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NOTE

Characterization of a spontaneous flocculation mutant derived from *Candida glabrata*: A useful strain for bioethanol production

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A spontaneous flocculation mutant (Cgflo1) was obtained from *Candida glabrata*. Flocculation of Cgflo1 was dependent on divalent cations such as Ca^{2+} and Mg^{2+} and was inhibited by galactose. Cgflo1 is believed to be a useful strain for bioethanol production based on its flocculation ability at higher temperatures.

[**Key words:** *Candida glabrata*; Flocculation; Bioethanol production; Divalent cations; Lectin-sugar complex; Galactose]

The production and utilization of bioethanol as an alternative fossil fuel have attracted considerable attention in the effort to combat global warming and improve energy reserves. Bioethanol production generally utilizes derivatives from food crops such as corn grain and sugar cane (1). The development of effective bioethanol production processes from the lignocellulose contained in wood and plant stems is currently being attempted by many research groups (2, 3). To facilitate these efforts, both saccharification and fermentation processes must be improved.

Flocculation is the asexual and reversible aggregation of yeast cells to form flocs, which rapidly form a sediment at the bottom of a liquid substrate (4). There have been many studies to date on a flocculent *Saccharomyces cerevisiae* strain for bioethanol production (5–7). Industrial fermentation for bioethanol production involves repeated-batch fermentation or a continuous fermentation process. In the batch fermentation process, the fermented yeast cells must be removed prior to further processing. The separation of cells in suspension may have to be carried out by centrifugation or filtration, both of which are expensive procedures. However, if flocculent strains are used, the separation of cells can be achieved without such expensive procedures by using batch fermentation systems. Kida et al. report a highly efficient continuous fermentation system that utilizes flocculent yeast strains (5). Thus, it is possible that flocculent yeast could be advantageous in industrial fermentation as part of the process of bioethanol production.

Calcium-dependent flocculation mechanisms in *S. cerevisiae* have been studied in detail (8). Flocculation in *S. cerevisiae* is mediated by specific cell-surface lectins (flocculins) that possess the ability to bind directly to the mannose residues of mannan in neighboring cells. To date, two different flocculation phenotypes have been characterized on the basis of their sensitivities to sugar inhibition: Flo1 (mannose-sensitive) and NewFlo (mannose- and glucose-sensitive) in *S. cerevisiae* (8). In *Schizosaccharomyces pombe*, cell-surface galactosylation is essential for nonsexual flocculation (9).

We previously suggested that *Candida glabrata* may be useful for bioethanol production under acidic conditions to prevent bacterial contamination (10). Because the strain is relatively acid- and heat-tolerant and enables effective ethanol conversion, *C. glabrata* is expected to be useful in the development of an effective fermentation process. It is already known that several *Candida* strains do not grow sufficiently to produce ethanol under anaerobic conditions (11). In contrast, *C. glabrata* is able to contribute to ethanol production since it is able to grow under anaerobic conditions; the characteristics of *C. glabrata* with respect to ethanol production are similar to those of *S. cerevisiae* (12).

In the present report, we describe the characteristics of a flocculation mutant isolated from *C. glabrata*. The mechanisms of adhesion to human cells and biofilm formation of *C. glabrata* have been analyzed previously in terms of pathogenesis (13), but to the best of our knowledge, no studies have been carried out to date with the aim of characterizing flocculation mutants of *C. glabrata*.

We discovered that a certain number of cells would form a sediment during the cultivation of *C. glabrata* NFRI3165 (wild-type strain) in YPD liquid medium (1% yeast extract, 2% peptone and 2% glucose) at 30 °C. We extracted the sedimented fraction and spread it onto YPD agar medium. The flocculation ability of the selected strains observed in YPD medium was estimated by test-tube assay. Briefly, cells grown in YPD medium for 24 h at 30 °C were left in test tubes (diameter, 16 mm; length, 150 mm) at room temperature for 2 min and sedimentation was then compared by visual observation. Most of the isolated strains sedimented within 2 min, while the wild-type strain required more than 6 h for sedimentation. A mutant exhibiting typical sedimentation among mutant strains was denominated Cgflo1 and this strain was used for further analysis.

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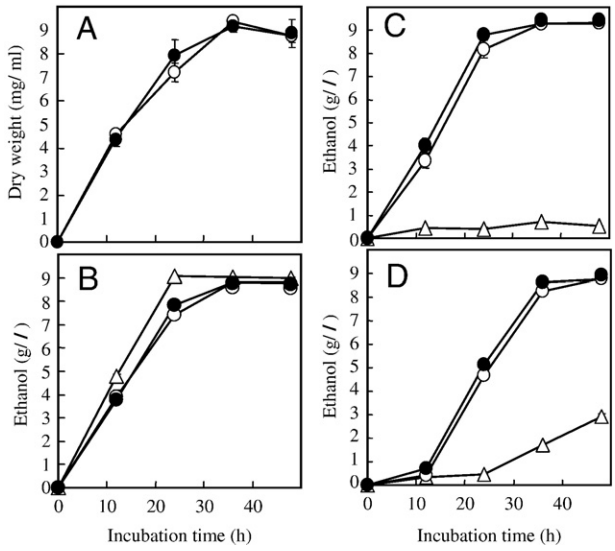

FIG. 1. The growth (A) and ethanol production (B–D) of the wild-type strain and the Cgflo1 mutant under various conditions. (A) The wild-type (open circles) and Cgflo1 (closed circles) strains were cultivated in YPD medium at 30 °C, pH 6.2, with shaking, and the dry weight of the cells was measured. The wild-type (open circles), Cgflo1 (closed circles) and *S. cerevisiae* (open triangle) strains were cultivated in YPD medium at (B) 30 °C (pH 6.2), (C) 42 °C (pH 6.2), and (D) 30 °C (pH 2.5) in static culture, and the concentration of ethanol that accumulated in the media was monitored.

To assess the influence of the mutation on the growth of Cgflo1, we measured time-dependent changes in the dry weight of cells cultured in YPD medium with shaking at 120 rpm (pH 6.2, 30 °C) (Fig. 1A). To determine the dry weight of cells, the centrifuged cell pellets was washed twice with water and then air-dried at 105 °C. The growth of the wild-type and Cgflo1 strains were almost identical (Fig. 1A). In a static culture, both Cgflo1 and the wild-type cells sedimented on the bottom during the incubation.

We also examined the ethanol productivities of the wild-type and Cgflo1 strains compared with those of *S. cerevisiae* (NBRC 0224) in YPD medium at various temperatures (30 °C and 42 °C) (Fig. 1B and C) and pH values (6.2 and 2.5) (Fig. 1B and D). The ethanol concentrations were measured using a high-performance liquid chromatography (HPLC) system containing a refractive index detector (Prominence UFLC; Shimadzu, Kyoto, Japan) equipped with a fermentation monitoring column (Bio-Rad Laboratories, Hercules, CA, USA). No significant differences in ethanol production between the wild-type strain and Cgflo1 were observed under any tested conditions (Fig. 1B, C and D). However, *C. glabrata* is more tolerant to high temperatures and low pH than *S. cerevisiae* (Fig. 1C and D). These data suggest that the flocculation mutation in Cgflo1 does not affect its growth and fermentation ability.

To investigate the dependence of the flocculation ability on divalent cations, we examined the flocculation ability of Cgflo1 in the presence of various cations (Fig. 2A). Stationary-phase cells grown in YPD medium were washed with 10 mM of ethylenediaminetetraacetic acid (EDTA) (pH 5.0) four times and with deionized water three times. Cgflo1 did not exhibit flocculation after the EDTA treatment, which suggests that flocculation depends on divalent cations. The washed cells were suspended in deionized water with 0.5 mM of various divalent cations at a cell density of $OD_{600} = 20$. As shown in Fig. 2A, the flocculation of Cgflo1 was found to be dependent on the addition of Ca^{2+}, Co^{2+}, Mg^{2+}, Zn^{2+}, or Fe^{2+} while the wild-type strain did not flocculate. In contrast, the addition of Mn^{2+} or Cu^{2+} did not induce flocculation in either Cgflo1 or the wild-type strain (data not shown). The dependence of Cgflo1 on Ca^{2+} flocculation was similar to that of the flocculation strain of *S. cerevisiae* (8). It thus appears that flocculation in Cgflo1 involves lectin-sugar complex formation on the cell surface, which is known to depend on divalent cations (14).

Next, we investigated the effects of the addition of a monosaccharide on the flocculation of Cgflo1. Stationary-phase cells were washed with EDTA and with deionized water as described above, and various monosaccharides at a concentration of 100 mM were added to the samples. Fig. 2B shows that the flocculation of Cgflo1 was strongly inhibited by the addition of galactose, while the addition of glucose, maltose or mannose did not inhibit flocculation. It was previously shown that flocculation is sensitive to mannose in any type of flocculation strain of *S. cerevisiae* (15). The present data suggest that Cgflo1 flocculation is independent of the binding of mannose residues of mannan to neighboring cells. The observed inhibition by galactose implies that binding to galactose residues is required for Cgflo1 flocculation. In the case of *S. pombe*, it has been reported that cell-surface galactosylation is required for the flocculation of a *gsf1* mutant (9). Thus, there may be some similarities between the flocculation mechanisms of *S. pombe* and *C. glabrata*. Cormack et al. report that the adherence of *C. glabrata* to human cells is inhibited by galactose (13). Additionally, it has been reported that the hydrolyzates of wood and rice straw contain 18.7 mM and 1 mM of galactose, respectively (16, 17). The concentration of galactose in wood hydrolyzate slightly inhibited the flocculation of Cgflo1 while that in rice straw hydrolyzate did not (data not shown). Thus, Cgflo1 would be suitable for the fermentation of rice straw hydrolyzate.

In *C. glabrata*, several adhesion-like genes including *EPA* (epithelial adhesion) family have been previously identified (13). *EPA1* is primarily expressed during the log phase. Log-phase cells of Cgflo1 (OD_{600} value of less than 3) showed aggregation but not sedimentation, while the concentrated cells (OD_{600} value of more than 20)

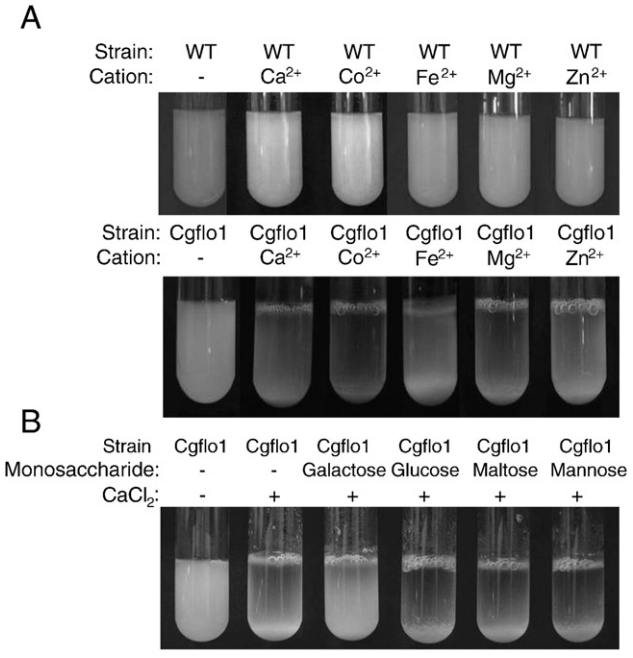

FIG. 2. Effects of divalent cations (A) and monosaccharide (B) on flocculation ability in the wild-type strain (WT) and Cgflo1 mutant. (A) Stationary-phase cells of the wild-type strain or the Cgflo1 mutant were pre-treated with 10 mM of EDTA (for deflocculation) and suspended in deionized water ($OD_{600} = 20$), and various metal chlorides were added to the suspensions at a final concentration of 0.5 mM. The suspensions were left to stand at room temperature for 2 min. (B) Stationary-phase cells were pre-treated with 10 mM of EDTA and were suspended in a monosaccharide solution containing 100 mM of galactose, glucose, maltose or mannose ($OD_{600} = 20$). The suspensions were left to stand at room temperature for 2 min.

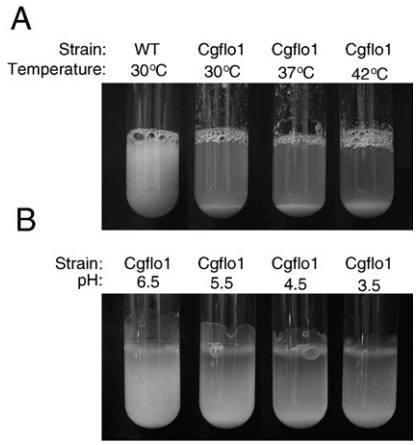

FIG. 3. Effects of cultivation temperature (A) and pH (B) on the flocculation ability of the Cgflo1 mutant. (A) The wild-type strain (WT) and the Cgflo1 mutant were cultivated in YPD medium at the indicated temperatures for 24 h. The cultures were left to stand at room temperature for 2 min. (B) Stationary-phase cells pre-treated with 10 mM of EDTA were suspended in 50 mM potassium acetate buffer at pH values of 6.5, 5.5, 4.5 or 3.5 ($OD_{600} = 20$). The suspensions were left to stand at room temperature for 2 min.

showed aggregation and rapid sedimentation (data not shown), indicating that the flocculation ability of Cgflo1 is not affected by growth phase but is highly influenced by cell density. These results are consistent with the expression pattern of the *EPA1* gene.

To investigate the potential of Cgflo1 for bioethanol production, the dependence of its flocculation ability on temperature and pH was examined (Fig. 3). The dependence of flocculation on cultivation temperature was examined as follows. Cgflo1 and the wild-type strain were cultivated at various temperatures for 24 h. The cultures were then left to stand at room temperature for 2 min. Fig. 3A suggests that flocculation occurred even in Cgflo1 cells that had been cultivated at 42 °C. It has been reported that flocculation was not observed in a flocculation strain of *S. cerevisiae* when the cultivation temperature reached or exceeded 37 °C (18). The flocculation ability of Cgflo1 at high temperatures may provide a great advantage for bioethanol fermentation, because not only would cooling costs be reduced, but also a fermentation offset due to high temperatures could be avoided. We confirmed that the ethanol fermentation ability of *C. glabrata* at 42 °C was almost identical to those at 30 °C (Fig. 1C) and 37 °C (data not shown). To determine the effects of pH on the flocculation ability of Cgflo1, we examined this strain at various pH values (Fig. 3B). Stationary-phase cells were washed with EDTA and with deionized water as described above, and the cells were suspended in 50 mM potassium acetate buffer in samples with pH values ranging from 3.5 to pH 6.5. The suspension was left to stand at room temperature for 2 min and the flocculation ability of the cells was examined. The results presented in Fig. 2B suggest that the flocculation ability of Cgflo1 is maintained at pH values ranging from 3.5 to 5.5. However, flocculation was greatly inhibited at pH 6.5. This pattern of pH dependence in Cgflo1 is similar to that in *S. cerevisiae* (19). Because bioethanol fermentation is generally carried out at a weakly acidic pH value, Cgflo1 is expected to be suitable for either repeated batch fermentation or continuous fermentation processes.

In the present study, we characterized a flocculation mutant obtained from *C. glabrata* by spontaneous mutation. A number of other groups have previously carried out pathogenicity studies of *C. glabrata* (13, 20), while our aim was to focus on *C. glabrata* as a potentially useful strain of yeast for bioethanol production, given that this yeast strain has been shown to be more acid- and heat-tolerant than most other strains of yeast examined in this context to date (10). These characteristics are generally considered to be important for the bioethanol production process and, to the best of our knowledge, this is the first study to provide the flocculation characteristics and ethanol production ability of a flocculation mutant of *C. glabrata*. Flocculation is advantageous for both repeated-batch fermentation and continuous fermentation ethanol production processes. We are currently attempting to develop practical fermentation systems using various flocculation mutants of *C. glabrata*.

In *S. cerevisiae*, the Flo1-type flocculation strain (mannose-sensitive) and the NewFlo-type flocculation strain (mannose- and glucose-sensitive) have been characterized to date (4). Interestingly, Cgflo1 was found to be sensitive to galactose but insensitive to mannose and glucose (Fig. 2B), strongly suggesting that the flocculation mechanisms of Cgflo1 differ greatly from those of the flocculation strains of *S. cerevisiae*, even though *C. glabrata* is taxonomically closely related to *S. cerevisiae* (21). Such galactose sensitivity implies that cell-surface galactosylation is essential for the flocculation of Cgflo1. It has been reported that the adherence of *C. glabrata* to human cells is inhibited by galactose, but not by glucose or mannose (13, 22); it is therefore possible that there are mechanistic similarities between flocculation and adherence to human cells in *C. glabrata.* There are 67 adhesion-like proteins in *C. glabrata*, including the lectin-like Epa family and glycosylphosphatidylinositol (GPI) proteins (22). The cell walls of the *C. glabrata* contained 50% more protein than those of *S. cerevisiae*. We speculate that differences in the amount of cell-wall proteins cause different flocculation patterns, such as galactose sensitivity. Iraqui et al. report the isolation of biofilm-forming mutants from *C. glabrata* (23). A correlation between flocculation and biofilm mutants remains to be determined. We are currently attempting to clone the gene that is determinative of the flocculation phenotype.

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