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Alpha Mating Type-Specific Expression of Mutations Leading to Constitutive Agglutinability in *Saccharomyces cerevisiae*

SYUICHI DOI* AND MASAO YOSHIMURA

Department of Legal Medicine, Kinki University School of Medicine, Sayama-cho, Minamikawachi-gun, Osaka 589, Japan

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Two mutants of *Saccharomyces cerevisiae* have been isolated and characterized. The mutants were constitutively agglutinable at 36°C, the temperature at which wild-type cells agglutinate only after induction by mating pheromone. The mutant cells had other properties specific for the normal α cell type, i.e., conjugation with *a* cells, response to *a* mating pheromone, and production of α mating pheromone. The two mutations, *cag1* and *cag2*, were recessive and expressed only in α cells. *cag1* is linked very closely to the *MAT* locus, but *cag2* is unlinked to the *MAT* locus. These *cag* mutations complemented *ste3-1*. These results indicate that *CAG* genes are novel α-specific genes involved in the regulation of sex agglutinin synthesis.

Saccharomyces cerevisiae cells of different mating types (*MAT***a** and *MAT*α) specifically adhere to each other with sex specific glycoproteins (sex agglutinins) on the cell surface (5, 11, 22). Agglutinating clumps of cells eventually sort themselves into pairs and fuse to produce zygotes. There are two types of sexual agglutinability in *S. cerevisiae*, constitutive and inducible. Constitutive strains synthesize sex agglutinins without induction by mating pheromone, and inducible strains produce them only after induction by mating pheromone (1–4, 12, 15, 21).

Sexual agglutinability can be easily reversed by changing physiological conditions such as the growth temperature (3) and carbon source (21). Sexual agglutinability of wild-type cells is usually constitutive when cells are cultured at 25°C in a medium with glucose, but it is inducible at 36°C in the same medium (3).

We have isolated constitutive agglutinable mutants at 36°C and characterize in this paper two mutations specific for α cells.

MATERIALS AND METHODS

Yeast strains. The genotypes and sources of the *S. cerevisiae* strains used in this study are listed in Table 1.

Media and culture conditions. Modified YPD (2) and minimal media (3) were used for yeast culture and for prototroph recovery, respectively. MV medium (8) was used for preparing culture filtrates.

When cells were cultured at 25°C, 0.1 ml of 2-day-old culture was inoculated into 50 ml of YPD medium in a conical flask (300 ml) and then incubated with shaking on a reciprocal shaker. When cells were cultured at 36°C, 0.01 ml of the culture was inoculated into 50 ml of YPD medium and cultured with shaking. Tester cells (K21-1A and K21-1C) were always grown at 25°C in YPD medium with shaking.

Isolation of constitutive mutants for agglutinability. Exponentially growing S288C cells were washed with sterile water and suspended in 0.1 M phosphate buffer (pH 8.0) at a density of ca. 2×10^8 cells per ml. Ethyl methanesulfonate (3% [vol/vol]) was added, and incubation was continued at 25°C for 60 min with gentle shaking. Then, an equal volume of 12% (wt/vol) sodium thiosulfate was added. After 10 min, portions (0.2 ml) were inoculated into 5 ml of YPD medium and incubated overnight at 25°C with shaking, followed by spreading of cells on YPD plates to form colonies at 25°C. Each colony was transferred to YPD plates and cultured overnight at 25°C to prepare master plates. The colonies on master plates were again transferred to fresh YPD plates by replica plating and cultured overnight at either 25 or 36°C. Cells (late-logarithmic or early-stationary phase) were then suspended in 10 mM phosphate buffer (pH 5.5) at an optical density (530 nm) of 1. The agglutination test was carried out by mixing the cell suspension (0.25 ml) with the same volume of tester cell suspension (A_{530} = 1) in a well on a titer plate at 25°C. Candidates which showed extensive agglutination at both 25 and 36°C were retested after culturing cells at either 25 or 36°C in liquid YPD medium in conical flasks. Finally, from ca. 4,000 colonies tested, eight clones which showed prominent agglutination in phosphate buffer even when grown at 36°C were selected as constitutive mutants, and the mutation was denoted as *cag* (constitutive agglutinability).

Sexual agglutination test. Cells at late-logarithmic phase of growth were harvested, washed twice, and suspended in phosphate buffer at an optical density (530 nm) of 10. Portions (0.5 ml) of the cell suspension to be tested and of the tester cells (A_{530} = 10) were mixed with 9 ml of phosphate buffer in a 50-ml conical flask and incubated on a reciprocal shaker (120 rpm) at 25°C. The degree of sexual agglutination was determined by measuring the optical density of the suspension before and after brief sonication as described previously (3). Sexual agglutination of tetrad segregants was performed as follows. One loopful of a culture (2 days at 25°C) of each segregant was inoculated into 5 ml of YPD medium in test tubes and incubated on a reciprocal shaker (80 rpm) at 36°C until the culture reached the late-logarithmic or early-stationary phase of growth. Cells were harvested, washed twice, and suspended in phosphate buffer at A_{530} = 1. One milliliter of the segregant cell suspension was mixed in test tubes with 1 ml of tester cell suspension (A_{530} = 1) of opposite mating type, and the tubes were shaken reciprocally (80 times per min) at 25°C.

Mating reaction. Cells prepared in the same way as those used in the agglutination test were mixed with cells of the opposite mating type in YPD medium and shaken at 25 or 36°C. Agglutination intensity and zygote formation were determined as described previously (3).

* Corresponding author.

TABLE 1. Strains used

Strain	Genotype	Source
K21-1A	*MAT***a**	Our stocks
K21-1C	*MAT*α	
K61-3C	*MAT*α *leu2 his4 thr4*	
CM1-8A	*MAT*α *leu1 lys5 cyh2*	
K120-17D	*MAT***a** *leu2 his4 thr4*	
K169-7B	*MAT*α *arg4 trp1 met14*	
K169-12A	*MAT***a** *arg4 trp1 met14*	
S288C	*MAT*α	R. K. Mortimer
K164-1B	*MAT*α *cag1*	This study
S288C-S4	*MAT*α *cag2*	
K171-7A	*MAT*α *cag1 arg4 trp*	Meiotic segregants from
K171-7B	*MAT*α *cag1 met14*	K164-1B × K169-12A
K180-3A[a]	*MAT***a**′ *cag1 trp1*	Meiotic segregants from
K180-3B[a]	*met14*	K171-7A × K171-7B
	*MAT***a**′ *cag1 arg4*	
K179-1A	*MAT*α *cag2 leu2 his4*	Meiotic segregants from
K179-1B	*MAT*α	S288C-S4 × K120-17D
K179-1C	*MAT***a** *cag2 leu2 his4 thr4*	
K179-1D	*MAT***a** *thr4*	
G107-94D	*matα1-5 matα2-4 ade6 trp5 leu1*	S. Harashima, originally from I. Herskowitz
K185-4	*MAT*α *ste3-1 thr1 ura3 arg1 trp1 his4*	*ste3-1* was derived from G213-15C from I. Herskowitz

[a] *MAT***a**′ was derived from the silent locus encoding mating type information, *HMR* or HML of the parent, either K171-7A or K171-7B, by rare mating. Hence, the *MAT* locus of **a**-type segregants was designated *MAT***a**′.

Mating pheromone assay. Mating pheromone activity was monitored by growth inhibition. Response to **a** mating pheromone in the mutant cells was examined. Culture filtrate was prepared by culturing **a** (K21-1A) or α (K21-1C) cells in MV medium for 48 h at 25°C. Exponentially growing cells to be tested were harvested, washed with water, and suspended in water (A_{530} = 1). The assay mixture was composed of 4 ml of culture filtrate, 0.5 ml of 10× YPD, and 0.5 ml of the cell suspension. The mixture was incubated at the temperature at which cells were grown.

Production of α mating pheromone by mutant cells was also studied. Cells were cultured in MV medium at 25 or 36°C for 48 h. Mating pheromone activity in the culture filtrate was assayed at 25°C, using K21-1A cells as tester. The composition of the assay mixture was the same as that described above. Buds with diameters larger than half the diameter of the mother cell were counted as cells.

Spheroplast formation, fusion, and regeneration. Cells were harvested from 10 ml of exponentially growing culture, washed once with 1 M sorbitol solution, and suspended in 2 ml 0.1 M citrate buffer (pH 6.8) containing 1 M sorbitol and 10 mM EDTA at a density of 10^8 cells per ml. A lytic enzyme, Zymolyase 60000 (Seikagaku Kogyo Co. Ltd., Tokyo), was added to a final concentration of 30 μg/ml, and the culture was then incubated at 25°C with gentle shaking for 60 min. Spheroplasts thus obtained were washed with 1 M sorbitol, fused in 34% polyethylene glycol 4,000 containing 20 mM $CaCl_2$ and 1 M sorbitol, and then incubated at 25°C. After 60 min, fused spheroplasts were washed with 1 M sorbitol and spread on minimal plates with 1 M sorbitol for regeneration of cells. The plates were incubated at 25°C until colonies appeared (usually 3 to 5 days).

Genetic analysis. Methods and media used in genetic analyses were described previously (3).

RESULTS

Phenotypic characterization. Sexual agglutination of the mutant strains K164-1B and S288C-S4, which bear *cag* mutations (*cag1* and *cag2*, respectively), was studied (Fig. 1). In phosphate buffer, induction of sexual agglutination by mating pheromone did not occur (2). Cells of wild-type strain S288C grown at 25°C started agglutination shortly after incubation, whereas agglutination did not occur when the cells were cultured at 36°C. In contrast, cells of each mutant agglutinated extensively even when cultured at 36°C. Figure

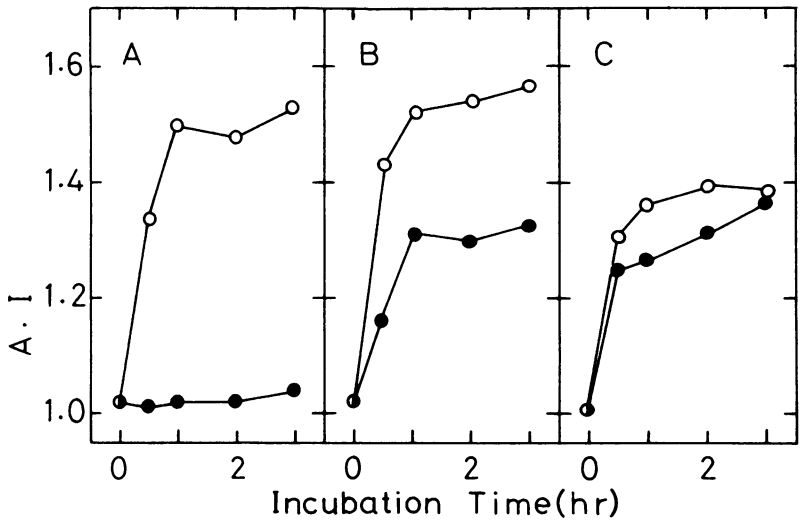

FIG. 1. Sexual agglutination of constitutive mutants bearing *cag* mutations. (A) Wild type strain S288C. (B) Constitutive mutant K164-1B bearing *cag1*. (C) Constitutive mutant S288C-S4 bearing *cag2*. Cells of each strain were grown in YPD medium at either 25°C (○) or 36°C (●) and tested for sexual agglutinability in 10 mM phosphate buffer (pH 5.5) at 25°C. A.I., Agglutination index.

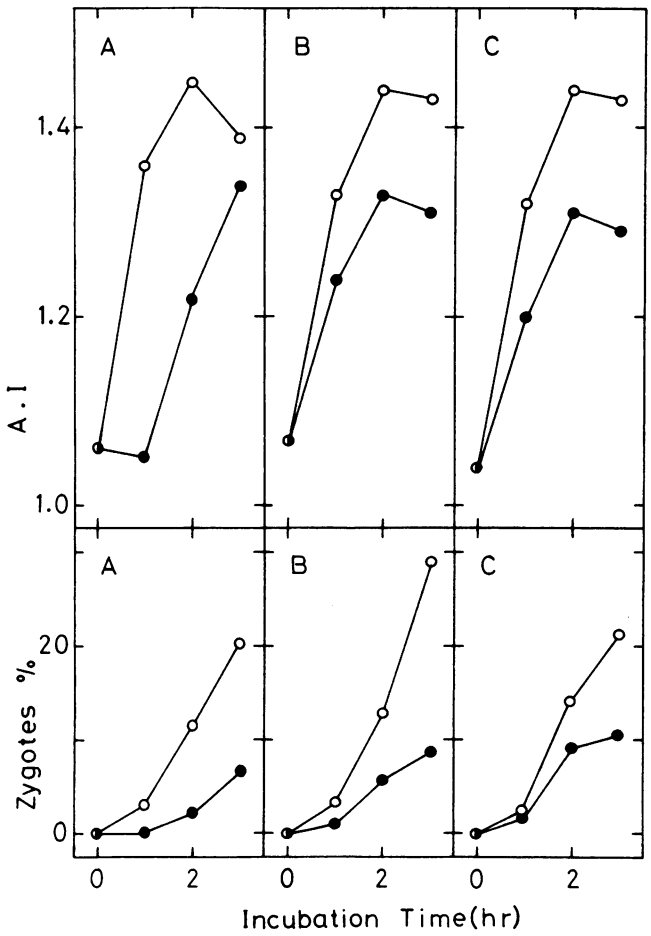

FIG. 2. Mating reaction of constitutive mutants in YPD medium. (A) Wild type strain S288C. (B) Strain K164-1B bearing *cag1*. (C) Strain S288C-S4 bearing *cag2*. Cells of each strain grown in YPD medium at either 25°C (○) or 36°C (●) were assayed for their mating ability in YPD medium at the temperature at which cells were grown. A.I., Agglutination index.

2 shows the mating reaction of each mutant in YPD medium at either 25 or 36°C. At 36°C, sexual agglutination in the mutants began immediately after incubation, whereas that in the wild type started somewhat later. This delay in sexual agglutination is due to the induction by mating pheromone. Normal zygote formation was observed in these mutants at 36°C, except that it occurred about 1 h earlier than that in the wild type. These results indicate that *cag* mutations do not affect conjugation ability at 25 and 36°C.

Response of the mutants to **a** mating pheromone was studied (Fig. 3). Mating type **a** culture filtrate retarded cell division in both wild-type and mutant cells at 25 or 36°C. Kinetics of the inhibition of cell division were similar among the wild type and the mutants. Mating type α culture filtrate did not retard cell division of the wild type and the mutants at each temperature. These results indicate that the mutants do not change their response to **a** mating pheromone.

Production of α mating pheromone by the mutants was assayed (Fig. 4). Cell division of **a** tester cells (K21-1A) was inhibited when they were exposed to α mating culture filtrates prepared from cultures of both the wild type and the mutants at 25 or 36°C. However, these culture filtrates did not inhibit cell division of α-type tester cells (K21-1C). The culture filtrates of each strain had ca. 2 to 4 U of α mating pheromone per ml as determined by serial twofold dilutions. These results show that *cag* mutations do not affect production of α mating pheromone.

Recessiveness of the *cag* mutations. *MATα/MATα* diploids carrying the *cag* mutation in a heterozygous state were tested for their sexual agglutinability. The diploids were obtained by spheroplast fusion or by crossing the mutants with the *matα1-5 matα2-4* double mutant G107-94D, which behaves like an **a**-type cell (19). These diploids extensively agglutinated at 25°C in phosphate buffer or YPD medium (Table 2). In contrast, they agglutinated only in YPD medium after the induction, indicating that their agglutinability is constitutive at 25°C but inducible at 36°C. The mutations are, therefore, recessive.

Segregation of sexual agglutinability. Tetrad data are shown in Table 3. In the cross K164-1B × K120-17D, sexual agglutinability at 36°C was segregated 2:2 (constitutive:inducible). On the other hand, the cross S288C-S4 × K120-17D gave irregular segregation for sexual agglutinability at 36°C. In the cross S288C × K120-17D, all segregants had inducible agglutinability at 36°C. At 25°C, the sexual agglutinability of any segregant from these crosses was constitutive. Other genetic markers, such as mating type and nutritional requirements, segregated normally (data not shown). In the cross K164-1B × K120-17D, any α-type

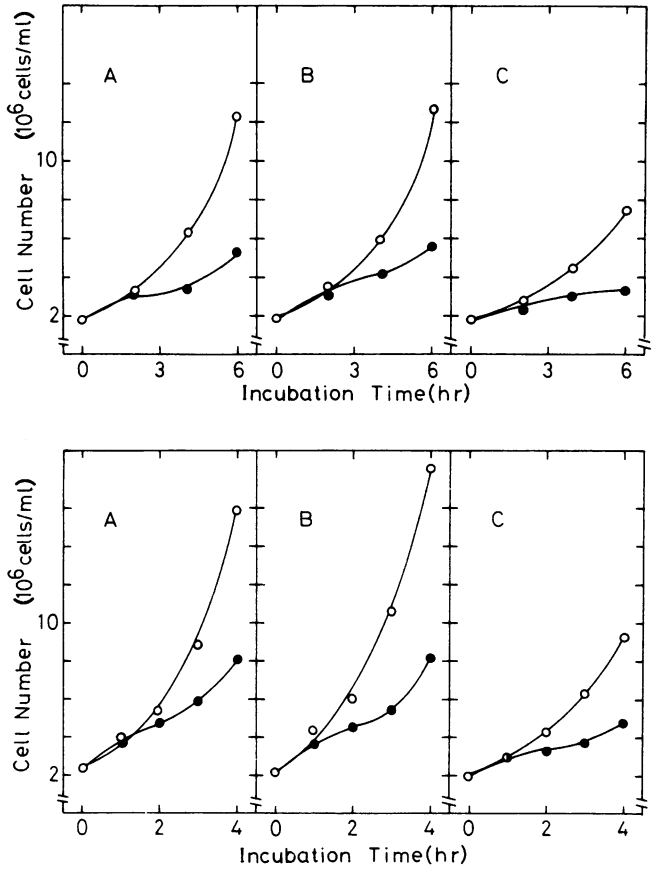

FIG. 3. Response of constitutive mutants to **a** mating pheromone. (A) Wild type strain S288C. (B) Strain K164-1B bearing *cag1*. (C) Strain S288C-S4 bearing *cag2*. Cells of each strain were grown at 25°C (upper) or 36°C (bottom) and exposed to **a** (●) and α (○) culture filtrate at the temperature at which cells were grown.

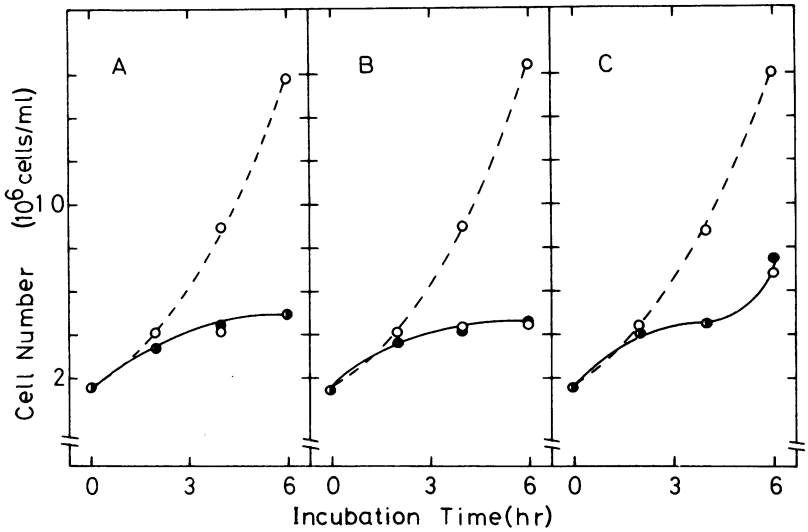

FIG. 4. Production of α mating pheromone by constitutive mutants. (A) Culture filtrate from wild type strain S288C. (B) Culture filtrate from strain K164-1B. (C) Culture filtrate from S288C-S4. Tester cells of K21-1A (**a**) (solid line) or K21-1C (α) (dotted line) grown at 25°C were exposed at 25°C to culture filtrates of mutant cells obtained 48 h after incubation in MV medium at either 25°C (○) or 36°C (●). Culture filtrates obtained from the 25 and 36°C cultures had identical effects on the cell growth of strain K21-1C.

segregant had constitutive agglutinability (α^c) and any **a** type segregant had inducible agglutinability ($\mathbf{a}^i$) at 36°C (58 tetrads analyzed). In the cross S288C-S4 × K120-17D, both constitutive (α^c) and inducible (α^i) agglutinabilities in α-type segregants were observed. No constitutive segregants of **a** type ($\mathbf{a}^c$) were obtained in this cross (2 $\mathbf{a}^i$:2 α^c, 4; 2 α^i:1 α^i:1 α^c, 10; 2 $\mathbf{a}^i$:2 α^i, 2). These results indicate (i) that *cag1* in K164-1B is tightly linked to the *MAT* locus or is in the locus itself and (ii) that *cag2* in S288C-S4 is expressed specifically in α mating-type cells. The mutation *cag2* is unlinked to the *MAT* locus, because tetrad distribution of the *MAT* locus and *cag2* was 3 parental ditype:2 nonparental ditype:11 tetratype (Table 4).

Mating-type specificity of *cag* mutations. Since *cag1* is closely linked to *MATα*, **a**-type cells harboring *cag1* were obtained as segregants from diploids by rare mating between cells of the same mating type. Mating-type conversion by illegitimate mating is known to be due to changes in the *MAT* cassette (13, 14). The resultant diploid (K171-7A/K171-7B) sporulated well and yielded four viable spores. Mating type

TABLE 2. Sexual agglutinability of *MATα*/*MATα* diploids carrying heterozygous *cag* mutations

Diploid	Relevant genotype	Agglutination[a] at:			
		25°C		36°C	
		Phosphate buffer	YPD	Phosphate buffer	YPD
K171-7A / K61-3C	*MATα cag1* / *MATα*	1.44	1.39	1.01	1.28
K171-7A / G107-94D	*MATα cag1* / *matα1-5 matα2-4*	1.36	1.34	1.00	1.34
K179-1A / K61-3C	*MATα cag2* / *MATα*	1.41	1.38	1.03	1.32
K179-1A / G107-94D	*MATα cag2* / *matα1-5 matα2-4*	1.33	1.42	1.01	1.30

[a] Cells grown at 25 or 36°C were tested for sexual agglutinability at the same temperature in phosphate buffer or YPD medium. At 25°C, agglutination intensity was determined after 1 h of incubation. At 36°C, the intensity was determined after 2 h of incubation, because wild-type cells need a time lag during which agglutinability is induced by mating pheromone.

TABLE 3. Segregation of sexual agglutinability at 36°C

Cross	Relevant genotype	No. of tetrads (constitutive:inducible) segregating at				
		4:0	3:1	2:2	1:3	0:4
S288C × K120-17D	*MATα* × *MATa*	0	0	0	0	8
K164-1B × K120-17D	*MATα cag1* × *MATa*	0	0	58	0	0
S288C-S4 × K120-17D	*MATα cag2* × *MATa*	0	0	4	10	2

and nutritional requirements were segregated 2:2 (data not shown). Sexual agglutinability at 36°C was segregated 2 $\mathbf{a}^i$:2 α^c (eight tetrads analyzed). This result suggests two possibilities: (i) mutation *cag1* is in the *MAT*α locus and eliminated by the insertion of *MAT***a** cassette and (ii) *cag1* is tightly linked to the *MAT*α locus (Table 4) and its expression is specific for the α mating type. The diploid resulting from rare mating between cells of **a** mating type ($\mathbf{a}^i$ segregants from K171-7A/K171-7B) yielded four viable spores whose mating type was 2 **a**:2 α. Segregation of sexual agglutinability at 36°C was 2 $\mathbf{a}^i$:2 α^c in 20 tetrads analyzed. Thus, constitutive agglutinability was restored only in α progenies from an $\mathbf{a}^i \times \mathbf{a}^i$ cross, indicating that *cag1* is expressed specifically in α cells. In addition, these results suggest that *cag1* is not in the *MAT* cassette.

To confirm the mating type specificity of mutation *cag2*, each segregant from the tetratype tetrad (2 $\mathbf{a}^i$:1 α^i:1 α^c) from the cross S288C-S4 × K120-17D was crossed with wild-type cells ($\mathbf{a}^i$ or α^i) (Table 5). If *cag2* is α specific and unlinked to the *MAT* locus, one of the two $\mathbf{a}^i$ segregants should give α^c spores. K179-1A (α^c) showed irregular segregation for the agglutinability at 36°C, similar to that in the original mutant. Two segregants, K179-1B (α^i) and K179-1D ($\mathbf{a}^i$), gave no spores constitutive for agglutinability at 36°C. On the other hand, one $\mathbf{a}^i$ segregant, K179-1C, yielded constitutive segregants (α^c). Distribution of segregation type in the ascus was essentially the same as that for the original mutant. These results indicate that one of the $\mathbf{a}^i$ segregants carries the *cag2* mutation, which was cryptic in **a**-type cells.

Complementation test. *MAT*α/*MAT*α diploids prepared by spheroplast fusion were examined for sexual agglutinability (Table 6). If two mutations are distinctive from each other, diploids should behave as normal α cells, with constitutive agglutinability at 25°C but inducible agglutinability at 36°C. In YPD medium, these diploids agglutinated and produced zygotes at 25 or 36°C (data not shown). In phosphate buffer, all diploids could agglutinate extensively at 25°C, but not at 36°C. This shows that sexual agglutinability of these diploids is constitutive at 25°C but inducible at 36°C. Thus, *cag* mutations complemented each other and an α-specific *ste3-1* mutation identified by MacKay and Manney (8, 9).

DISCUSSION

We have characterized two mutants bearing *cag* mutations, which were derived from a *MAT*α strain. These mutants agglutinated extensively in phosphate buffer even when grown at 36°C, indicating that they produced sex agglutinins constitutively at 36°C. The mutant cells exhibited other α-specific properties, namely, they conjugated normally with **a** cells, responded to **a** mating pheromone, and secreted α mating pheromone. Thus, α mating type-specific functions were not affected by the *cag* mutations. These results indicate that *cag* mutations are different from α-specific mutations *ste3* (9, 17), *ste13* (17), *kex2* (6), and *tup1* (7, 10, 18, 20), which have already been identified.

Response to **a** mating pheromone and production of α mating pheromone were similar among the wild-type and *cag* mutant cells (Fig. 3 and 4). Cells which did not synthesize sex agglutinins at 36°C could respond to **a** mating pheromone and produce α mating pheromone (Fig. 1, 3, and 4). These facts suggest that regulation of synthesis of sex agglutinin is different from that of response to and production of mating pheromone. Hence, it is likely that *CAG* genes are specifically involved in regulation of sex agglutinin synthesis.

Genetic analyses indicate that the *cag* mutations are recessive and expressed specifically in α cells. These two mutations were mapped to different loci, *cag1* and *cag2*. *cag2* is unlinked to the *MAT* locus, whereas *cag1* is tightly linked to the *MAT* locus, but not in the locus itself. However, the possibility that *cag1* lies within the mating cassette in one of the regions of homology is not excluded completely, because a conversion need not lead to replacement of the entire homologous X and Z regions of the cassette. Complementation tests demonstrated that both *cag1* and *cag2* were distinctive from *ste3*, *matα1*, and *matα2* (Tables 2 and 6).

These α-specific *cag* mutations reported here may help to elucidate the mechanism of genetic control of sex agglutinin synthesis.

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TABLE 4. Segregation data from crosses of *cag* mutants with wild-type strain K120-17D

Mutant	Relevant genotype	cag vs mating type[a]		
		PD	NPD	T
K164-1B	*MAT*α *cag1*	58	0	0
S288C-S4	*MAT*α *cag2*	3	2	11

[a] PD, NPD, and T refer to parental ditype, nonparental ditype, and tetratype asci, respectively.

TABLE 5. Segregation of agglutinability at 36°C in crosses of tetratype tetrad segregants from S288C-S4 × K120-17D and wild-type strains

Cross	Agglutinability[a] at 36°C	Segregation of agglutinability (constitutive:inducible)				
		4:0	3:1	2:2	1:3	0:4
K179-1A × K120-17D	$\alpha^c \times \mathbf{a}^i$	0	0	1	2	1
K179-1B × K120-17D	$\alpha^i \times \mathbf{a}^i$	0	0	0	0	10
K179-1C × K169-7B	$\mathbf{a}^i \times \alpha^i$	0	0	1	3	1
K179-1C × CM1-8A	$\mathbf{a}^i \times \alpha^i$	0	0	1	5	2
K179-1D × K169-7B	$\mathbf{a}^i \times \alpha^i$	0	0	0	0	5
K179-1D × CM1-8A	$\mathbf{a}^i \times \alpha^i$	0	0	0	0	7

TABLE 6. Complementation test

Diploid	Relevant genotype	Agglutination at:[a]	
		26°C	36°C
K171-7A / K179-1A	*MAT*α *cag1* / *MAT*α *cag2*	1.45	1.01
K171-7B / K185-4	*MAT*α *cag1* / *MAT*α *ste3-1*	1.43	1.02
K179-1A / K185-4	*MAT*α *cag2* / *MAT*α *ste3-1*	1.37	1.01

[a] Agglutination intensity was determined after 1 h of incubation in phosphate buffer (10 mM, pH 5.5) at 25°C and was expressed as the agglutination index.

G213-15C and G107-94D, respectively. We also thank Hyogo Sinohara, Kinki University, and Kazuyuki Tanabe, Osaka City University, for their suggestions during this work.

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