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Novel Strategy for Isolating Suppressors of Meiosis-deficient Mutants and Its Application for Isolating the *bcy1* Suppressor

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A novel strategy was developed for isolating suppressors from sporulation-deficient mutants. The mutation in the *BCY1* gene, which codes for the regulatory subunit of cAMP-dependent protein kinase, when homozygous, results in diploids being meiosis and sporulation deficient. Two plasmids, YCp-MATα and YEp-SPOT7-lacZ, were introduced into *MATa BCY1*+ or *MATa bcy1* haploid cells. The transformant of the *BCY1*+ haploid cell produced β-galactosidase under nutrient starvation, but the *bcy1* transformant did not. Using this system, the mutagenesis experiment performed on the *bcy1* transformant strain resulted in a number of sporulation mutants that produced β-galactosidase under nutrient starvation. One complementation group, *sob1*, was identified from the isolated suppressor mutants and characterized as a single recessive mutation by tetrad analysis. Genetic analysis revealed that the *sob1* mutation suppressed the sporulation deficiency, the failure to arrest at the G1 phase of the cell cycle, and the sensitivity to heat or nitrogen starvation caused by the *bcy1* mutation. However, the *sob1* mutation did not suppress the sporulation deficiency of *ime1* and of *ime2* diploids. These results suggest that the *sob1* mutation affects a gene which functions as a downstream regulator in both meiosis and cell cycle regulation.

Key words: Yeast, sporulation, meiosis, suppressor, *bcy1*

The sporulation of yeast cells is a program of cellular differentiation that includes genetic recombination, meiotic division, and spore formation (3). Two signals are required for meiosis and sporulation in yeast *Saccharomyces cerevisiae*: nutrient starvation and MAT product a1 and α2, which determine the a/α cell type (7, 10, 15). Sporulation is restricted to one type of cell, the *a*/α diploid cell which expresses both *MATa* and *MAT*α genes. The other two types of cells, α or *a* haploid cells, express an inhibitor of meiosis, *RME1*, that blocks the initiation of meiosis (16). The expression of *RME1* gene is repressed by *MATa1*/α2 gene products in a/α diploid cells (2,8). Two positive factors, *IME1* and *IME2*, in the initiation of meiosis were isolated by their ability to promote a sporulation, when present on multicopy plasmid, regardless of the expression of the *RME1* gene (4, 8, 17, 18, 22).

RAS/cAMP pathway seems to be involved in the initiation of meiosis (11, 13). Mutations in the adenylate cyclase gene, *CYR1*, and in two of its regulators, *CDC25* and *RAS2*, result in low levels of cellular cAMP (13, 23). In these mutants, meiosis and sporulation take place in rich medium. Mutations in the *BCY1* gene, which codes for the regulatory subunit of cAMP-dependent protein kinase, suppress the mutations, *cyr1*, *cdc25*, and *ras2* by producing the protein kinase, independent of cAMP (1, 12, 23). The mutation *bcy1*, when homozygous, results in diploids being meiosis and sporulation deficient (12). The $RAS2^{val19}$ mutation results in a high level of cellular cAMP and constitutively activates the cAMP-dependent protein kinase (23); this dominant mutation also results in the diploid being meiosis and sporulation deficient. This implies that high activity of the protein kinase inhibits meiosis, and that the reduced activity might be a necessary intermediate step in the regulation of meiosis by nutrient starvation (14, 21).

Over-expression of the *IME1* gene completely overcomes MAT defects but may not circumvent all nutritional control. Kassir *et al* (8) found that overexpression of *IME1* allowed sporulation in the presence of glucose and nitrogen. They also found induced expression of the *IME1* gene in temperature-sensitive *cdc25* diploids shifted to high temperatures in rich media. Matsuura *et al* (14) found that *IME1* over-expression overcomes the meiosis deficiency of *bcy1* and of $RAS2^{val19}$ diploids. Double

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mutants *ime1 cyr1* and *ime1 ras2* failed to sporulate (14). These results may suggest that the *IME1* gene serves as the merging point for the signals representing the two basic requirements for meiosis and sporulation, mating type control and nutrient starvation. While it is clear that *IME1* is a necessary positive regulator of meiosis, responding both to mating type and nutritional conditions, it is not clear if it is sufficient (15, 17, 18). Over-expression of the *IME1* gene does not block sporulation in most strains and does not allow expression of late meiosis-specific genes. It is possible that other genes are involved downstream of cAMP-dependent protein kinase in the response to starvation. Here we report a novel strategy for isolating suppressors of the sporulation-deficient mutant, *bcy1*.

Materials and Methods

Strains

Genotypes of the yeast strains are listed in Table 1. All yeast strains contained the *ho* mutation that resulted in a deficiency of the mating type switch. Strains of AM242-1A and AM242-1D were used as tester strains for identification of the mating type of yeast cell (21).

Media

The complete medium (YPD), pre-sporulation medium (YPA), and minimal medium (SD) were made as described by Sherman, Fink, and Hicks (19). Two different sporulation media were used in this study. One contained 1% potassium acetate, 0.1% bacto-yeast extract, and 0.5% dextrose, and used to sporulate diploid cells and to determine sporulation efficiency. The other one, a rich sporulation medium, is basically the same except for the supplementation of amino acids, purine, and pyrimidine required by auxotrophic mutation, and was used for isolating the *bcy1* suppressors. The composition of sulfur-free medium was the same as the synthetic medium (19), except that all salts containing sulfates were replaced by those containing chloride, and yeast extract was omitted. The nitrogen-free medium was composed of 0.16% Bacto yeast nitrogen base (Difco) and 2% dextrose. SC-Leu, SC-Ura and SC-Leu/Ura media were used for selection of yeast transformants.

Table 1. Yeast strains

Strain	Genotype
R19-4C	*MATα bcy1-1 leu2 ura3 trp1*
R9-4C (1-9)	*MATα bcy1-1 sob1-9 lue2 ura3 trp1*
R31-1A	*MATα leu2 ura3 trp1*
R31-1B	*MATα sobl-9 ura3 trp1*
R31-1C	*MATα bcy1-1 sob1-9 ura3 trp1*
R31-1D	*MATa leu2 ura3 trp1*
DE-SB-1	*MATa leu2 ura3 trp1/MATα leu2 ura3 ade8*
DE-SB-11	*MATa bcy-1 ura3 trp1/MATα bcy1-1 ura3 trp1 lys2*
DE-SB-16	*MATa bcy1-1 Sob1-9 ura3 trp1/MATα bcy1-1 sobl-9 ura3 trp1*
DE-SB-16	*MATa sobl-9 leu2 ura3 lys2/MATα sobl-9 ura3 trp1*

Genetic methods and transformation

Standard genetic procedures of cross, sporulation, spore dissection, and tetrad analysis were followed as described by Sherman, Fink, and Hicks (19). Diploids were isolated by prototrophic selection if possible. When prototrophic selection could not be employed, diploids were identified after single colony isolation by testing an ability to sporulate. In the case of sporulation-deficient strains, they were selected for the inability to mate.

Transformation of yeast cells was performed by the lithium acetate method (5). *E coli* strain, DH1, was used for transformation and amplification of the plasmids as described by Maniatis, Fritsch, and Sambrook (9).

Plasmids construction and DNA methods

For construction of YEp13-SPOT7:lacZ, YEp13 was digested with SalI and *Hind*III and prepared for ligation with *SPOT7* promoter and *lacZ* coding sequence. *SPOT7* promoter was obtained from digestion of 125L-184 with *Hind*III and *Bal*31. A coding sequence of *lacZ* was obtained from *Sma*I/*Sal*I digestion of pMC1430. Ycp19-MATa was contructed by ligation of *Sal*I/*Bam*HI digested YCp-19 with *Sal*I/*Bam*HI DNA containing the *MATα* gene.

Determination of sporulation efficiency, G1 arrests, sensitivity to heat or nitrogen starvation

The cells grown on YPD plate were transferred to SPO plates, and incubated at 30°C. After 3 days, the sporulation efficiency and the proportion of unbudded cells were determined under a light microscope. At least 600 cells were counted for each determination.

The exponentially growing cells in YPD medium were exposed to 52°C for 4 min or transferred to nitrogen-free medium. The cultures heat-treated or incubated in the nitrogen-free medium for 48 hours were then spread onto YPD plates with proper dilution and incubated at 25°C for 3 days. The viability was determined by comparison with non-treated culture.

Results and Discussion

Isolation of *bcy1* suppressor mutants

To isolate the bcy1 suppressor mutants efficiently, an isolation method was designed as illustrated in Fig. 1. The *SPOT7-lacZ* fusion gene was used as a reporter to monitor whether the cells sporulated or not. The expression of the *SPOT7* gene is induced only during meiosis (25). Diploid cells carrying the *SPOT7-lacZ* fusion gene produce β-galactosidase, and consequently, colonies of the cells develop a blue color on sporulation medium containing X-gal. The initiation of meiosis requires the information of MAT locus, the function of a1-α2 products as well as starvation (10). However, the recessive suppressor mutations can be efficiently isolated only from the haploid cells. We found that introduction of both *MATα* and *SPOT7-lacZ* genes into the *MATa BCY1*$^+$, but not into *MATa bcy1*, haploid cells results in production of β-galactosidase on sporulation media. According to this novel strategy, we introduced two plasmids, YCp19-MATα and YEP13-*SPOT7-lacZ*, into the *MATa bcy1* (R19-4C) mutant. The transformants were mutagenized with ethylmethanesulfonate (EMS) and spread on YPD plates to give a density of approximately 300 colonies per plate. The master plates were replica plated to sporulation plates containing 40 mg/l of X-gal, and incubated at 30°C. After 3 or 4 days, some colonies had developed a blue color. By this method, fifteen colonies were selected.

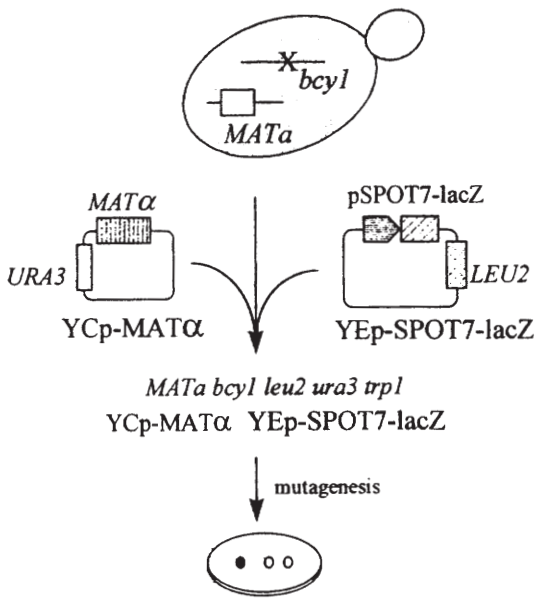

Fig. 1. A novel strategy for isolation of the *bcy1* suppressors. R19-4C (*MATa bcy1*) was transformed with YCp-MATa and YEp-SPOT7-*lacZ*. The transformants were mutagenized with ethylmethanesulfonate and spread on YPD plates to give 300 colonies per plate. The master plates were replica-plated to sporulation media containing 40 mg/l of X-gal. After 3 or 4 days, blue colonies were selected for further genetic analysis.

Genetic analysis of *bcy1* suppressors

The strategy of genetic analysis for the isolated fifteen colonies that developed the blue color on the SPO-Xgal plate is illustrated in Fig. 2. To select a bcy1 suppressor from among the fifteen isolated colonies, the cells of each isolate were transformed with YEp-HO, which expresses a endonuclease and functions in the mating type switch (6) to permit the mating type switch and subsequent diploidization. The transformants of each isolate were separately grown on SC-Leu/Ura medium to a stationary phase, and the diploids were selected from the culture. Three diploids sporulated, others did not. This suggests the majority of blue colonies may carry a mutation that derepresses the expression of the *SPOT7-lacZ* gene, or other defects. Three diploids that are capable of sporulating can be expected to carry a mutation that suppresses the sporulation deficiency by the *bcy1* mutation. The sporulated cells were dissected, and germinated. All spores obtained from a diploid were expected to carry the same genetic background except the mating type locus. The spores of *MATa* from each diploid were identified by using the mating type tester strains, and crossed to other original isolates from R19-4C (*MATα bcy1*). The diploids were selected, and examined for sporulation ability. By this complementation test, one group was identified among the isolated colonies. To determine whether the isolated suppressor mutation is recessive or dominant, and single or multiple mutations, the cells of original colonies were crossed with the wild type cells. The resultant diploids

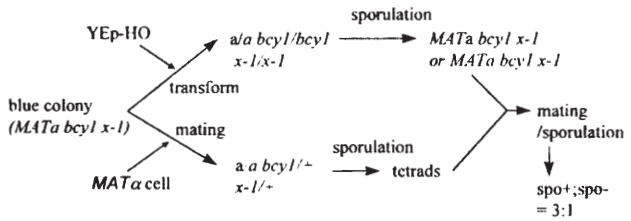

Fig. 2. Genetic analysis of *bcy1* suppressors. The blue colonies selected from SPO-Xgal plates were transformed with plasmid YEp-HO or mated with *MATα* cells. Diploids homozygous for *bcy1* and *x-1* (unknown mutation) were isolated from the YEp-HO transformed culture. The a or α haploid cells carrying *bcy1* and *x-1* mutations were obtained from tetrads from the sporulated cells. These cells were used for mating with haploid cells from tetrads of sporulated diploids heterozygous for *bcy1* and *x-1* mutations.

Table 2. Sporulation and G1 arrests

Strain	Genotype	Sporulation (%)	Unbudded cell (%)			
			Grow	SPO	NH_4^+	SO_4^-
DE-SB-1	+/+	42	34	92	91	94
DE-SB-16	*bcy1/bcy1*	0	32	32	34	41
DE-SB-11	*bcy1 sob1/bcy1 sob1*	11	35	85	78	91
DE-SB-17	*sob1/sob1*	36	36	94	95	93

Cells of each diploid grown on the YPD plates were transfered into YPA plate, and incubated for 24 hours, then in the sporulation medium for 3 days. Sporulation efficiency and proportion of the G1 arrested cells was determined by counting sporulated cells or unbudded cells, respectively. To determine G1 arrest of the cells after starvation of nutrient, exponentially growing cells in YPD medium were transfered into nitrogen- or sulfur-free medium, and incubated for 3 days. Grow, exponentially growing cells; SPO, sporulation medium; NH_4^+, nitrogen-free medium; SO_4^-, sulfur-free medium.

were sporulated, and dissected. Each spore of the obtained tetrads was crossed with a or α *bcy1* suppressor mutants that were previously obtained. The diploid cells were selected from each culture crossed, and examined the sporulation ability. The sporulation ability of each tetrad mainly segregated in a 3+:1- (spo+:spo-) ratio. These results suggest that the isolated suppressor mutants carry a single and recessive mutation (*sob1*; suppressor of *bcy1*). One of the isolates, *sob1-9*, was further analyzed.

Characterization of *sob1* mutation

The sporulation efficiency of the diploids homozygous for *bcy1* and *sob1* mutations was compared to the wild type or *bcy1* diploids. While the *bcy1* diploids failed to sporulate, the b*cy1 sob1* diploids were able to sporulate efficiently (Table 2). Wild type yeast cells were arrested at the G1 phase under nutrient starvation, but the *bcy1* mutant was not (12, 21). The proportion of unbudded cells was significantly higher in *bcy1 sob1* mutant culture on nitrogen- or sulfur-free or sporulation medium (Table 2).

The yeast cells arrested in the G1 phase are more resistant to heat treatment than dividing cells, and retain a viability under nutrient starvation (20, 23). The *bcy1* mutants were sensitive to heat treatment, and rapidly lost viability under the nitrogen- or sulfur- free media (20). In contrast, the dividing cyr1-2 or ras2 mutants that produce a low level of cAMP are more resistant to heat treatment and nutrient starvation than normal cells. The *bcy1 sob1* mutants showed about 50 times more resistance to heat treatment and 25 times higher viability under nitrogen starvation than that of *bcy1* mutants (Table 4).

Table 3. Sensitivity to nitrogen starvation and heat treatment

Strain	Genotype	Viability (%)	
		NH_4^+ for 48h.	52°C for 4 min.
R31-1D	*wt*	42	8×10^{-2}
R31-1B	*bcy1*	0.1	2×10^{-4}
R31-1C	*bcy1 sob1*	3	5×10^{-3}
R31-1A	*sob1*	38	3×10^{-2}

The exponentially growing cells at 25°C were transfered into the nitrogen-free medium, and incubated for 48 hours or exposed to lethal temperature, 52°C, for 4 min.

Table 4. Functional relationship between *SOB1* and *IME1* genes

Genotype	Sporulation	
	YPA	SPO
+/+	<0.1	32
bcy1 sob1/bcy1 sob1	12	35
sob1 ime1::TRP1/sob1 imel::TRP1	<0.1	<0.1
sob1 ime2::LEU2/sobl ime2::LEU2	<0.1	<0.1
bcyl sob1 imel::TRP1/bcy1 sob1 imel::TRP1	<0.1	<0.1
bcy1 sob1 ime2::LEU2/bcy1 sob1 ime2::LEU2	<0.1	<0.1

The disruption mutants of *IME1* or *IME2* genes was obtained from tetrads of *sob1* or *bcy1 sob1* diploids transformed with pHS113 or pAM403-1 that were obtained from Dr. A.P. Mitchell.

Over-expression of the *IME1* gene overcame the sporulation deficiency of *bcy1* or $RAS2^{val19}$ diploids, but failed to arrest at the G1 phase when the cells were starved for nutrients (14). Double mutants *ime1 cdc25* and *ime1 ras2* are sporulation deficient (14). However, unlike the over-expression of *IME1*, the *sob1* mutation suppresses not only sporulation deficiency, but also the failure of G1 arrest under nutrient starvation and partial starvation and heat sensitivity of *bcy1* mutation. We constructed double mutants *sob1 ime1* and *sob1 ime2*. The mutants were deficient in meiosis (Table 3). Downstream of the protein kinase seemed to branch to control meiosis through *IME1*, and to control G1/Go arrest, independently of *IME1*. The *SOB1* gene acts as a downstream regulator in both meiosis and cell cycle regulation and upstream of *IME1*.

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pAM403-1.

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