Short Communication

Molecular Cloning of a Glucoamylase-producing Gene in the Yeast *Saccharomyces*

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Saccharomyces diastaticus carrying any one of the unlinked *STA* genes (*STA1*, *STA2*, or *STA3*) produces glucoamylase-isozymes I, II, or III, respectively, in the culture medium and ferments starch.^{1,2)} *S. diastaticus* is most closely allied to *Saccharomyces cerevisiae* and may be considered to be derived from the latter species by the acquisition of glucoamylase activity.³⁾ Recently, we reported that both glucose in the culture medium and a mating type constitution of *MATa/MATα* repress the production of glucoamylase I.⁴⁾ Details will be published elsewhere (manuscript submitted). To extend our understanding of regulatory mechanisms for glucoamylase production (synthesis and secretion) and moreover to provide some information on polymorphism of the *STA* genes and on the mechanisms of gene evolution in the two

yeast species (*S. diastaticus* and *S. cerevisiae*), we have attempted to clone the *STA* genes which may be the structural genes of the glucoamylases. In this paper we describe conversion of a starch-non-fermenting yeast strain to a starch-fermenting one by an autonomously replicating plasmid vector containing a nuclear *STA1* gene fragment of *S. diastaticus*. Relevant genotypes of the yeast and bacterial strains used are listed in Table I.

When the cells of *S. diastaticus* and *S. cerevisiae* were separately cultured on a YPSB plate (1% yeast extract, 2% peptone, 3% soluble starch, $3.3 \times 10^{-3}\%$ bromocresol purple and 2% agar) at 28°C for 4~6 days, followed by incubation at 4°C for 1~2 days, *S. diastaticus* formed halos around its colonies, while *S. cerevisiae* did not. The two experiments described below confirmed that halo formation reflects both starch-fermentation ability and glucoamylase activity. First, in asci from a diploid constructed by the cross between *S. diastaticus* and *S. cerevisiae*, all tetrad segregants which showed starch-fermentation ability (*Sta*⁺) by the classical Durham-tube method¹⁾ formed halos on the YPSB plate, and *vice versa*. Second, when a series of diluted solutions containing the glucoamylase activity were incubated in wells prepared on the YPSB plate at 50°C (an optimal temperature for the enzyme) for 1 hr, followed by incubation at 4°C overnight, halos were formed around the wells, with the size of the halo increasing with

TABLE I. YEAST AND BACTERIAL STRAINS

Organism	Strain	Genotype	Source
<i>S. diastaticus</i>	5106-9A	<i>a leu2 arg4 STA1</i>	H. Tamaki
	YIY2-11A	<i>a leu2-3,112 his2 STA1</i>	This work
	YIY257	<i>a leu2-3,112 his2 his4 STA1</i>	This work
	YIY269	<i>α leu2-3,112 his2 his4 arg4 lys2 STA1</i>	This work
	5301-17B	<i>α lys7 STA3</i>	H. Tamaki
	YIYD	<i>a leu2-3,112 his4 lys7 sta</i> ^o	This work
<i>S. cerevisiae</i> ^a	YIY4B-3	<i>α leu2-3,112</i>	This work
Recombinant ^a	YIY2-12B	<i>a leu2-3,112 his4 ura3</i>	This work
<i>E. coli</i>	JA221	<i>F⁻ recA1 leuB6 ΔtrpE5 lacY hsdR⁻ hsdM⁺</i>	A. Toh-e

^a YIY2-12B is one of the *Sta*⁻ segregants derived from the cross between *S. diastaticus* and *S. cerevisiae*. Genotypes for starch fermentation of *S. cerevisiae* and the recombinant strains will be published elsewhere (manuscript submitted).

increasing enzyme activity. Our scheme for cloning a starch-fermentation gene was based on the hope that cells which formed no halos would be converted to halo-forming ones when a plasmid vector containing the starch-fermentation gene is introduced.

We constructed a recombinant plasmid library using as a vector the plasmid pYII, which had been previously constructed in our laboratory.⁵⁾ The plasmid, which replicates in both *Escherichia coli* and a yeast, confers ampicillin and tetracycline resistance upon bacteria and complements *leu2* and *ura3* mutations in yeast and *leuB* and *pyrF* mutations in bacteria. The total genomic DNA from a strain, 5106-9A, of *S. diastaticus* carrying the *STA1* gene was partially digested with a restriction endonuclease *Sau3A*, and fragments larger than 5 kilobases (kb) were recovered and inserted by ligation into the *Bam*HI site of the cloning vehicle, essentially as described by Carlson and Botstein.⁶⁾ The resulting recombinant DNA molecules were used to transform *E. coli* to ampicillin resistance. We obtained more than 20,000 ampicillin-resistant clones, 95% of which were tetracycline sensitive. Selection for plasmids containing the starch-fermentation gene was carried out in yeast. A recipient strain, YIYD, was one of the *Sta*⁻ segregants derived from the cross between two strains of *S. diastaticus*; one (YIY257) carrying *STA1* and another (5301-17B) carrying *STA3*. In asci from diploids constructed by the cross between the recipient strain YIYD and a *S. diastaticus* strain (YIY269 or 5301-17B), each carrying *STA1* or *STA3*, respectively, starch-fermentation ability was segregated as 2 (fermenting): 2 (non-fermenting), suggesting that the recipient strain YIYD carries no active starch-fermenting genes, and thus it was designated as *sta*^o. Recombinant plasmid DNA prepared from the library was used to transform the strain YIYD (*a leu2-3, 112 his4 lys7 sta*^o) to a leucine prototroph by the yeast transformation method described previously.^{5,7)} The selection medium was a synthetic minimal medium⁵⁾ containing 1% glucose, 2% soluble starch, 1.2 M sorbitol (as stabilizer

of protoplasts), 20 mg/ml histidine, and 30 mg/ml lysine. Transformants carrying a plasmid capable of complementing *sta*^o were then selected by their ability to form halos on the selection plates. Seven halo-forming transformants were obtained from about 30,000 *Leu*⁺ transformants, and one was chosen for further analysis. The halo-forming clone showed starch-fermentation ability and produced glucoamylase activity in the culture medium. Production of glucoamylase by the *Sta*⁺ clone required the presence of a plasmid in the cells: colonies grown non-selectively on the YPSB plate that lost the *Leu*⁺ plasmid also lost halo-forming ability. The recombinant plasmid carried by the *Sta*⁺ clone was recovered and transferred again to bacteria by transformation. Plasmid DNA was purified from bacterial culture and re-introduced back to the *sta*^o recipient. In this case, all *Leu*⁺ transformants formed halos around their colonies and showed both starch-fermenting and glu-

FIG. 1. Physical map of pSTA1.

DNA sequences are diagrammed as follows: thin solid line, 2- μ m DNA; thick solid line, pBR322; wavy line, *LEU2* gene; zigzag line, *URA3* gene; closed circle, yeast replication origin; closed triangle, bacterial replication origin. The tetracycline and ampicillin resistance genes are represented by *Tc*^r and *Ap*^r, respectively. Restriction sites are marked as follows: *Eco*RI (E), *Bam*HI (B), *Hind*III (H), *Sal*I (S), *Pst*I (Pt), *Kpn*I (K), *Pvu*II (Pv) and *Hpa*I (Hp). One of the two joints of the vector DNA and yeast DNA insert, indicated by B/Sau (*Sau*3A), is not cut by *Bam*HI. The size of DNA fragments in the insert is given in kilobases. The inverted arrows indicate the DNA sequence for the *Sta*⁺ determinant.

TABLE II. CHARACTERIZATION OF GLUCOAMYLASE PRODUCED BY THE TRANSFORMANT WITH pSTA1

Enzyme	Optimal conditions ^a		Heat stability (half period, min) ^b at				
	pH	Temperature (°C)	40°C	50°C	55°C	60°C	65°C
Glucoamylase I	5.3	50	— ^c	195	33	10	3
Glucoamylase produced by the transformant	5.3	50	— ^c	193	34	10	3

^a Cells of the strain YIY2-11A of *S. diastaticus* carrying the *STA1* gene and the transformant (*S. cerevisiae* with pSTA1) were separately cultured in YPS medium (1% yeast extract, 2% peptone and 3% soluble starch) at 28°C for 3 days. The culture supernatant was obtained by centrifugation, dialyzed extensively against deionized water and assayed for glucoamylase activity. The reaction mixture (final volume, 0.5 ml) contained 0.4% soluble starch, 80 mM acetate buffer (pH 4.6~5.6), and the enzyme solution (0.1 ml). The mixture was incubated at 35~60°C for up to 60 min, and every 10 min the reducing sugar (glucose) formed was determined by the standard Somogyi-Nelson method.²⁾ The optimal conditions for the enzyme activity were estimated from the initial rate of the reaction.

^b The dialyzed enzyme solution (1.0 ml) was incubated at the temperatures indicated. Aliquots (0.1 ml) were removed and the remaining activity was immediately assayed at 40°C for 20 min at the optimal pH (5.3); under these conditions, the activity proceeded linearly at least for 60 min.

^c The activity was not inactivated at least for 2 hr at 40°C.

coamylase-producing abilities. The plasmid, named pSTA1, appears to contain a determinant that allows the *sta*^o recipient to produce glucoamylase activity. The restriction map of the plasmid pSTA1 (13.9 kb) is shown in Fig. 1. The plasmid contained a 5.3 kb insert at the *Bam*HI site of the cloning vector. Location of the DNA sequence conferring the *Sta*⁺ phenotype in the insert was easily determined as described below. The plasmid pSTA1 was digested with *Kpn*I, and a subsequent fragment of 10.5 kb containing a yeast replication origin (origin of 2 μ m DNA) and the *URA3* gene (see Fig. 1) was purified by low-melting-point agarose gel electrophoresis. The linear DNA fragment, thus purified, was directly used to transform a *Sta*⁻ strain YIY2-12B carrying the *ura3* mutation to *Ura*⁺ by the method previously described by us.^{5,7)} The *Ura*⁺ transformants were obtained at a high frequency (more than 10³ transformants per μ g of the linear DNA), and all *Ura*⁺ transformants formed halos around their colonies, suggesting that the *Sta*⁺ determinant is located at a region from the *Kpn*I site to the *Bam*HI/*Sau*3A site in the insert, indicated by the inverted arrows in Fig. 1. Restriction anal-

ysis of the plasmid DNA recovered from the *Sta*⁺ *Ura*⁺ transformants indicated that the linear DNA molecule used for the transformation was correctly ligated *in vivo* so as to produce an autonomously replicating circular plasmid.

The plasmid pSTA1 also transformed a strain, YIY4B-3, of *S. cerevisiae* to *Sta*⁺. The transformant produced 5~10-fold the amount of glucoamylase activity compared with that secreted from a strain of *S. diastaticus* carrying only one copy of the *STA1* gene on the chromosomal DNA. When the transformant cells were cultured without shaking in YPS medium at 28°C for 6 days, glucose and ethanol were accumulated in the medium at concentrations of 0.2 and 0.5%, respectively, detected by high-pressure liquid chromatography. Both optimal conditions (pH and temperature) and thermodenaturation kinetics of the glucoamylase produced by the transformant were the same as those of the glucoamylase I secreted from strain YIY2-11A of *S. diastaticus* carrying the *STA1* gene (Table II).

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