicate determinations. The maximum variation between tubes was 3%, with a mean standard error of 2%. Control values ranged from 96 to 100% aggregated cells. Figures in parentheses indicate *MIG1* copy number. Plasmid pRS316 was not tested because it had no effect on flocculation intensity (see Table 2).

Inhibitor	Flocculation intensity (%) with <i>MIG1</i> expressed on plasmid:	
	pRS426 (10–40)	pBM2220 (100–200)
None	100	100
250 mM EDTA	0	0
1 mg Pronase E ml ⁻¹	23	82
3 mg Pronase E ml ⁻¹	0	20
500 mM Mannose	70	85
1 M Mannose	5	18
500 mM α -Methyl D-mannoside	60	80
1 M α -Methyl D-mannoside	0	10

at two levels in YM 4134, having a single copy of *SSN6* and *TUP1*, resulted in an additive response with respect to flocculation intensity. It appeared that *MIG1* overexpression caused increased synthesis of the flocculation protein, since the concentration of mannose or α -methyl D-mannoside required to cause inhibition of flocculation in such strains was higher, and more Pronase E was needed to destroy the flocculation protein in these cells (Table 3).

A role for *MIG1* gene dosage as a cause of differential flocculation intensity in the wild-type strains NCYC 227

Fig. 1. Growth of non-flocculent (1, NCYC 431; 3, SD 164) and flocculent (2, NCYC 227; 4, NCYC 1119) strains of *S. cerevisiae* after 24 h on YP glucose (a) and YP raffinose (b) agar plates.

and 1119 was also studied. *MIG1* overexpression has been shown to cause repression of *SUC2* (invertase) gene expression (Nehlin & Ronne, 1990). Growth experiments with flocculent and non-flocculent strains showed slow growth of the flocculent strains NCYC 227 and 1119 when raffinose was used as the carbon source, while growth on glucose was unaffected (Fig. 1). When the corresponding invertase levels were determined (Table 4), it was found that reduced invertase levels in flocculent strains affected their growth on raffinose. These observations were further confirmed by Northern analysis which indicated higher concentrations of *MIG1* mRNA in the cells of flocculent strains compared with non-flocculent strains (data not shown). This suggested constitutive overexpression of *MIG1* in flocculent strains. Growth of the highly flocculent strain NCYC 1119 was extremely slow in YP glycerol broth. This could be due to the effect on glycerol utilization of *MIG1* overexpression. These results could also explain variations in flocculation among strains of *S. cerevisiae* (Stratford & Carter, 1993), with some strains showing higher flocculation intensity compared with the others (Stratford & Assinder, 1991).

Table 4. Whole-cell invertase and *MIG1* mRNA in strains of *S. cerevisiae*

Invertase levels are given as a percentage (100% = 14.45 U). Invertase was assayed in whole cells as described by Trumbly (1988) and specific activity was expressed as $\mu\text{mol glucose formed min}^{-1} (\text{mg whole-cell protein})^{-1}$. For determination of whole-cell protein concentration, 0.5 ml cell suspension was taken in a test tube and boiled for 5 min with 0.5 ml 1 M NaOH. Total protein was estimated in the lysed suspension by the Lowry method. —, Non-flocculent; + + + +, highly flocculent.

Strain	Invertase (%)	Degree of flocculation
431	100	—
227	69	+ + +
1119	40	+ + + +

FLO genes may encode cell-surface flocculation lectins (Teunissen *et al.*, 1993b). Direct activation of these genes by *MIG1* could be postulated since Mig1 has stretches of glutamine-rich residues similar to the activator protein *Sp1* (Nehlin & Ronne, 1990). This appears improbable in the light of the report of the absence of transcriptional activation by glutamine residues of *Sp1* (Ponticelli *et al.*, 1995). Hence *MIG1* has a probable repressor function in flocculation gene expression. Thus, only positive regulation of the flocculation structural gene by an activator proposed by Stratford (1992) can be explained in relation to the *Mig1* activity. *FLO8* as an activator of genes involved in flocculation has been suggested recently (Kabayashi *et al.*, 1995). Since Mig1p is a repressor protein, a direct repression of the activator is inconceivable because *MIG1* disruption in flocculent *S. cerevisiae* caused the appearance of a non-flocculent phenotype. It can be presumed that the activator is controlled by an active repressor whose function is repressed by *MIG1*. In this model, it is conceivable that the number of *MIG1* transcripts determine the degree of inhibition of the repressor as has been shown in this study on differential *MIG1* expression, resulting in an additive response in respect to flocculation. However, further experimental evidence is required to substantiate this model.

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