JOURNAL OF BACTERIOLOGY, May 1982, p. 890–899
0021-9193/82/050878-10$02.00/0

Vol. 150, No. 2

Repression and Induction of Flocculation Interactions in *Saccharomyces cerevisiae*†

BRIAN L. A. MIKI,[1]* N. HUNG POON,[2]‡ AND VERNER L. SELIGY[2]

Molecular Genetics Group, Division of Biological Sciences, National Research Council, Ottawa, Ontario K1A 0R6,[2] and Genetic Engineering Section, Ottawa Research Station, Agriculture Canada, Ottawa, Ontario K1A 0C6,[1] Canada

Received 14 July 1981/Accepted 5 January 1982

The biological control of flocculation interactions by factors related to growth under different conditions of aeration was documented with a new assay for flocculence. The degree of flocculence expressed in a genetically defined *Saccharomyces cerevisiae* strain (*FLO1/FLO1 ade1/ade1*) remained constant during aerobic growth but varied with aeration. Flocculence was repressed in anaerobically growing cells but was induced in stationary cells or cells returned to aerobic growth. Repression was correlated with the selective inactivation of cell surface lectin-like components. The changes in flocculence were accompanied by changes in 16 extractable proteins separated by electrophoresis; however, a clear correlation between specific protein bands and flocculence could not be established. The study clearly demonstrated that the phenotypic expression of *FLO1* could be reproducibly manipulated for experimental purposes by aeration alone.

In yeasts, flocculation or the aggregation of cells due to cell surface interactions is of considerable importance to the brewing industry (9, 24, 28). The discovery and mapping of a single gene, *FLO1*, that governs flocculation (23, 26) has stimulated an interest in this system as a model for the genetic control of specific cell surface interactions (17). Although a mechanism for flocculation interactions has not been validated in detail, it appears that specific protein-carbohydrate interactions are involved (16, 17, 27) and that the specificity of these interactions is dependent on the protein (16, 17). Among several approaches to these studies, three are particularly useful: comparisons between flocculent and nonflocculent strains that are isogenic except at the *FLO1* locus (16, 17); mutagenesis of genetically defined flocculent strains (10, 11); and induction of flocculation within a strain during growth (15, 18–20). Although the last approach was used in many more studies than are cited here, it is very difficult to draw general conclusions on the nature of induction, because in general the strains used were not genetically defined, they were grown under a variety of conditions which alter flocculation properties, and methods for measuring flocculence were varied and possibly imprecise. These considerations are critical for a proper interpretation of data especially because the expression of *FLO1* may be altered by suppressor genes, not all of which have been described (11, 17).

Using an assay developed here, we have examined the control of repression and induction of flocculence by aeration with defined strains of *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Strains. The flocculent strain S646-1B and the nonflocculent strain S646-8D have been described (17). Flocculent strain S230-13B, a trisomic for chromosome I, was heterozygous for *FLO1* and an unlinked suppressor.

Cell growth. Yeast cells were grown in YEPD (2% dextrose, 2% peptone, 1% yeast extract, pH 6.2; Difco Laboratories, Detroit, Mich.). Aerobic growth by method A was achieved in a 5,000-ml Microferm fermentor (New Brunswick Scientific Co., Edison, N.J.) at 30°C with vigorous agitation and high aeration to ensure saturation of media with dissolved oxygen. Cells were also grown in a shaker incubator at 200 rpm in 500-ml conical flasks filled with 100 ml of YEPD (method B) or 400 ml of YEPD (method C) at 30°C or 100 ml of YEPD at 24°C (method D). Anaerobic growth was achieved by the BBL Microbiology Systems GasPak system (Becton, Dickinson & Co., Mississauga, Ont.) at 30°C, with agitation delivered by a magnetic stirrer. A methylene blue indicator strip was used to verify removal of oxygen from the chamber.

The progress of growth was monitored every 2 h by the absorbance at 660 nm of the cell suspension, which was converted to cell density from standard curves. Cells were harvested at various stages of growth by centrifugation at 940 × *g*, and the cell pellets were washed at least two times with excesses of deionized water.

Microscopy. Optical dark-field micrographs were

† National Research Council publication no. 19993.

‡ Present address: ens BIO LOGICALS Inc., Ottawa, Ontario K1Z 6W4, Canada.

taken with a Leitz microscope equipped with a dark-field condenser and Polaroid Land Camera loaded with type 52 film.

Settling profiles and quantitation of free and aggregated cells. Cells were suspended in 2 mM EDTA, pH 8.0, to a cell density of 6.5×10^7 per ml (16, 17). Samples of 0.8 ml were placed in a 1-ml cuvette (1-cm path length). The suspensions were adjusted to 5 mM $CaCl_2$ from a stock solution of 100 mM $CaCl_2$, and the cuvettes were agitated for about 15 s to promote flocculation. The cuvettes were quickly placed in a modified Gilford 240 spectrophotometer with the light beam (50-μm diameter) placed 3 mm below the surface of the suspension. Absorbance was monitored automatically at a dwell of 2 s, and parallel measurements from four samples were recorded on a calibrated chart recorder.

Flocs (aggregated cells) and free cells were collected separately after the flocs had settled. Flocs were washed twice with 5 mM $CaCl_2$ by cycles of settling and resuspended in 0.8 ml of 2 mM EDTA. The cell density of both fractions was determined by absorbance at 660 nm, using standard curves.

The interactions between flocculent cells and repressed cells were examined as described previously (17). Briefly, a constant amount of flocculent cells was mixed with increasing amounts of repressed cells before the initiation of flocculation with $CaCl_2$. Subsequent analysis followed the above description.

Protein extraction. Cells were extensively washed with distilled water, suspended in 2 volumes of 10% mercaptoethanol–25 mM EDTA–1 mM phenylmethylsulfonyl fluoride–10 mM Tris-hydrochloride (pH 7.0), and incubated for 90 min at 30°C with shaking. Supernatants were collected after centrifugation at $940 \times g$ for 10 min. This was repeated twice, and the combined supernatants were clarified by centrifugation at 12,000 $\times g$ for 10 min. Cells were suspended in 2 volumes of 1 M sorbitol–60 mM EDTA–100 mM acetate buffer, pH 7.0, and digested with 200 μg of zymolyase 60,000 (Kirin Breweries, Miyahara, Japan) per g of wet cells for 1 h at 35°C with shaking. Supernatants were collected after centrifugation at $940 \times g$ for 10 min, and the pellets were washed with 1 M sorbitol. The supernatants were combined. Both extracts were divided. One portion was precipitated with cold 10% trichloroacetic acid and washed with ethanol, ethanol-ether (1:1), and ether. The other portion was dialyzed overnight with several changes of 2 mM EDTA and, finally, distilled water for 20 min. Protein was determined by the method of Lowry et al. (13), with bovine serum albumin as a standard, and carbohydrate was determined by the phenol-sulfuric acid method (8), with mannose as the standard.

To test for competitive inhibition of flocculation, extracts were adjusted to 400 μg of protein per ml in 2 mM EDTA and combined with flocculent cells under standard assay conditions. To test for agglutination of nonflocculent cells, extracts were combined with nonflocculent cells as above, and aggregation was examined by microscopy after various periods of time ranging from 1 to 5 h.

Electrophoresis. Proteins were dissolved in 2% sodium dodecyl sulfate–5% mercaptoethanol–60 mM Tris (pH 7)–10% glycerol and boiled for 5 min. About 150 μg (total dry weight) was loaded per well and separated on 12.5% acrylamide gels with a 5% stacking gel (12), using the Bio-Rad model 220 vertical slab gel apparatus. Electrophoresis was run at 10 mA per gel overnight. Protein bands were stained with 2.5% Coomassie blue in 50% methanol–10% acetic acid, and gels were destained in 40% methanol–5% acetic acid.

RESULTS

The flocculent strain S646-1B (*FLO1/FLO1 ade1/ade1*) and the nonflocculent strain S646-8D (*flo1/flo1 trp1/trp1*) were isogenic, homothallic diploids differing only at the *FLO1*, *ade1*, and *trp1* loci (17). The flocculent strain could be distinguished by the large cell aggregates or flocs, which formed in the presence of Ca^{2+} ions; however, free cells which did not form stable interactions were also evident (Fig. 1b). Cells of the nonflocculent strain interacted weakly but did not form aggregates (Fig. 1a). A flocculent strain, S230-13B, was trisomic for chromosome I and heterozygous for *FLO1* and expressed genes which suppressed the expression of *FLO1*. Although less flocculent than strain S646-1B, it was similar with respect to the characteristics of flocculence described for strain S646-1B (16, 17).

cCD. Flocculation could be quickly and conveniently demonstrated by monitoring settling profiles which reflect the decrease in turbidity of a cell suspension with time (Fig. 2a and c; 16). Upon addition of $CaCl_2$ to cell suspensions, deflocculated with EDTA, flocculent strains S646-1B and S230-13B displayed two components of settling owing to the rapid settling of flocs, F, and slower sedimentation of free cells, S. When flocs and free cells were collected separately, adjusted to the same cell density, and reexamined as above, identical settling profiles were obtained for both fractions; therefore, free and aggregated cells did not differ in their capacity for cell-cell interactions (data not shown). Nonflocculent strains yielded only a single component, S, independent of cell density (16, 17). In the flocculent strains component F varied in size with cell density (Fig. 2a and c). Quantitation of free and aggregated cells demonstrated that the amount of cells participating in flocculation was a linear function of cell density above a critical cell density (cCD; Fig. 2b and d) which differed between the two strains. It was also noticed that floc size increased with cell density and with time. Consistent with this was the observation that the settling rates, indicated by the slopes of component F, increased with cell density, which in turn improved the resolution of components F and S (Fig. 2a and c). The density of free cells, however, was relatively constant and was equivalent to the cCD (Fig. 2b and d).

The cCD was reproducibly estimated from the settling profiles by converting the absorbance

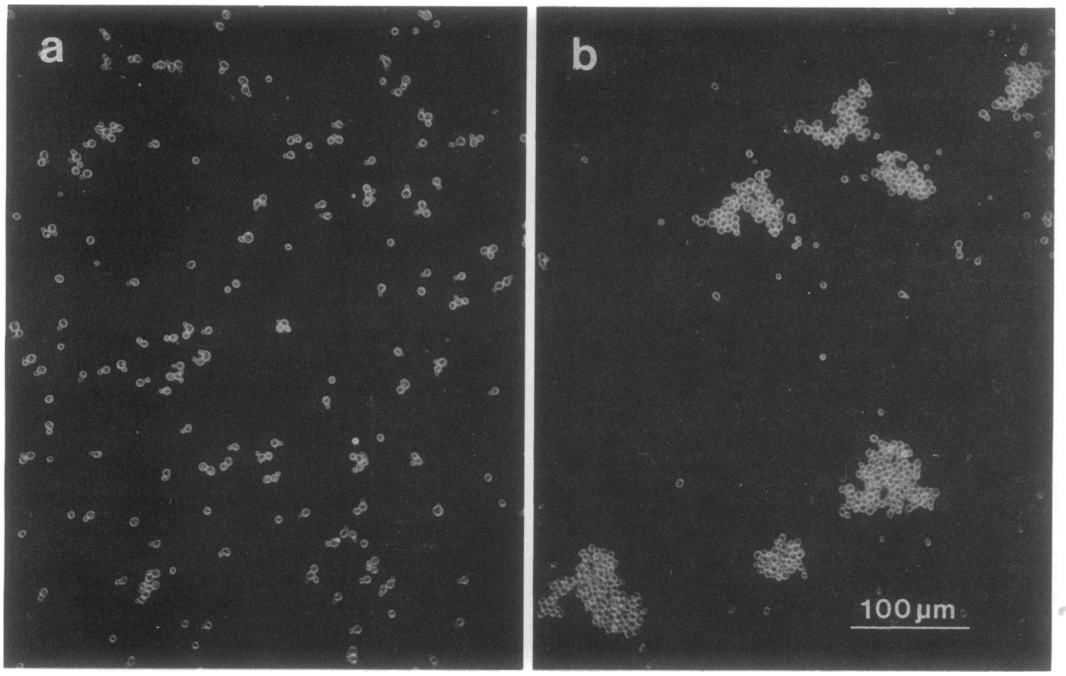

FIG. 1. Optical dark-field micrograph illustrating (a) the weak interactions among cells of the nonflocculent strain S646-8D and (b) the aggregation of flocculent cells of strain S646-1B, both in the presence of $CaCl_2$.

value at the intersection of components F and S to cell density, using standard curves (Tables 1 and 2). These values reflected visual differences in the degree of flocculence between strain S646-1B, which was strongly flocculent with a cCD of 7.4×10^6 cells per ml, and S230-13B, which was moderately flocculent with a cCD of 36×10^6 cells per ml (Table 2). For comparative purposes, the estimated value of the cCD provided a rapid and sensitive method for measuring degrees of flocculence.

In this assay, the composition of the suspending medium influenced the degree of flocculence considerably. The cCDs obtained for strain S646-1B in growth media were approximately reproduced in 2 mM EDTA (pH 8) and 100 mM sodium acetate (pH 4.5) after addition of 5 mM $CaCl_2$. In Tris buffer at pH 7.5, flocculence was inhibited, and in phosphate buffer at pH 7.0 it was enhanced (Table 1). Routine assays were therefore performed in 2 mM EDTA, pH 8, at cell densities of about 6.5×10^7 per ml with the addition of 5 mM $CaCl_2$ to initiate flocculation from the deflocculated state. These conditions optimized Ca^{2+}-dependent flocculation and minimized substitution of Ca^{2+} ions by other divalent cations (17). The use of cell densities that were about 10-fold greater than the cCD ensured clear resolution of the intersection of components F and S.

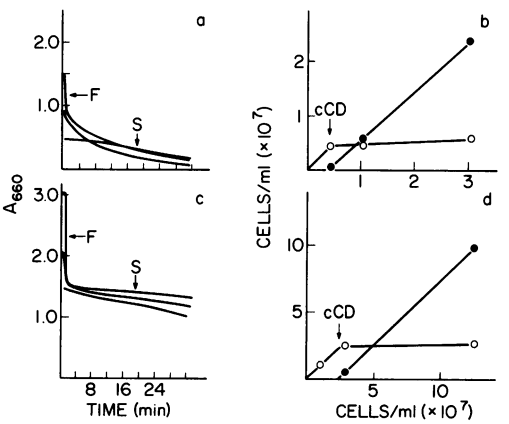

FIG. 2. Relationship between flocculation and cell density. Cells of strains S646-1B (a, b) and S230-13B (c, d) were harvested during aerobic logarithmic growth by method B. Cells were resuspended at different cell densities in 2 mM EDTA, and flocculation was initiated with 5 mM $CaCl_2$. (a, c) Settling profiles were recorded automatically by the change in absorbance at 660 nm (A_{660}) with time. Component F, due to the rapid settling of flocs, and component S, due to the slow sedimentation of free cells, are indicated. (b, d) The amounts of free (○) and aggregated (●) cells were measured separately (ordinate) after 3 to 5 min of settling for each sample, varying in cell density (abscissa). The cCDs are indicated.

TABLE 1. Variation of flocculence in various buffers

Buffer	cCD (cells/ml × 10^6)[a]
YEPD, pH 5.7[b]	8.0
10 mM sodium phosphate, pH 7.0	2.9
100 mM sodium acetate, pH 4.5	8.8
2 mM EDTA, pH 8.0	6.4
25 mM Tris, pH 7.5	NF[c]

[a] Estimated from the intersection of components F and S in settling profiles of strain S646-1B.

[b] Flocculation was measured in YEPD before cell harvest without addition of 5 mM $CaCl_2$. In other samples $CaCl_2$ was used to initiate flocculation.

[c] NF, Nonflocculent.

Relationship of flocculation to aeration and growth stage. The degree of flocculence in logarithmically grown cells of strain S646-1B varied with the aeration of the culture media. Forced aeration in a fermentor (method A) yielded the greatest flocculence, indicated by the low cCD, 2.1 × 10^6 cells per ml (Table 2). Shaking in partially filled flasks (method B) and filled flasks (method C) yielded higher cCDs of 7.4 × 10^6 and 11 × 10^6 cells per ml, respectively, reflecting lower degrees of flocculence (Table 2). In anaerobically growing cells, flocculence was completely repressed (Table 2; Fig. 3). Glucose repression could not account for the relationship between aeration and flocculation since aerobic growth in media varying from 2 to 20% glucose was not correlated with a decrease in flocculence (Table 2).

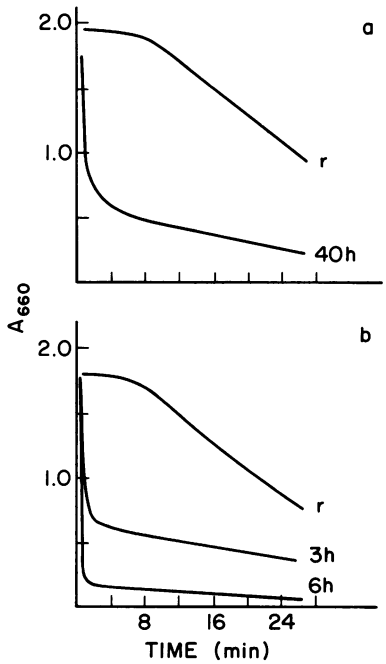

FIG. 3. Induction of flocculence illustrated by settling profiles. Flocculence was repressed in strain S646-1B during logarithmic anaerobic growth (r) but was induced (a) in stationary cells grown anaerobically for 40 h or (b) in growing cells returned to aerobic growth for 3 and 6 h by method A. Settling profiles were recorded as in the legend to Fig. 2. A_{660}, Absorbance at 660 nm.

TABLE 2. Variation of flocculence in logarithmically grown cells under different conditions of aeration and glucose concentrations

Strain	Growth condition[a]	cCD (cells/ml × 10^6)[b]
S230-13B	Aerobic (B)	36.0 ± 4.0
S646-1B	Aerobic (A)	2.1 ± 0.4
	Aerobic (B)	7.4 ± 1.3
	Aerobic (C)	11.3
	Aerobic (D)	5.0
	Anaerobic	NF
	Anaerobic induction[c]	7.4
	Aerobic induction (A)[d]	1.6
	Aerobic induction (B)	7.2
	2% glucose (B)[e]	8.7
	5% glucose (B)	8.3
	10% glucose (B)	6.5
	15% glucose (B)	8.1
	20% glucose (B)	5.7

[a] The method of aerobic growth is shown in parentheses.

[b] Measured in 2 mM EDTA as described in Table 1. NF, Nonflocculent.

[c] Anaerobically induced flocculence was measured in stationary cells grown for 40 h, as in the legend to Fig. 3.

[d] Aerobic induction was measured in cells growing logarithmically for 6 h, as in the legend to Fig. 3.

[e] The growth medium was YEPD varying in glucose concentration.

It is generally reported that flocculation appears in the stationary growth stage (9). Strain S646-1B, however, was equally flocculent at all stages of aerobic growth when assayed in 2 mM EDTA or 100 mM sodium acetate (Table 3). When assayed in growth media before cell harvest, flocculence was absent during early logarithmic growth and appeared during logarithmic growth. This apparent onset reflected the attainment of the cCD by the cell culture and not induction. During growth, the pH of the culture media (Table 3) did not vary sufficiently to alter flocculence (17). Furthermore, concentration of early-log-phase cells to levels above the cCD in the same media resulted in flocculation (data not shown). Thus, aerobic growth of strain S646-1B was not accompanied by changes in flocculence. The same results were obtained when cells were grown aerobically in 0.67% yeast nitrogen base–

TABLE 3. Expression of flocculence during cell growth under aerobic and anaerobic conditions

Growth condition	Strain	Growth stage	Time (h)	Medium pH	Cell density (× 10^6/ml)	cCD (cells × 10^6/ml)[a]		
						YEPD	2 mM EDTA	100 mM acetate
Aerobic[b]	S646-1B	Early log	4.5	6.1	5	NF	9.4	9.8
		Mid-log	7.8	5.7	26	7.7	9.3	9.5
		Stationary	23.0	5.3	78	10.2	8.8	10.0
	S646-8D	Early log	4.5	5.9	11	NF	NF	NF
		Mid-log	7.8	5.3	54	NF	NF	NF
		Stationary	23.0	5.9	170	NF	NF	NF
Anaerobic	S646-1B	Log	19.0	5.6	8	NF	NF	NF
		Stationary	42.0	5.3	17	6.5	7.0	8.0
	S646-8D	Log	19.0	5.7	4	NF	NF	NF
		Stationary	42.0	5.2	5	NF	NF	NF

[a] Measured as in Table 1.
[b] Aerobic growth was by method B.

2% dextrose–20 μg of adenine per ml (data not shown). Strain S646-8D was nonflocculent under all of the above conditions; however, it grew more quickly and attained eight generations before reaching stationary stage, compared with seven for the flocculent strain (Table 3).

A very different situation was observed during anaerobic growth. Repression of flocculence in growing cells was reversed in stationary cells (Fig. 3a; Table 3). The degree of flocculence was comparable to that obtained by aerobic growth by method B but did not attain the levels achieved by cells grown by method A (Tables 2 and 3). The nonflocculent strain S646-8D remained nonflocculent under anaerobic growth conditions (Table 3). The growth of both strains was significantly retarded during anaerobiosis (Table 3).

Induction of flocculence was also achieved in growing cells by switching from anaerobic to aerobic growth (Fig. 3b; Table 2). The level of flocculence attained depended on the method of aeration (Table 2). The cCDs obtained were comparable to those of cells grown continuously under aerobic conditions, suggesting that the induction of flocculence was complete. By controlling aeration of the culture alone, it was possible to obtain cells from the same strain with various degrees of flocculence and examine the induction of flocculence under two different conditions.

Selective repression of lectin-like activity. At least two distinct components of the cell surface seem to be involved in the mechanism of flocculation (16, 17). A lectin-like protein may be associated with cells in which *FLO1* is expressed, and a carbohydrate structure associated with the mannan layer of the yeast cell wall may provide recognition and binding sites. The former was distinguished by its selective sensitivity to proteinase K and mercaptoethanol. The latter remained available for interactions with flocculent cells. Similarly, repression by anaerobiosis was characterized by the selective loss or inactivity of the lectin-like component since repressed cells were capable of extensive interactions with flocculent cells. When increasing amounts of repressed cells were allowed to interact with a constant amount of flocculent cells, the proportions of repressed cells sequestered into flocs exceeded the proportions achieved by aerobically grown nonflocculent cells of strain S646-8D (Fig. 4; 16) or flocculent cells of strain S646-1B selectively inhibited by mercaptoethanol or proteinase K (17). This did not indicate residual lectin-like activity since the proportion of interacting nonflocculent cells grown anaerobically also exceeded those grown

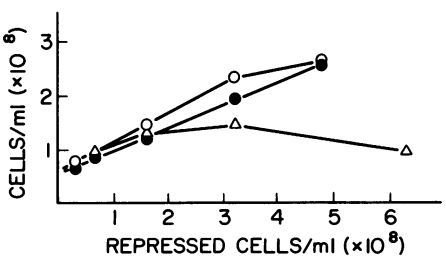

FIG. 4. Interactions between repressed cells and flocculent cells of strain S646-1B. In the presence of 2 mM EDTA, a constant amount of flocculent cells of strain S646-1B, grown aerobically, was mixed with different amounts (abscissa) of repressed cells of strain S646-1B (●) or S646-8D (○) grown anaerobically. After flocculation was initiated with $CaCl_2$, the amount of aggregated cells was measured (ordinate). For comparative purposes the interactions between flocculent cells and aerobically grown nonflocculent cells (△) are included (16).

aerobically (Fig. 4; 16). The enhanced interactions may therefore reveal other general alterations in the cell wall resulting from the different conditions of growth.

Examination of proteins. Proteins were examined from cells in which flocculation was repressed under anaerobic conditions and during the induction of flocculation in aerobically growing cells or stationary cells grown anaerobically. Proteins were extracted by mercaptoethanol and zymolyase since flocculation was irreversibly lost after these treatments but not after a variety of others (16). Both extracts consisted predominantly of proteins after dialysis; however, about 30 to 40% carbohydrate was also present. Zymolyase extracted about 18 mg of protein per g of wet cells from both strains, whereas mercaptoethanol extracted about 11 mg per g of wet cells from repressed cells, which decreased to 7 mg per g of wet cells during aerobic induction of the flocculent strain S646-1B. The nonflocculent strain S646-8D yielded 11 mg of protein per g of wet cells after parallel growth, confirming that flocculent strains undergo a more rigid structuring of their cell walls than nonflocculent strains (25). Different subsets of proteins were extracted by zymolyase and mercaptoethanol (Fig. 5). After the induction of flocculation by aerobiosis or in stationary anaerobically grown cells, 16 protein bands changed in relative intensity. Eleven of these were revealed in extracts by mercaptoethanol (M1 to M11) and five were revealed in extracts by zymolyase (Z1 to Z5). Bands M1, M2, M3, M4, M6, and M10 accumulated during aerobic induction in both strains (Fig. 5, left) but were barely visible in extracts from anaerobically grown stationary cells (Fig. 5, right). These bands were therefore not correlated with the onset of flocculation in stationary cells and may have been associated with other processes related to aerobiosis. Bands M5 and M7 also could not be correlated with the onset of flocculation. Although these bands decreased in relative proportion during both aerobic and anaerobic induction of flocculation in the flocculent strain, parallel changes occurred in the nonflocculent strain (Fig. 5). Band M8 was not correlated with flocculation since it did not change in relative intensity during aerobic or anaerobic induction in our flocculent strain (Fig. 5); however, it was greatly depleted in extracts from the nonflocculent strain grown to station-

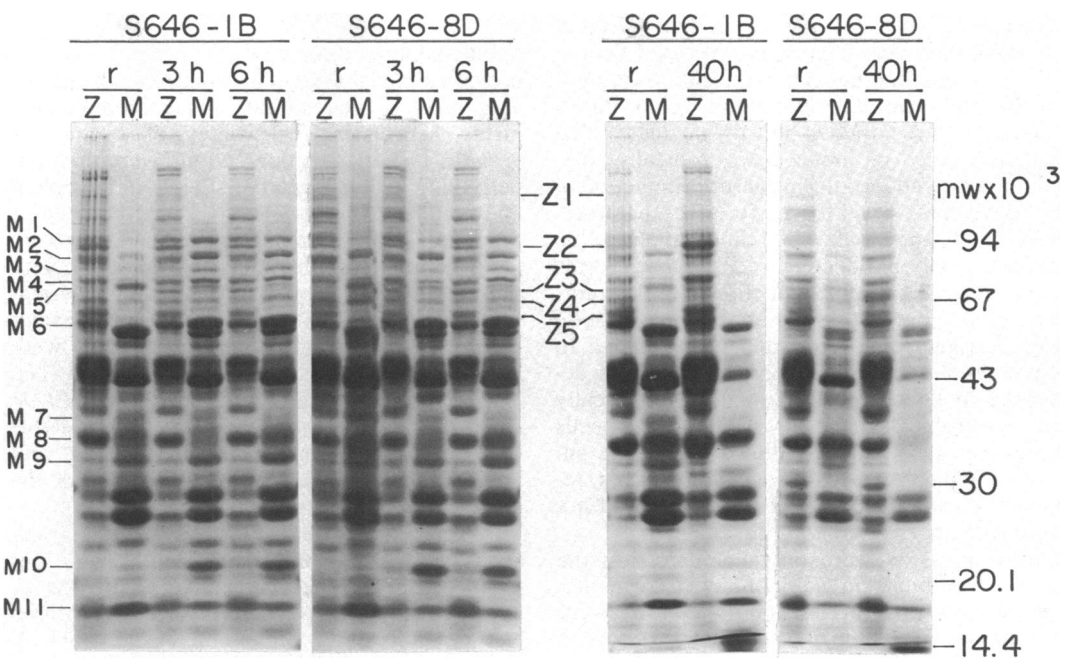

FIG. 5. Electrophoretic separation of proteins on 12.5% polyacrylamide gels (12). Proteins were extracted by zymolyase (Z) and mercaptoethanol (M) from strains S646-1B and S646-8D grown under repressive conditions (r) and during (left) aerobic induction for 3 and 6 h or (right) anaerobic induction for 40 h as described in the legend to Fig. 3. Proteins M1 to M11 were identified among mercaptoethanol extracts, whereas Z1 to Z5 were identified among zymolyase extracts. The mobilities of the molecular weight standards, phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin, are indicated in descending order by their molecular weights.

ary stage anaerobically (Fig. 5, right). Similarly, the relative proportions of band M9 remained unaltered during aerobic induction in flocculent and nonflocculent strains (Fig. 5, left), but were reduced in anaerobically grown stationary cells of both strains (Fig. 5, right); therefore, its presence seemed to be correlated with logarithmic growth rather than flocculation. Band M11 decreased during aerobic induction (Fig. 5, left) but remained elevated during anaerobic induction in both strains (Fig. 5, right); therefore, it seemed to be correlated with anaerobic processes other than flocculation. On the basis of apparent molecular weights, bands M5, M7, and possibly M2 and M3 were not extracted by zymolyase; however, within parallel zymolyase extracts, bands comparable to M1, M4, M6, M8, and M11 were present in approximately similar yields and bands comparable to M9 and M10 were present in much lower yields. These bands, however, did not change in relative intensity in zymolyase extracts, suggesting that the ease of their extractions by mercaptoethanol was due to structural rearrangements in the cell wall under the various growth conditions.

Zymolyase extracts contained five bands which changed in relative intensity during aerobic and anaerobic induction. The disappearance of band Z1 was correlated with the induction of flocculation. Band Z1 was not a major protein but was relatively elevated in repressed flocculent cells only and became reduced during both aerobic and anaerobic induction (Fig. 5). Bands Z2 and Z5 were enriched only in flocculent cells, were repressed or induced anaerobically (Fig. 5), and were reduced during aerobic induction of the flocculent strain (Fig. 5, left); therefore, these bands appeared to be related to anaerobic processes only in flocculent cells. Band Z3 was enriched during aerobic induction of both strains (Fig. 5, left) but not during anaerobic induction (Fig. 5, right); therefore, it may be related to processes involved in aerobiosis. Band Z4 decreased in flocculent cells induced aerobically and anaerobically and in nonflocculent cells during aerobic induction; however, during anaerobic induction of nonflocculent cells it remained elevated (Fig. 5). Therefore, it did not seem to be correlated with flocculence.

Since proteins were only compared on the basis of mobility in sodium dodecyl sulfate, several control experiments not shown were performed. Exposure of zymolyase extracts to mercaptoethanol and mercaptoethanol extracts to zymolyase did not alter the mobilities of bands. Proteases were detected in extracts of repressed cells but were inhibited by phenylmethylsulfonyl fluoride and did not alter the protein patterns during prolonged incubations. Phenylmethylsulfonyl fluoride did not inhibit flocculation. Equivalent banding patterns were obtained from extracts collected by trichloroacetic acid precipitation and by dialysis, suggesting that selective losses were not responsible for the observed differences in protein bands. Changes in protein extractability accompanied induction, but similar banding patterns were obtained in duplicated experiments and during repeated extractions of the same cell sample, suggesting that the differences were meaningful.

Attempts were made to detect lectin activity in crude extracts (400 μg/ml) by competitive inhibition of flocculation and aggregation of nonflocculent cells. Massive precipitation of the extracts upon addition of $CaCl_2$, however, hampered the estimations of cCD; therefore, visual and microscopic observations were subjectively interpreted. Nonflocculent cells were not aggregated by any of the extracts, as might be expected if multivalent lectin was present. Marginal inhibition of flocculation by zymolyase extracts of both aerobically and anaerobically induced flocculent cells was visually apparent, but the specificity of inhibition was not certain; therefore, an unambiguous recovery of active proteins had not been achieved.

DISCUSSION

Flocculation, directed by the gene *FLO1*, may provide a model system for studies on the genetic control of cell surface recognition mechanisms (17). During the progress of aerobic cell growth, flocculation interactions were unmodified, allowing comparative studies of flocculent and nonflocculent strains (16, 17). In this report the repression and induction of flocculation were controlled by growth conditions, and the potential of these techniques for providing information on the mechanism of flocculation was examined. The strains of *S. cerevisiae* used were homothallic diploids as described earlier (17). The flocculent strain S646-1B (*FLO1/FLO1 ade1/ade1*) and the nonflocculent strain S646-8D (*trp1/trp1*) were isogenic except for *FLO1* and the marker genes and were derived from the same parental lab strain.

A primary requirement for these studies was an assay which could distinguish varying degrees of flocculence. Subjective evaluation of flocculence during aerobic growth in cultures can be misleading because a cCD, which is strain dependent, must be attained before flocculation is observed. Subsequent growth appears to be accompanied by an intensification of flocculence but in fact reflects the linear relationship between the amount of cells participating in flocculation and cell density. Although flocculence appeared to be induced during aero-

bic growth, it was in fact unmodified. Furthermore, strain differences in cCD could determine differences in the time of the apparent induction.

The apparent induction of flocculence in aerobic growth media may be erroneously indicated by several assays, without a clear documentation of the relationships between flocculation measurements and cell density. Generally, assays are variants of a few basic types which rely on the size of flocs, proportions of aggregated and free cells, rates of sedimentation, subjective evaluation, or specific equipment (5). The results presented here suggest that floc size or proportions of aggregated to free cells vary with cell density. Similarly, settling rates reflect floc size, which is cell density dependent. For meaningful comparisons of flocculence by these methods, uniform initial cell densities must be used. Even then, however, the measurements become progressively less discriminating as densities exceed the cCD; therefore, the choice of the cell density used relative to the cCD of each population of flocculent cells has a significant effect on the measurements and interpretations of results. Other factors, such as the composition of the media in which the assay is performed, also affect the degree of flocculence. Unfortunately, standard assay conditions are not in use, and comparisons of degrees of flocculence among studies are impossible.

These findings stress the need for a reliable and simple assay for flocculence. The value of cCD appears to reflect the intensity and stability of flocculation interactions, and it is independent of the variability associated with cell density. The cCD can be estimated from the density of cells remaining free in suspension shortly after the flocs have settled or at the intersection of components F and S in settling profiles. Free cells appear to aggregate into primary flocs randomly and progressively until the density of free cells is lowered to the cCD. Thereafter free cells may be unable to form stable interactions. The use of cell densities 10-fold greater than the cCD assures clear delineation of the intersection of components F and S in settling profiles. Only small amounts of cell suspension are required, and several measurements can be performed simultaneously. The composition of the suspending medium affects flocculence and the cCD value. Routine assays were performed in 2 mM EDTA to ensure that cells were not interacting before initiating flocculation by the addition of $CaCl_2$. The use of pH 8 rather than the pH of the growth media (pH 5 to 6) ensured Ca^{2+}-dependent flocculence and minimized substitution by other divalent cations (17). Unlike other assays, measurements of flocculence by cCD may be standardized among different laboratories so that consistency in the literature can be achieved. Woof (29) described a conceptually similar assay which measured the free cells as a terminal count.

The repression and induction of flocculence during growth were examined under aerobic and anaerobic conditions. In strain S646-1B, repression was only observed among anaerobically growing cells. Induction occurred in stationary cells or growing cells returned to aerobic conditions. This finding appears to be novel since repression and induction during aerobic growth are generally reported (9), and a variety of factors that affect the onset of flocculation has been studied (1, 2, 9, 15, 18–20). When assays were performed in samples of uniform cell density (18–20, 22), apparent induction did not seem to account for these differences in results. Since aeration alone has such a profound influence on flocculation and is a highly variable parameter in other studies, although the degree of flocculence varied, repression in logarithmically growing cells was confined to anaerobically grown cells; therefore, aeration methods also could not account for the difference. Similarly, variations in the pH of growth media between 5 and 6 and growth temperature between 24 and 30°C did not alter these observations. Glucose repression, as reported for the sex-directed flocculation interactions of *Schizosaccharomyces pombe* (4, 7), did not apply to flocculation in strain S646-1B. Other factors, such as the cation components of the growth media (1, 20, 22), were not examined; however, YEPD components were not involved. The number of genes which suppress the expression of *FLO1* is not known; however, care was taken to segregate extraneous suppressor genes from strain S646-1B. The repression of flocculation in other strains during logarithmic growth may be due to genes not encountered in our strains. Whether the expression of genes controlling the repression of flocculence in anaerobically growing cells of our strain can be dissociated from fundamental cellular processes is not yet known. Some studies imply that mitochondria may participate in the expression of flocculence in *S. cerevisiae*, although the results are not yet clear (11, 20), and in the induction of sex-directed flocculation in *S. pombe* (3, 6).

Although evidence suggests that proteins are important in flocculation (21) and differ in extracts of flocculent and nonflocculent cells (10, 14), views on the nature of their participation vary greatly (2, 16, 17, 27). In earlier reports we considered a mechanism for flocculation interactions which involved protein-carbohydrate interaction (16, 17). Certain mannan carbohydrates common to several yeast strains were believed to act as specific binding sites for a lectin-like protein found only on flocculent cells. During repression of flocculence these carbohydrates

were more available for interaction than under nonrepressive conditions; however, the lectin-like activity was either inhibited or absent. The proteins extracted from flocculent and nonflocculent strains were examined by electrophoresis during repression or induction achieved aerobically or anaerobically. Our analysis of the data was based on the assumption that flocculence induced aerobically or anaerobically required the same critical proteins in the cell surfaces which differ from those on nonflocculent cells. A clear picture of the nature of repression and induction did not emerge. The relative intensities of 16 protein bands, extracted by zymolyase and mercaptoethanol, changed during the induction of flocculation by aerobiosis and anaerobiosis. Most of these changes could be correlated with events other than flocculation. A few were strain specific; however, changes in the relative intensity of only band Z1 could be correlated with flocculence, and it decreased as flocculence increased. It is possible that specific proteins govern the repression of flocculation in anaerobically growing cells. Bands specific to flocculent cells and their accumulation under both conditions of induction could not be observed. A protein has been isolated from alkali extracts of flocculent cells which was not present in extracts from nonflocculent mutants (10). Similar differences were not obtained with our strains (unpublished data).

Whether active lectin-like proteins were extracted is not known because the activity could not be detected in the extracts or on the cells after exposure to mercaptoethanol and zymolyase. Several alternative explanations may account for these observations. The lectin-like activity may depend on the constraints imposed by the cell wall for activity. When associated with the cell wall, it was stable and could not be dissociated by urea, sodium dodecyl sulfate, Triton X-100, boiling, boiling in sodium dodecyl sulfate, EDTA, or mild treatment with NaOH (16). It may have been inhibited by the conditions of extraction whether extracted from the cell wall or not. It may have been competitively inhibited by coextracted carbohydrates or glycoproteins. The recovery of proteins active in flocculation has not been achieved in any studies but is necessary proof for the model presented earlier (17); therefore, this subject must be examined in greater depth in a separate report.

In this study, the control of the phenotypic expression of *FLO1* by aeration and by factors expressed only during anaerobic growth has been documented in strain S646-1B. Although the control mechanism remains unknown, the potential for studying differential gene expression and control of cell surface recognition processes exists. We have presented aspects of the mechanism and control of flocculence here and earlier (17); however, more in-depth studies are required. We are currently examining the interactions at the cell surface with biophysical techniques and the transcriptional and translational products of the flocculent and nonflocculent cells during changes in flocculation activity.

ACKNOWLEDGMENT

We are grateful to M. Dove for the electrophoresis of proteins.

LITERATURE CITED

1. **Amri, M. A., R. Bonaly, B. Duteurtre, and M. Moll.** 1979. Interrelation between Ca^{2+} and K^{+} ions in the flocculation of two brewers yeast strains. Eur. J. Appl. Microbiol. Biotechnol. **7**:235–240.
2. **Beavan, M. J., D. M. Belk, G. G. Stewart, and A. H. Rose.** 1980. Changes in electrophoretic mobility and lytic enzyme activity associated with development of flocculating ability in *Saccharomyces cerevisiae*. Can. J. Microbiol. **25**:888–895.
3. **Calleja, G. B.** 1973. Role of mitochondria in the sex-directed flocculation of a fission yeast. Arch. Biochem. Biophys. **154**:382–386.
4. **Calleja, G. B.** 1974. On the nature of the forces involved in the sex-directed flocculation of a fission yeast. Can. J. Microbiol. **20**:797–803.
5. **Calleja, G. B., and B. F. Johnson.** 1972. A comparison of quantitative methods for measuring yeast flocculation. Can. J. Microbiol. **23**:68–74.
6. **Calleja, G. B., and B. F. Johnson.** 1979. Temperature sensitivity of flocculation induction, conjugation and sporulation in fission yeast. Antonie van Leeuwenhoek J. Microbiol. Serol. **45**:391–400.
7. **Calleja, G. B., B. F. Johnson, and B. Y. Yoo.** 1980. Macromolecular changes and commitment to sporulation in the fission yeast *Schizosaccharomyces pombé*. Plant Cell Physiol. **21**:613–625.
8. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
9. **Geilenkotten, I., and E. J. Nyns.** 1971. The biochemistry of yeast flocculence. Brew. Dig. **April**:64–70.
10. **Holmberg, S.** 1978. Isolation and characterization of polypeptide absent from nonflocculent mutants of *Saccharomyces cerevisiae*. Carlsberg Res. Commun. **43**:401–413.
11. **Holmberg, S., and M. C. Kielland-Brandt.** 1978. A mutant of *Saccharomyces cerevisiae* temperature sensitive for flocculation. Influence of oxygen and respiratory deficiency on flocculence. Carlsberg Res. Commun. **43**:37–47.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Marfey, P., S. C. Sørensen, and M. Ottesen.** 1977. Studies on yeast flocculation. Comparison of enzymatic digests of flocculent and nonflocculent cells of *Saccharomyces carlsbergensis*. Carlsberg Res. Commun. **42**:353–367.
15. **Masschelein, C. A., C. Jennehomme-Ramos, C. Castiau, and A. Devreux.** 1963. Mechanism of phenotypic variations in the flocculence character of yeast. J. Inst. Brew. **69**:332–338.
16. **Miki, B. L. A., N. H. Poon, A. P. James, and V. L. Seligy.** 1980. Flocculation in *Saccharomyces cerevisiae*: mechanism of cell-cell interaction, p. 165–170. *In* G. Stewart and I. Russell (ed.), Current development in yeast research. Pergamon Press Canada Ltd., Toronto.
17. **Miki, B. L. A., N. H. Poon, A. P. James, and V. L. Seligy.**

1982. Possible mechanism for flocculation interactions governed by the gene *FLO1* in *Saccharomyces cerevisiae*. J. Bacteriol. **150**:878–889.

18. **Mill, P. J.** 1964. The effect of nitrogenous substances on the time of flocculation in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **35**:53–60.
19. **Nishihara, H., T. Toraya, and S. Fukui.** 1976. Factors affecting flocculation of brewer's yeast. J. Ferment. Technol. **54**:351–355.
20. **Nishihara, H., T. Toraya, and S. Fukui.** 1976. Induction of floc-forming ability in brewer's yeast. J. Ferment. Technol. **54**:356–360.
21. **Nishihara, H., T. Toraya, and S. Fukui.** 1977. Effect of chemical modification of cell surface components of a brewer's yeast on the floc-forming ability. Arch. Microbiol. **115**:19–23.
22. **Porter, A. M., and R. J. Macaulay.** 1965. Studies on flocculation. I. A relationship between the pH and calcium content of the growth medium. J. Inst. Brew. **71**:175–179.
23. **Russell, I., G. G. Stewart, H. P. Reader, J. R. Johnston, and P. A. Martin.** 1980. Revised nomenclature of genes that control yeast flocculation. J. Inst. Brew. **86**:120–121.
24. **Stewart, G. G.** 1975. Yeast flocculation. Practical implications and experimental findings. Brew. Dig. **March**:42–62.
25. **Stewart, G. G., and I. F. Garrison.** 1972. Some observations on co-flocculation in *Saccharomyces cerevisiae*. Am. Soc. Brewers, Chem. Proc., p. 118–131.
26. **Stewart, G. G., and I. Russell.** 1977. The identification, characterization, and mapping of a gene for flocculation in *Saccharomyces* sp. Can. J. Microbiol. **23**:441–447.
27. **Taylor, N. W., and W. L. Orton.** 1978. Aromatic compounds and sugars in flocculation of *Saccharomyces cerevisiae*. J. Inst. Brew. **84**:113–114.
28. **Windisch, S.** 1968. Flocculation of brewing yeasts. Brew. Dig. **November**:62–66.
29. **Woof, J. B.** 1962. An approach to yeast sedimentation characteristics. J. Inst. Brew. **68**:315–317.