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α-FACTOR-DIRECTED SYNTHESIS OF *BACILLUS STEAROTHERMOPHILUS* α-AMYLASE IN *SACCHAROMYCES CEREVISIAE*

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Promoter and leader sequence of *Bacillus stearothermophilus* α-amylase gene were removed and the gene was joined in-frame to sequences encoding the leader region of *Saccharomyces cerevisiae* mating pheromone α-factor on plasmid p69A (a hybrid of pBR322 and *S. cerevisiae* 2-μm plasmid). *S. cerevisiae* cells were transformed with plasmids containing the hybrid genes, obtaining yeast transformants which exhibit a significant extra-cellular amylolytic activity in solid medium, but not in liquid medium. Levels of α-amylase activity in solid medium were found to depend on the mode of fusion of the α-amylase gene to the α-factor leader region.

α-amylase is produced by many organisms in animals, plants and microorganisms. Among them, *Bacillus stearothermophilus*, a Gram-positive thermophilic bacterium, is known to produce a highly thermostable α-amylase (1). Thermostability, being a very desirable enzymatic property in many industrial processes, makes *B. stearothermophilus* α-amylase a potential candidate for industrial application. Its gene has already been cloned and sequenced (2,3). We have attempted to introduce it into the yeast *S. cerevisiae* by using an expression vector, based on the masculine pheromone α-factor (MFα1) gene which had proved to be very useful in the secretion of active foreign proteins (4,5). In this paper, we wish to report a successful expression of *B. stearothermophilus* α-amylase gene in yeast under the control of the MFα1 promoter.

MATERIALS AND METHODS

Strains and culture conditions. *B. stearothermophilus* A-10 was kindly provided by Dr. T. Oshima of this institute. It was grown at 60°C in Luria-Bertani (LB) medium containing 0.5% soluble starch. *Escherichia coli* HB101 (F^-, *hsdS20* ($r_B^-m_B^-$), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(Sm^r), *xyl-5*, *mtl-1*, *supE44*, λ)(6) was grown in LB medium containing 60 μg of ampicillin/ml and 15 μg

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Abbreviation: kb, kilobase pair(s); bp, base pair(s).

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of tetracycline/ml, when required. LB agar was made by addition of Difco Bacto-agar (1.5%, w/v). *S. cerevisiae* 5064 (*Matα, leu2*, [KIL-K]) was kindly provided by Dr. K. Ouchi of Kyowa Hakko Kogyo Co. Ltd.. Yeast cells were grown in YPD medium (7). Minimal medium (SD medium) for selection of yeast transformants and detection and determination of α-amylase activity was prepared as described by Sherman et al. (7). Regeneration agar was SD medium containing 22% sorbitol and 3% agar.

Plasmid DNA preparation and transformation. Plasmid DNA from *B. stearothermophilus* was prepared as described by Mielenz (8) and used as a source of α-amylase gene. Vector plasmids were extracted from *E. coli* by alkaline lysis method (Maniatis et al. (6)) and purified by CsCl-ethidium bromide gradient centrifugation (Davis et al. (9)). Small-scale preparations of *E. coli* plasmid DNA were made by the above alkaline lysis method. Transformation of *E. coli* was performed by the method described by Maniatis et al. (6). Preparation of plasmid DNA from *S. cerevisiae* and transformation of the yeast were performed according to the procedure of Beggs (10).

Chemicals and enzymes. Tetracycline and ampicillin were purchased from Sigma Chemical Co., St. Louis, Mo.. Restriction endonucleases and DNA modifying enzymes were purchased from Takara Shuzo Co., Kyoto, Japan and used according to the supplier's instruction.

Gel electrophoresis. Agarose gel electrophoresis was performed in horizontal slab gels in Tris-acetate buffer (pH 8.0) and the gels were photographed according to the method described previously (11). Polyacrylamide gel electrophoresis was done in Tris-borate buffer (pH 8.3) according to the method described by Maniatis et al. (6). DNA was recovered from agarose gel with GENECLEAN KIT of Bio101 Inc., La Jolla, Cal. according to the maker's recommendation.

Detection and determination of amylase activity. Amylase-producing colonies were detected on LB (*E. coli*) and SD (*yeast*) media-agar plates containing 0.5% blue starch (amylase screening plates). The blue starch was prepared as follows. Cibachron Blue FR, kindly provided by Dr. P. Gibson of Ciba-Geigy Co., was covalently ligated to free hydroxyl groups of starch according to the manufacture's protocol. The starch was dialyzed against tap water, centrifuged, and washed with 70% ethanol until no color remained in the supernatant. Amylase-producing colonies were easily detected on such plates after several hours of incubation at the desired temperature, owing to the appearance of a light blue halo surrounding them, in contrast to the deep blue color of the medium. Determination of amylase activity in the cell cultures was done by direct measuring the starch degradation as described by Wilson and Ingledew (12).

DNA sequencing. DNA sequencing was done according to the method of Sanger (13) and Messing (14), using a Takara M13 sequencing kit (Takara Shuzo Co.). ^{32}P-labeled CTP was purchased from Amersham.

RESULTS AND DISCUSSION

B. stearothermophilus α-amylase is known to be plasmid-borne (8). *B. stearothermophilus* contains four plasmids with sizes from 12 kb to over 108 kb. A mixture of the plasmids (20 μg) was digested completely with *Hin*dIII and electrophoresed in an agarose gel. The DNA fragments corresponding to about 5.2 kb were extracted from the gel and the purified fragments (0.5 μg) were ligated into the *Hin*dIII site of pBR322 (0.5 μg). *E. coli* HB101 was transformed with the ligation mixture. Out of 100 ampicillin-resistant colonies tested on amylase screening plates, four proved to have high amylolytic activity. All the four transformants contained a chimeric plasmid of 9.6 kb carrying a 5.2 kb-insert. This recombinant plasmid was named pAmy1 (see Fig.1).

Ihara et al. (3) have determined the complete nucleotide sequence of *B. stearothermophilus* α-amylase gene. The α-amylase gene (1644 bp) was found to be

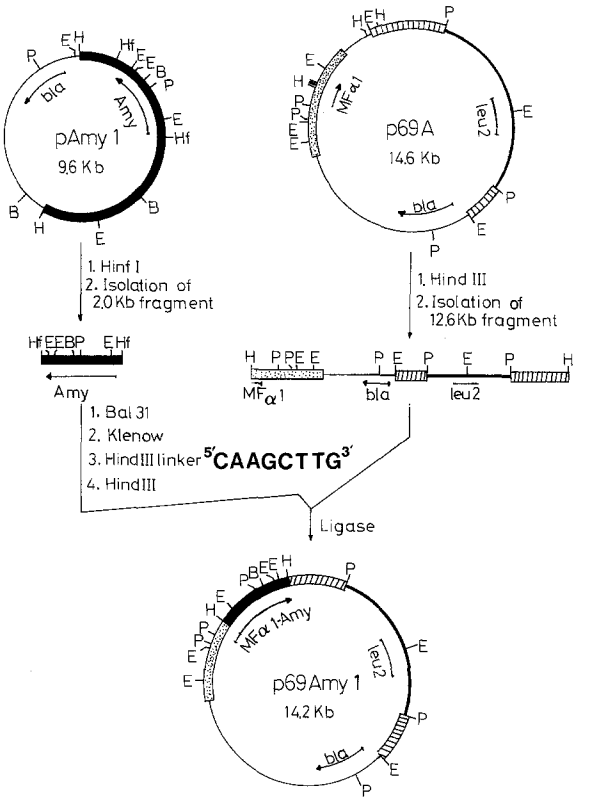

Fig.1. Schematic representation of the construction of α-amylase secretion plasmid p69Amy1.
——, pBR322 DNA; ——, *S. cerevisiae* chromosomal DNA containing *leu2* gene; ▥, 2-μm DNA; ▨, *S. cerevisiae* chromosomal DNA containing MFα1 gene; ■, *B. stearothermophilus* plasmid DNA carrying α-amylase gene. B, *Bam*HI restriction site; E, *Eco*RI; H, *Hind*III; Hf, *Hin*fI; P, *Pst*I. For the details, see text.

flanked by two *Hin*fI sites; one is 180 bp upstream from the start codon GTG and the other, 200 bp downstream from the stop codon TGA. Using this information, pAmy1 (10 μg) was digested with *Hin*fI, and a 2.0 kb fragment was electrophoretically isolated from an agarose gel. This fragment (1 μg) was made blunt-end by a treatment with Klenow fragment of *E. coli* DNA polymerase I, ligated to *Hin*dIII linker (5'-CAAGCTTG-3') and inserted into the *Hin*dIII site of pBR322 (1 μg). The resulting DNA sample was introduced into *E. coli* HB101, showing that the 2.0 kb fragment carries the entire functional α-amylase gene. Restriction maps of the cloned 2.0 kb fragment were identical to those reported by Ihara et al. (3) and the nucleotide sequence around 1 kb upstream from *Bam*HI site located inside the α-amylase structural gene was the same as that determined previously (3).

The α-amylase secretion plasmids were constructed as shown in Fig.1. The 2.0 kb fragment carrying the entire α-amylase gene (2 μg) was digested with Bal 31 nuclease (0.5 U) at 30°C for 2 min to remove its promoter and leader sequence encoding a signal peptide consisting of 34 amino acids (3). During this

time about 200 bp were expected to be removed from each end of the DNA fragment. The ends of the fragment were completely filled up by a treatment with Klenow fragment, and ligated to the above *Hin*dIII linker. After digestion with *Hin*dIII, the DNA was ligated to *Hin*dIII-digested plasmid p69A (2 μg). The resulting DNA sample was introduced into *E. coli* HB101 and the cells were selected for ampicillin-resistance. A DNA pool, prepared from *E. coli* transformants, was used to transform *S. cerevisiae* 5064 (the mating type α) to Leu^+ prototrophy. Out of 8000 transformants tested on the amylase screening plates, only one was found to show a sign of amylase production, A plasmid maintained in this colony was recovered in *E. coli* cells and analyzed. The results showed that the plasmid carries a 1.7 kb-insert, and this recombinant plasmid (14.2 kb) was named p69Amyl. The structure of p69Amyl at the junction between MFα1 and the α-amylase DNA sequences was determined by nucleotide sequencing. As shown in Fig.3-(I), the construction resulted in a *Hin*dIII gene fusion consisting of the MFα1 leader coding sequence, an artificially generated cystein codon, 13 codons remaining from the α-amylase signal peptide coding sequence and the mature α-amylase coding sequence. Analysis of the 3'-flanking region of the α-amylase gene verified that 59 bp after the stop codon were left.

p69Amyl conferred some amylase activity in amylase screening plates to *S. cerevisiae* (Fig.4). We have examined whether removing the above extra sequences corresponding to the 13 amino acids and the created cystein codon results in an increase of the α-amylase activity. In the experiments, the structure of 3'-flanking sequence of the α-amylase gene remained unchanged. Procedures for such construction are shown in Fig.2. Briefly, the 5'-part of the α-amylase gene from which around 40 bp had been nibbled away from the 5' end by Bal 31 was joined to its intact 3'-counterpart at the *Bam*HI site. The rearranged α-amylase structural gene was recovered through pBR322 in *E. coli* cells and the extent of 5'-nibbling was checked electrophoretically on polyacrylamide gel. Out of 94 samples tested, 12 samples showed a suitable extent of nibbling. These samples were then submitted to nucleotide sequencing. Two proved to have been nibbled in-frame; one has one codon remaining from the α-amylase signal peptide coding sequence and the other has just the mature α-amylase gene. Their structures are shown in Fig.3-(II) and (III), respectively. In both cases, the 3'-terminal amino acid of the MFα1-part is a tryptophan. This codon was derived from the *Hin*dIII linker; however, tryptophan is the first amino acid residue of the mature pheromone tridecapeptide (15). The two ideally shortened genes with *Hin*dIII-cohesive ends were ligated to *Hin*dIII-digested p69A, obtaining in *E. coli* the corresponding composite plasmids which were named p69Amyl.1 and p69Amyl.2 for Construction II and III, respectively. *S. cerevisiae* 5064 was transformed with the two composite plasmids, obtaining amylase-positive yeast transformants with a high frequency.

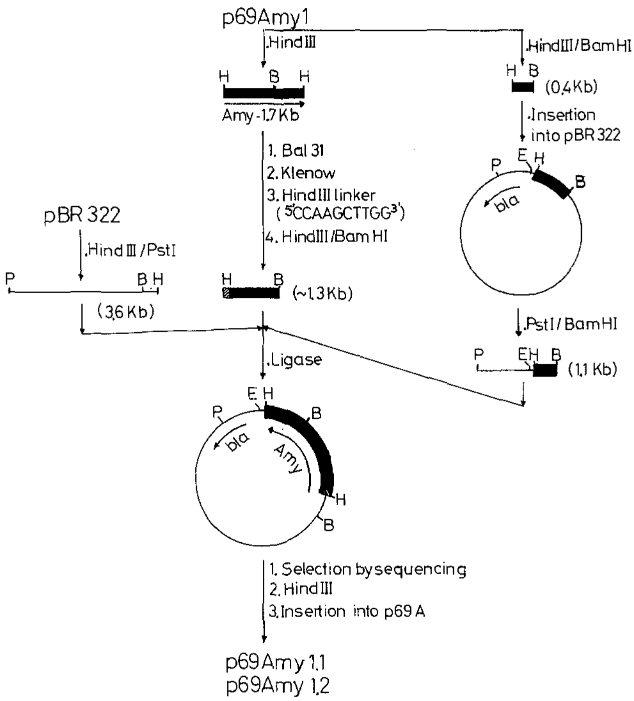

Fig.2. Construction of p69Amyl derivatives p69Amyl.1 and p69Amyl.2. For the details, see text and Fig.1.

I

240 250 260 (Hind III) 70 80

-GGG GTA TCT TTG GAT AAA AGA GAG GCT GAA GCT TGT *GCC TCG CTG TTC TGC CCA ACA-*

.gly val ser leu asp lys arg glu ala glu ala *cys ala ser leu phe cys pro thr...*

-13

II

240 250 260 (Hind III) 110 120

-GGG GTA TCT TTG GAT AAA AGA GAG GCT GAA GCT TGG *GCT GCC GCA CCG TTT AAC GGC-*

.gly val ser leu asp lys arg glu ala glu ala trp *arg ala ala pro phe asn gly...*

-1 +1

III

240 250 260 (Hind III) 110 120

-GGG GTA TCT TTG GAT AAA AGA GAG GCT GAA GCT TGG *GCC GCA CCG TTT AAC GGC ACC-*

.gly val ser leu asp lys arg glu ala glu ala trp *ala ala pro phe asn gly trp...*

+1

Fig.3. Nucleotide sequences at the fusion junction between the MFα1 and α-amylase genes of p69Amy-series plasmids.

Construction I, p69Amyl; II, p69Amyl.1; III, p69Amyl.2. Normal characters refer to the nucleotide sequence of the MFα1 gene; italics, the α-amylase gene; open characters, the sequence derived from the *Hin*dIII linker (this sequence is also the same as the naturally occurring one (15)). Small numbers over the nucleotide sequences refer to the nucleotide position relative to the first nucleotide of the start codon from the MFα1 or α-amylase genes. The numbers below the amino acid sequences refer to the position of amino acid residues in relation to the first amino acid residue (+1) of the mature α-amylase. The alanine and tryptophan residues positioned at the *Hin*dIII fusion site are the 3'-terminal amino acid of the MFα1 signal peptide and the first amino acid of the mature pheromone tridecapeptide, respectively.

The yeast transformants carrying p69Amy1, p69Amy1.1 and p69Amy1.2 formed halos in amylase screening plates. In supernatants from their cultures only very slight amylase activity could be found ($\lesssim$1 U/ml), although thermostability of the enzyme was retained. To compare amounts of amylase production in the transformed yeast cultures, the negatives of the halos pictured in Fig.4 yielded a densitometric measurement. The relative amounts of amylase production conferred by p69Amy1, p69Amy1.1 and p69Amy1.2 were estimated to be 55, 93 and 100%, respectively (Fig.4), indicating that the construction where all the original leader sequence of the α-amylase was removed conferred the highest amylolytic activity. The results suggest that the extra amino acid residues remaining from the α-amylase signal peptide may be harmful for secretion of amylase. However, there is also the possibility that the artificially added cystein residue alters the conformation of amylase protein to decrease its enzymatic activity.

As for the fact that very slight amylase activity was found in supernatants from liquid cultures, the following observations are worth of note. (1) The plasmids could be rescued from those yeast transformants. (2) The plasmid-harboring yeast cells pre-cultured in liquid medium presented enough amount of amylase activity when assayed on solid medium. (3) Amylase activity in liquid cultures was higher in the early stages of growth, and intra- and extra-cellular activity were similar in all the growth conditions tested. (4) The same results were obtained in experiments using other *S. cerevisiae* (mating type α) strains.

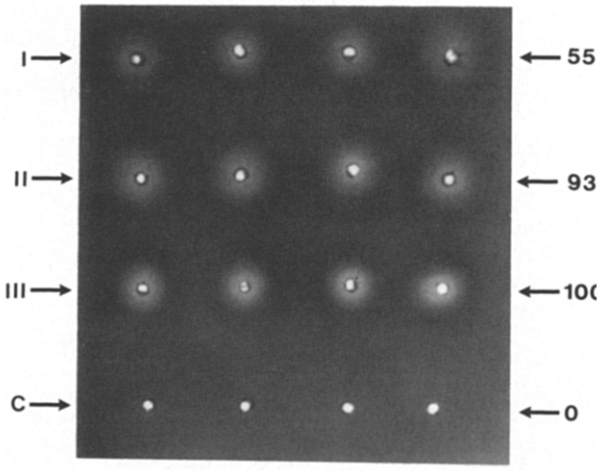

Fig.4. α-amylase production by *S. cerevisiae* cells carrying p69Amy-series plasmids.

Four sets of holes of 1 mm in diameter were made in an amylase screening plate, and each received around 10^5 cells of *S. cerevisiae* harboring the plasmids. The plate was incubated overnight at 30°C, and the intensity of the halos formed was densitometrically measured. I, *S. cerevisiae* 5064[p69Amy1]; II, 5064[p69Amy1.1]; III, 5064[p69Amy1.2]; C, 5064[p69A], used as a negative control. Numbers given at right are the relative amylolytic activity presented by each of the yeast transformants according to the results of the densitometric analysis.

These observations support the explanation that a (proteolytic) inactivation of the amylase protein occurs rapidly in liquid medium.

Recently it has been reported that *S. cerevisiae* cells carrying α-amylase genes from wheat (16) and mouse (17) could secrete α-amylase, efficiently hydrolysing starch presented in the liquid culture medium. To explain this difference between prokaryotic and eukaryotic sources of amylases, a more detailed analysis is necessary.

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