Isolation of Glucoamylase-non-producing Mutants in the Yeast *Saccharomyces diastaticus*

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Saccharomyces diastaticus produces extracellular glucoamylases and ferments starch. We have isolated glucoamylase-non-producing (*amy*) mutants from wild-type strains of *S. diastaticus* carrying a *STA1* gene after mutagenesis with ethylmethane sulfonate. All *amy* mutations were recessive and were divided into two complementation groups, *amy1* and *amy2*. The *amy1* group may be located in the *STA1* gene. The *amy2* mutation was involved in both glucoamylase production and flocculation.

Saccharomyces diastaticus carrying any one of the unlinked *STA* genes *STA1*, *STA2*, or *STA3*, secretes glucoamylase-isozymes I, II, or III, respectively, in the culture medium and ferments starch.^{1,2)} The secreted enzymes are glycosylated.²⁾ We have been interested in the genetic and biochemical control of synthesis and secretion of the glycosylated enzymes³⁾ and have isolated glucoamylase-non-producing (*amy*) mutants from *S. diastaticus* strains carrying the *STA1* gene. In this paper, we give a genetic analysis of the *amy* mutants. *S. diastaticus* strains used and their relevant genotypes are listed in Table I. The strains are highly flocculent since they carry a dominant flocculation gene *FLO8*, a novel flocculation gene derived from a *S. diastaticus* strain 5106-32D, kindly provided by H. Tamaki, and the details of the gene will be published elsewhere (manuscript No. 3-355).

MATERIALS AND METHODS

Strains. Yeast strains used and their relevant genotypes are listed in Table I.

Culture media. YPS medium contained 1% yeast extract, 2% peptone, and 3% soluble starch. YPSB medium was YPS medium containing $3.3 \times 10^{-3}\%$ bromocresol purple. YEPD medium contained 1% yeast extract, 2% peptone,

and 2% dextrose. If necessary, the media were solidified by 2% agar.

Fermentation test for starch. Fermentation test was carried out by the standard Durham-tube method.¹⁾ Cells were cultured in a test tube (1.8 × 18 cm) containing 5 ml of YPS medium at 28°C for up to 3 weeks.

Flocculation test. Cells were cultured with shaking in a test tube (1.5 × 10.5 cm) containing 1 ml of YEPD medium at 28°C for 1~2 days. Flocculating cultures were easily distinguished from non-flocculating ones because the former produced a sediment of cells.

Protoplast fusion. Fusion of protoplasts was carried out with selection for prototrophy as described previously.⁴⁾

Assay for glucoamylase activity. Each reaction mixture (final volume, 0.5 ml) contained 0.4% soluble starch, 80 mM sodium acetate buffer (pH 5.0), and an enzyme solution. The reaction mixture was incubated at 50°C for 20 min, and the reducing sugar (glucose) formed was determined by the standard Somogyi-Nelson method.²⁾ Under the condition used here, the activity proceeded linearly for at least 30 min.

Genetic analysis. Genetic techniques including tetrad analysis were described previously.⁴⁾

RESULTS AND DISCUSSION

When cells of *S. diastaticus* were cultured on a YPSB plate at 28°C for 4~6 days and then

TABLE I. *S. diastaticus* STRAINS

Strain	Genotype ^a	Source
YIY2-11A	<i>a leu2-3,112 his4 STA1 FLO8</i>	This work
YIY2-12D	<i>a leu2-3,112 his2 lys2 STA1 FLO8</i>	This work
YIY11-1B	<i>a leu2-3,112 STA1 FLO8</i>	This work
YIY4-3A	<i>α his4 arg4 STA1 FLO8</i>	This work
YIY256	<i>a arg4 STA1 FLO8</i>	This work
YIYF	<i>a leu2-3,112 his4 sta° FLO8</i>	This work

^a Recently, we found that *S. cerevisiae* carries an inhibitor gene (*INH1*) against glucoamylase production, and then the genotype of *S. diastaticus* is proposed to be *STA1 inh°*. Details will be published elsewhere (manuscript submitted).

FIG. 1. Halo Formation on the YPSB Plate. The parent strains (top, YIY11-1B; bottom, YIY2-11A) formed halos around colonies. The *Amy⁻* mutant strains (top, YKF12; bottom, YKF2) formed no halo.

FIG. 2. Flocculation in YEPD medium. The parent strain YIY11-1B (left) and the *Amy⁻ Flo⁺* mutant strain YKF2 (center) were highly flocculent and cells were immediately sedimented to the bottom of the test tubes. The *Amy⁻ Flo⁻* mutants strain YKF12 (right) were non-flocculent.

TABLE II. GLUCOAMYLASE ACTIVITY AND THE OTHER PHENOTYPES OF THE PARENT AND THE MUTANT STRAINS

Strain	Glucoamylase activity ^a	Halo ^b	Fermentation ^c	Flocculation ^d
Parent				
YIY2-11A	16.3	+	+	+
YIY2-12H	16.3	+	+	+
YIY11-1B	15.0	+	+	+
Mutant				
YKF2	0.6	—	—	+
YKF5	0.5	—	—	+
YKF12	0.6	—	—	—
YKF13	0.7	—	—	—

^a Cells were cultured in YPS medium at 28°C for 3 days. The culture supernatants were obtained by centrifugation, dialyzed extensively against de-ionized water, and assayed for glucoamylase activity. The activity is presented as increased absorbance at 660 nm for 20 min per 0.1 ml of enzyme solution.

^b A small amount of cells were smeared on the YPSB plate, and the plate was incubated successively at 28°C for 6 days and at 4°C for 2 days. +, halo-forming; —, halo-non-forming.

^c +, fermentable; —, non-fermentable.

^d +, flocculating; —, non-flocculating.

plate was incubated at 4°C for 1~2 days, a halo was formed around each colony (Fig. 1). The halo formation reflected the glucoamylase activity, since when a series of diluted enzyme solution were incubated in wells in a YPSB plate at 50°C (an optimal temperature for the enzyme activity) for 1 hr, followed by incubation at 4°C overnight, halos were formed around the wells with the size of the halo increasing with the increasing activity present in the wells. Thus, in tetrad analysis, we scored glucoamylase-producing ability (producing, *Amy⁺*; non-producing, *Amy⁻*) by means of halo formation on YPSB plates. This method has allowed us to determine glucoamylase-producing ability of the large number of cultures necessary for genetic studies. The tetrad data were consistent with those obtained from a starch fermentation test by a classical Durham-tube method.¹⁾

Glucoamylase-non-producing (*Amy⁻*) mutants were isolated from three wild-type strains

YIY2-11A, YIY2-12D, and YIY11-1B (Table I), after mutagenesis with ethylmethane sulfonate (final concentration, 3%). Mutagenized cells (survival, 50%) were spread on the YPSB plates, followed by incubation at 28°C for 6 days and at 4°C for 2 days. Colonies forming no halo were picked up and subcultured to single colonies on the YPSB plate. We obtained 146 independent mutants. They showed no starch fermentation. Fifty-eight of the mutants were highly flocculent (Flo⁺), however, the remaining 88 mutants were non-flocculent (Flo⁻). Four mutant strains were chosen for further analysis: YKF2 and YKF5 are Amy⁻ Flo⁺, and YKF12 and YKF13 are Amy⁻ Flo⁻ (Figs. 1 and 2). The mutant strains YKF2 and YKF5 were derived from the parent strains YIY2-12D and YIY11-1B, respectively, and the mutant strains YKF12 and YKF13 were derived from the parent strain YIY2-11A. Table II summarizes glucoamylase activity secreted and the other phenotypes of the parent and the mutant strains. All mutant strains produced greatly diminished glucoamylase activity. These mutants were crossed with a wild-type strain YIY4-3A, and the resulting diploids were subjected to tetrad analysis after sporulation, of which the results are presented in Table III. The tetrad data suggest that the mutant phenotypes, *i.e.* Amy⁻ Flo⁺ and Amy⁻ Flo⁻, are caused by single muta-

tions. Determination of dominant or recessive phenotype of the mutations and their complementation tests were carried out in strains homozygous for a mating type allele (*MAT*), since the mating type constitution *MATa/MATα* represses glucoamylase production. Details will be published elsewhere (manuscript No. 3-355). Strains homozygous for *MAT* were constructed by a standard protoplast fusion method⁴⁾ with selection for prototrophy. Most of the fusants are expected to be diploids, since, in the fusion conditions used, all six fusants examined were diploids, as estimated by tetrad analysis using a standard diploid strain homozygous for *MAT*. To determine dominant or recessive phenotype of the mutations, fusants were constructed using two haploid strains carrying *MATa*: one was a wild-type strain, YIY256, and the other was one of the mutant strains. Table IV shows the following abilities of the fusant strains: glucoamylase activity, halo formation, starch fermentation, and flocculation. The phenotypes of all fusant strains heterozygous for the mutant alleles were converted to the wild type, indicating that all mutations are recessive. Complementation tests were carried out in fusant strains constructed by combination of two haploid mutant strains carrying *MATa*. Table V shows the following abilities of the fusant strains: glucoamylase activity, halo for-

TABLE III. TETRAD ANALYSIS IN THE CROSS BETWEEN THE WILD-TYPE AND THE MUTANT STRAINS

Cross	Segregation	No. of asci
YIY4-3A × YKF2	2 (Amy ⁺ Flo ⁺) : 2 (Amy ⁻ Flo ⁺)	24
YIY4-3A × YKF5	2 (Amy ⁺ Flo ⁺) : 2 (Amy ⁻ Flo ⁺)	24
YIY4-3A × YKF12	2 (Amy ⁺ Flo ⁺) : 2 (Amy ⁻ Flo ⁻)	12
YIY4-3A × YKF13	2 (Amy ⁺ Flo ⁺) : 2 (Amy ⁻ Flo ⁻)	12

TABLE IV. RECESSIVE PHENOTYPE OF THE MUTATIONS

Fusant	Glucoamylase activity ^a	Halo ^b	Fermentation ^c	Flocculation ^d
YIY256 × YKF2	4.3	+	+	+
YIY256 × YKF5	5.6	+	+	+
YIY256 × YKF12	9.2	+	+	+
YIY256 × YKF13	11.4	+	+	+

^{a-d} See Table II (footnotes a~d, respectively).

TABLE V. COMPLEMENTATION TEST

Mutant ^a	YKF2				YKF5				YKF12				YKF13			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
YKF2-1	0.6	—	—	+	0.5	—	—	+	3.0	+	+	+	2.7	+	+	+
YKF5-1					0.9	—	—	+	3.6	+	+	+	3.6	+	+	+
YKF12-1									0.6	—	—	—	0.7	—	—	—
YKF13-1													0.7	—	—	—

^a Mutant strains YKF2-1, YKF5-1, YKF12-1, and YKF13-1 were derived from the crosses between the wild-type strains YIY4-3A and each of the original mutant strains YKF2, YKF5, YKF12, and YKF13.

Lane A, glucoamylase activity; Lane B, halo formation; Lane C, fermentation; and Lane D, flocculation. See footnotes in Table II.

TABLE VI. TETRAD ANALYSIS IN THE CROSS BETWEEN THE *sta*^o STRAIN AND THE *amy* MUTANT STRAINS

Cross		No. of asci ^b		
Genotype	Strain ^a	0 ⁺ :4 ⁻	2 ⁺ :2 ⁻	1 ⁺ :3 ⁻
<i>sta</i> ^o × <i>amy</i> 1-1	YIYF × YKF2-2	18	0	0
<i>sta</i> ^o × <i>amy</i> 1-2	YIYF × YKF5-2	18	0	0
<i>sta</i> ^o × <i>amy</i> 2-1	YIYF × YKF12-2	1	2	7
<i>sta</i> ^o × <i>amy</i> 2-2	YIYF × YKF13-2	3	2	7

^a Mutant strains YKF2-2, YKF5-2, YKF12-2, and YKF13-2 were derived from the crosses between the wild-type strain YIY4-3A and each of the original mutant strains YKF2, YKF5, YKF12, and YKF13.

^b Amy phenotype was determined by means of halo formation as described in Table II, footnote b.

mation, starch fermentation and flocculation. The results indicate that the four mutant alleles are divided into two complementation groups: the mutant alleles present in the mutant strains YKF2 and YKF5 were designated *amy*1-1 and *amy*1-2, respectively, and the other alleles present in the mutant strains YKF12 and YKF13 were designated *amy*2-1 and *amy*2-2, respectively.

A strain YIYF is one of the glucoamylase-non-producing segregants derived from the cross between two strains of *S. diastaticus*: one carrying the *STA*1 gene and the other carrying a *STA*3 gene. In asci from diploids constructed by the crosses between the strain YIYF and *S. diastaticus* strains carrying either *STA*1 or *STA*3, glucoamylase-producing ability is segregated as 2(producing): 2 (non-producing), suggesting that the strain YIYF carries no active glucoamylase-producing genes, so it is designated *sta*^o. Mutant strains carrying each of the four mutant alleles *amy*1-1, *amy*1-2,

*amy*2-1, and *amy*2-2, were crossed with the strain YIYF and the resulting diploids were subjected to tetrad analysis after sporulation. The results are presented in Table VI. In the crosses between the strain YIYF and the *amy*1 mutant strains, all segregants were Amy⁻, suggesting that the *amy*1 mutation may be located in the *STA*1 gene, which may be the structural gene for glucoamylase I. In the crosses between the strain YIYF and the *amy*2 mutant strains, three different ascus types appeared, suggesting that the *amy*2 mutant strains carry the intact *STA*1 gene, and the product of the *AMY*2 gene was simultaneously involved in both production of glucoamylase I and flocculation governed by the *FLO*8 gene, since the *amy*2 mutation made yeast cells Amy⁻ Flo⁻.

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