Genetically Controlled Self-Aggregation of Cell-Surface-Engineered Yeast Responding to Glucose Concentration

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We constructed an arming (cell-surface-engineered) yeast displaying two types of agglutinin (modified a-agglutinin and α -agglutinin) on the cell surface, with agglutination being independent of both mating type and pheromones. The modified a-agglutinin was artificially prepared by the fusion of the genes encoding Aga1p and Aga2p. The modified a-agglutinin could induce agglutination of cells displaying Aga1p (α -agglutinin). The upstream region of the isocitrate lyase gene of *Candida tropicalis* (*UPR-ICL*), active at a low glucose concentration, was used as the promoter to express the modified a-agglutinin- and α -agglutinin-encoding genes. The arming yeast displaying both agglutinins agglutinated and sedimented in response to decreased glucose concentration. When the glucose concentration was high, the arming yeast grew normally. In the late log phase, when the glucose concentration became very low, agglutination occurred suddenly and drastically and yeast cells sedimented completely. Sedimentation was confirmed by weighing the aggregated cells after filtration of the broth. Strains in which aggregation can be genetically controlled can be used in industrial processes in which the separation of yeast cells from the supernatant is necessary.

In brewing, fermentation, and some bioprocesses, separation of cells from broth is essential (2). Centrifugation of the broth is commonly used to achieve complete separation, but the device and its operation are often complicated and expensive. In many microorganisms, aggregation results from interactions of molecular components on the cell surface. Flocculation and agglutination occur in *Saccharomyces cerevisiae*, which is used in the production of foods and medical substances and is considered to be safe for human consumption. Mannose-sensitive and pH- and calcium-dependent flocculation require the production of flocculin, encoded by *FLO1* (9, 13), and depend upon a protein-carbohydrate interaction between flocculin and mannan present in the cell wall. Agglutination in *S. cerevisiae*, on the other hand, is a mating-type-specific cell-cell aggregation that occurs between mating partners through a protein-protein interaction of α -agglutinin, encoded by the *AGA1* gene, and a-agglutinin, composed of Aga1p and Aga2p, encoded by *AGA1* and *AGA2* (4, 7). Aga2p, which is the binding subunit of a-agglutinin, is linked with two cysteines to the cell wall attachment protein Aga1p, while α -agglutinin is a part of the flexible fimbrial cell wall coats of yeast cells. For agglutination as a prelude to mating, haploid cells of *S. cerevisiae* synthesize mating-type-specific cell surface proteins, agglutinins (2). α -Agglutinin is expressed by α cells, and *AGA2* is expressed only in a cells (7). The initiation of sexual agglutination occurs only when it is induced by the pheromone peptide produced by the corresponding mating partners.

The three genes encoding both agglutinins are pheromone inducible and regulated by different promoters. During mating,

they are controlled through a cascade of signal transduction. To control the aggregation artificially, promoters other than the intrinsic ones are needed. We developed a heterologous gene expression system in *S. cerevisiae* by using the 5' upstream region of the isocitrate lyase (ICL) gene (*UPR-ICL*) from the alkantrophic yeast *Candida tropicalis* (6). *UPR-ICL*-mediated transcription is repressed by glucose but strongly induced by nonfermentable carbon sources such as glycerol, acetate, and ethanol or under conditions of derepression (16, 17). When the intrinsic promoters of these agglutinins were changed to *UPR-ICL*, aggregation was expected to be controlled by the carbon source (17).

Normally, aggregation occurs only when two opposite-mating-type cells are mixed. Our original objective in this study was to manipulate the aggregation phenomenon to enable artificial aggregation of cells of the same mating type and to control the aggregation. To achieve this objective, we constructed a fusion protein of Aga1p-Aga2p and a new Aga1p which were regulated by the new promoter of *UPR-ICL*. The results showed that Aga1p-Aga2p fusion proteins exhibited the same activities as those of their natural forms and that aggregation could be controlled by the glucose concentration in the medium. Strains with this phenotype can be used in a wide range of bioprocesses in which separation of cells from the broth is difficult.

MATERIALS AND METHODS

Strains and media. We used *Escherichia coli* strain DH5 α [F^- *endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 λ^- recA1 gyrA96 Δ lacU169* (ϕ 80 *lacZ* Δ M15)] and *S. cerevisiae* strain MT8-1 (*MATa ade his3 leu2 trp1 ura3*) (14) as hosts. *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) containing 0.1% glucose. YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used to cultivate yeast cells. Yeast recombinant transformants were selected on SD plates (0.67% yeast nitrogen base without amino acid [Difco, Detroit, Mich.] but with appropriate supplements, to which 2% glucose was added as a carbon source).

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FIG. 1. Construction of the integrative plasmids pWI(-)A1A2, harboring the *AGA1-AGA2* fusion gene and *UPR-ICL* (A), and pWI(-) α , harboring the *AG α 1* gene and *UPR-ICL* (B). Small arrows along the *AGA1-AGA2* and *AG α 1* gene diagrams show the primers for PCR.

Construction of the *AGA1-AGA2* fusion gene. The *AGA1-AGA2* fusion gene was constructed as follows (Fig. 1A). A DNA fragment of *AGA2*, encoding the binding subunit of α -agglutinin (3), was prepared by PCR with the primers 5'-ACAGATCTAATTAAGATGCAGTTACTTC-3' and 5'-CCCCATGGAAA AACATACTGTGTG-3' and with *S. cerevisiae* MT8-1 genomic DNA (1) as the template. PCR was carried out to confirm the integration of the constructed genes into the chromosomes. The PCR mixture (100 μ l) was as follows: 0.8 μ g of genomic DNA, 100 pmol of each primer, 0.2 mM each deoxynucleoside triphosphate, the reaction buffer supplied by the vendor, and 5 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.). When the reaction was started, the mixture was held at 94°C for 5 min and then subjected to 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min. Then, in the final stage, the reaction mixture was kept for 10 min at 72°C and cooled to 4°C. All PCR amplifications were performed in a Perkin-Elmer Cetus model 480 thermal cycler (Applied Biosystems, Foster City, Calif.). The PCR fragment was digested with *Nco*I and *Bgl*II and inserted into plasmid pWI3ICL (6) digested with the same enzymes. A DNA fragment of *AGA1*, encoding the cell wall-anchoring subunit of α -agglutinin (4, 11), was prepared by PCR using the same template (primers 5'-TCCAT GGGGACATTATCTTTCGCTC-3' and 5'-TTCTCGAGATATTAAGTGAAT TACATTG-3'), digested with *Xho*I and *Nco*I, and then inserted into the *AGA2*-containing plasmid digested with *Xho*I and *Nco*I. The final recombinant plasmid was designated pWIA1A2. To examine whether the product of the fusion gene was functional or not, another recombinant plasmid designated pWI α , which contained the *AG α 1* gene, encoding α -agglutinin (8), was constructed using two primers: 5'-TCAGATCTTTTCAAAATGTCACTTTT-3' and 5'-CTCTCGAGTACCCGTTTGAATAGCT-3' (Fig. 1B).

Integration of the fusion gene into the yeast chromosomes. pWIA1A2 was cut by *Eco*RI to remove the 2 μ m fragment and self-ligated to generate a new recombinant plasmid, pWI(-)A1A2, which had *TRP1* as a selectable marker. The DNA fragment carrying *UPR-ICL* (6) and the *AG α 1*-coding region, obtained from partial digestion of plasmid pWI α with *Pvu*II, was transferred to the *Pvu*II-digested plasmid pRS406 (12) to generate a plasmid designated pWI(-) α , with *URA3* as a selectable marker.

Plasmids pWI(-)A1A2 and pWI(-) α were digested with *Hind*III and *Apa*I, respectively, and integrated into the genomic DNA of *S. cerevisiae* MT8-1 for stabilization of their expression using the yeast transformation kit YEAST-

MAKER (Clontech Laboratories, Inc., Palo Alto, Calif.). A coexpressing recombinant yeast strain was constructed by transforming and integrating both of the plasmids into a single yeast cell. The integration of both plasmids into the chromosomes was checked by PCR analysis (data not shown).

Genomic DNA of recombinant yeast cells was used as the template for PCR. In order to detect the integration of the constructed fusion gene of *AGA1-AGA2*, the primers 5'-CTCAAATGAAGTTCTTTACC-3' (*TERM-ICL* [6] primer) and 5'-ACAGATCTAATTAAGATGCAGTTACTTC-3' (*AGA2* primer) were used, while another pair of primers, the *TERM-ICL* primer and 5'-TCAGATCTTTT CAAAATGTCACTTTT-3' (*AG α 1* primer), were used to ensure the integration of the constructed *AG α 1*-encoding gene into the chromosome. The PCR conditions were the same as before. The PCR products were analyzed by electrophoresis using 1% agarose (data not shown).

Observation and measurement of cell aggregation with cultivated transformants. *S. cerevisiae* MT8-1, the yeast transformants 11-2 [MT8-1 harboring pWI(-)A1A2] and 4-1 [MT8-1 harboring pWI(-) α], and the cotransformant 141 (MT8-1 harboring both plasmids) were cultivated in YPD medium at 30°C until mid-log phase. Cells were harvested at the same growth phase, washed twice with water, transferred to a set of test tubes holding 10 ml of fresh YPD medium to give a final optical density at 600 nm of 0.025, and further cultivated. For the mixed culture, equal quantities of strains 11-2 and 4-1 were mixed to give the same optical density value. During cultivation, cells were sampled as indicated in the figures and observed under a microscope. The quantitative analysis of aggregation was carried out by filtration with an isopore polycarbonate membrane filter (Millipore Co., Bedford, Mass.) which has heat-resistant and constant weight properties. The pore size was 12 μ m, which allowed single cells to pass easily but prevented passage of the aggregated cell clumps. The filter can be dried completely at 80°C for 6 h without a significant change of weight. An aliquot of the broth (5 ml) was passed through the filter. The filters were dried at 80°C for 6 h and weighed, with the net increase in weight being attributed to the aggregated cells. Cell sediment was observed in the test tubes with the naked eye. Photographs were taken after mixing and allowing the culture to settle for 25 min.

Measurement of glucose concentration. An aliquot of culture broth was centrifuged to remove the cells. The glucose concentration of the supernatant was

FIG. 2. Time course of increase in cell weight on the filters (pore size, 12 μ m) and decrease in glucose concentration. Symbols: ●, strain MT8-1; △, strain 4-1; ▲, strain 11-2; □, mixed culture of 4-1 and 11-2; ○, glucose concentration in the medium. Mean values based on the results of five replicates are represented. Error bars are omitted because the variation was less than 10% of the value of each point.

measured with a D-glucose test kit (Boehringer Mannheim Co., Mannheim, Germany).

Characterization of disulfide linkages in the Aga1p-Aga2p fusion protein. Strain 11-2, which expresses the *AGA1-AGA2* fusion gene, was cultivated in YPD medium. The cells were harvested, washed twice with ice-cold 150 mM NaCl, and gently shaken in 50 mM Tris-HCl (pH 8.5) containing various concentrations of dithiothreitol (DTT) for 1 h at 4°C (18). Then, the suspension was mixed with an equal quantity of cells of strain 4-1, which expresses Aga1p, and shaken at 30°C for 30 min. The aggregation was measured by filtration as described above.

RESULTS

Expression of the Aga1p-Aga2p fusion protein and aggregation responding to glucose concentration. During the mixed cultivation of both 11-2 and 4-1 cells, the glucose concentration in the medium decreased with the cultivation time. When the glucose concentration decreased to about 7 mg/liter, the *UPR-ICL* promoter became active and expressed both the *AGA1* gene and the *AGA1-AGA2* fusion gene, leading to a carbon source-controlled aggregation. The weights of the mixed-culture cells of strains 11-2 and 4-1 on the filters increased significantly (Fig. 2). This phenomenon was also demonstrated by sedimentation of cells in the mixed culture (Fig. 3A and B). Microscopic observation confirmed that the two strains (11-2 and 4-1) of the same mating type (*MATa*) that carried their respective plasmids bound to each other by adhesion between expressed Aga2p and Aga1p on the cell surface, leading to aggregation without exposure to pheromones. In contrast, the individual cultures of 11-2 and 4-1 and the control strain (harboring the control plasmid) did not show any obvious aggregation, as was the case with the host strain, MT8-1. Although strain 11-2, expressing the Aga1p-Aga2p fusion protein, aggregated when it was mixed with the Aga1p-expressing strain 4-1, no aggregation occurred in the mixed cultivation when the DTT concentration was increased to 10 mM (Table 1). These

FIG. 3. Observation of cell aggregation by visual inspection of sedimentation in test tubes (A) and microscopic viewing (B and C). MT8-1, the host strain; 4-1, the strain expressing the *AGA1* gene; 11-2, the strain expressing the *AGA1-AGA2* fusion gene; 4-1+11-2; the mixed culture of 4-1 and 11-2; 141, the strain expressing both genes. The cells were cultivated to the stationary phase. (C) MT8-1 and 141 cultivated for 80 h were observed under a microscope. Bar, 10 μ m.

results indicate that the Aga1p-Aga2p fusion protein was correctly displayed on the cell surface and that it had the same function as the original a-agglutinin composed of Aga1p and Aga2p via disulfide linkages. The fusion of Aga1p and Aga2p did not affect the essential property of aggregation.

Construction of the self-aggregating yeast strain. Strain 141 was cultivated in YPD medium to determine its aggregation potential (Fig. 3C and 4). The net weight of the 141 cells on the filters increased in response to the decrease in glucose concentration in the medium, indicating that self-aggregation could be controlled by the selected promoters. The *UPR-ICL* promoter was very sensitive to the change in glucose level; i.e., when the concentration of glucose decreased to approximately 7 mg/liter, the *UPR-ICL* promoter became active and initiated the expression of the *AGA1-AGA2* fusion gene and the *AGA1* gene. The cells began to bind to each other by adhesion be-

TABLE 1. Effect of DTT treatment on aggregation of strains 11-2 and 4-1

DTT concn (mM)	Relative % of aggregated cells
0.....	100
0.1.....	100
0.5.....	33
1.....	28
5.....	17
10.....	3

FIG. 4. Time course of decrease in glucose concentration and increase in cell weight on the filters (pore size, 12 μ m) of the self-aggregating strain 141. Symbols: $\circ$, glucose concentration in the medium; Δ , dry weight of cells on the filters. Cells of MT8-1 and 141 were observed under a microscope or with the naked eye at 20 h (left grouping of three photographs) and 80 h (right grouping of three photographs) of cultivation. Bar, 10 μ m. Mean values based on results from five replicates are represented. Error bars are omitted because the variation was less than 10% of the value of each point.

tween Aga2p and Aga1p codisplayed on the cell surface. As a result, the cells started to aggregate, forming cell clumps, and all cells were completely precipitated by 100 h of cultivation.

DISCUSSION

In the sexual mating process, cells of different mating types aggregate with each other by means of adhesion between α - and α -agglutinins in response to pheromone induction (4). In α -agglutinin, only the AGA2-encoding peptide Aga2p can bind to the α -agglutinin produced by α -type cells. The Aga2p subunit is transferred to the yeast cell wall surface by attaching to Aga1p, which is a cell wall component encoded by AGA1, through two disulfide linkages (3). Although both AGA1 and AGA2 have been sequenced, their regulation in response to pheromones is not yet well understood (7, 15). This lack of understanding is at least part of the reason why agglutinin has not been widely used in the brewing industry.

In this work, we altered the original genetic regulation by using a different promoter, *UPR-ICL* (6), instead of the intrinsic one. Aggregation occurred in a mixed culture of strains 4-1 and 11-2 when the *UPR-ICL* promoter turned on transcription in response to decreased glucose concentration (Fig. 3A and B). We also constructed a strain, 141, which could self-aggregate in response to the glucose concentration in the medium

(Fig. 3C). This is the first report of aggregation of cells of the same mating type that responds only to the level of glucose in the medium and not to any pheromone induction.

Here, we used the upstream region of the isocitrate lyase (ICL) gene (*UPR-ICL*) as the regulator. ICL is one of the key enzymes of the glyoxylate cycle, which supplies C_4 compounds to the tricarboxylic cycle, especially when cells are growing on C_2 compounds such as ethanol and acetate (17). ICL is induced in *C. tropicalis* when cells are grown on a gluconeogenic carbon source such as acetate, *n*-alkane, or propionate (10). In *S. cerevisiae*, the *UPR-ICL* gene functions in the same manner as in *C. tropicalis* and is repressed by glucose and derepressed by nonfermentable carbon sources (5). Accordingly, these strains will not ferment the full range of sugars in the cultivation medium. In this study, *UPR-ICL*-induced transcription began when the glucose concentration decreased to 7 mg/liter; this response to concentration is similar to those previously observed in *C. tropicalis* and *S. cerevisiae*.

After aggregation began, the cell weights on the filters increased significantly (Fig. 2 and 4), indicating increases in the percentages of aggregated cells with time. Strain 141 produced larger cell clumps than the mixed cultivation of strains 4-1 and 11-2 (Fig. 3B and C). We hypothesize that cells in clumps of strain 141 bind more tightly since Aga1p and Aga2p were

displayed simultaneously on the surface of a single cell. We think that multiple binding reactions exceeding the number of binding events that normally happen during the mating processes may occur.

Our results demonstrate that cell aggregation can be controlled artificially. The strain can serve as a model for other similar types of strains in which different promoters are used to respond to different regulatory signals in the culture medium. The phenotype of genetically controlled cell aggregation might also be useful as an aid in the transferring of cells from a cultivation medium for various industrial applications.

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