Rapid Paper

Cloning of *Bacillus subtilis*
 α -Amylase Structural
Gene in Plasmid pUB110Yasutoshi TAKEICHI, Kazutaka OHMURA,
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α -Amylase is one of the major extracellular enzymes of *Bacillus subtilis*. The production of the enzyme is regulated by many kinds of regulatory genes such as *amyR*, *tmrA*, *pap9* and others.¹⁾ Introduction of a plural number of these regulatory genes into one cell by DNA mediated transformation causes hyperproduction of α -amylase by the synergistic effect of the regulatory genes introduced.²⁾ An extra-hyper α -amylase producing strain, T2N26, was constructed which contains at least 6 kinds of regulatory genes by stepwise transformation and mutation. The strain produced 1,000~2,000 times as much α -amylase as the original strain, *B. subtilis* 6160, a derivative of *B. subtilis* Marburg 168.³⁾ The production of *B. subtilis* α -amylase seems to be a good system for gene engineering of an active protein secretion system.

Blobel *et al.*^{4,5)} proposed the signal hypothesis for the secretion of proteins in which the signal peptide, an extension of the amino terminal end of the secretory protein, is required for the transfer of the protein across the membrane. The penicillinase gene from *B. licheniformis*^{6~8)} and the α -amylase gene from *B. amyloliquefaciens* N (9) were cloned and were also found to have leader sequences corresponding to 34 and 31 amino acids in their signal peptides, respectively. In order to find a signal peptide in *B. subtilis* α -amylase, we cloned the α -amylase structural gene of *B.*

subtilis NA64 in a plasmid pUB110. Using the recombinant plasmid, we will study the secretion mechanism of the enzyme and construct a secretion vector in *B. subtilis*.

B. subtilis NA64 (*purB6 metB5*), 207-21 (*hsdM leuA8 lys21 aroI906 metB5 amyE07*), 207-25 (*hsdM leuA8 lys21 aroI906 metB5 amyE07 recE4*), and M15-1 (*amyE15 aroI906 recE4*) are derivatives of *B. subtilis* Marburg 168. *B. subtilis* temperate phage $\rho 11$ was supplied by Dr. J. A. Hoch (Scripps Clinic and Foundation, La Jolla, CA, U.S.A.). $\rho 11\text{daroI}^+ \text{amyE}^+$ was constructed from $\rho 11\text{AA4}$.¹⁰⁾ Details of the construction of $\rho 11\text{daroI}^+ \text{amyE}^+$ will be described elsewhere. DNA mediated transformation experiments using competent cells and spheroplasts of *B. subtilis* were done by slightly modifying the methods of Wilson and Bott¹¹⁾ and Chang and Cohen,¹²⁾ respectively. Southern blotting¹³⁾ was performed using a Schleicher Shuell BA85 nitrocellulose sheet. A [³²P]-labeled DNA fragment containing the α -amylase structural gene from $\rho 11\text{daroI}^+ \text{amyE}^+$ for use as a probe was prepared using the nick translation kit of RCC Amersham, England. Restriction enzymes and T4 induced DNA ligase were purchased from Takara Syuzo Co. Ltd. (Kyoto, Japan). Conditions for agarose gel electrophoresis and methods for DNA preparations have been described previously.^{14,15)}

To clone the *B. subtilis* α -amylase structural gene, $\rho 11\text{daroI}^+ \text{amyE}^+$ DNA, which retains the α -amylase structural gene from *B. subtilis* NA64, was partially digested with restriction enzyme *Sau3A*. The DNA fragments generated were then ligated to *Bam*HI-cleaved pUB110. Hybrid plasmids were transferred into 207-25 according to the method of spheroplast transformation. Transformants were screened on plates for the expression of α -amylase activity and kanamycin (Km) resistance. One of the $\text{Amy}^+ \text{Km}^r$ transformants harbored a 6.8 kilo-base (Kb) plasmid which was designated as pTUB4 (Fig. 1). The plasmid contains a 2.3 Kb insert in pUB110. pTUB4 was transferred into 207-21, 207-25, M15-1, according to both the methods of

FIG. 1. Endonuclease Restriction Map of Plasmid pTUB4 DNA.

The 2.3 Kb (■) fragment carrying the *amyE*⁺ gene of *B. subtilis* was inserted into plasmid vector pUB110 (□).²⁰⁾

Sau3A/*Bam*HI are junction sites of pUB110 and the inserted DNA, which were not hydrolyzed by *Bam*HI.

FIG. 2. Hydrolysis of pTUB4 by Restriction Enzymes and Southern Hybridization of [³²P] Labeled *B. subtilis* α -Amylase Probe DNA Purified from ρ 11daroI⁺*amyE*⁺ to the DNA Fragments.

pTUB4 DNA was digested with *Bgl*II-*Sal*I (line 1), *Bgl*II-*Pst*I (line 2), *Bgl*II-*Cla*I (line 3), *Bgl*II (line 4), and *Eco*RI (line 5), electrophoresed in 1.0% agarose gel and blotted onto a nitrocellulose filter. A (line 1~5) is an UV photograph of the ethidium bromide stained gel and B (line 1~5) is an autoradiograph of the nitrocellulose filter hybridized with the probe.

competent cell transformation and spheroplast transformation. All Km^r transformants were Amy⁺.

TABLE I. Amy⁺ TRANSFORMING ACTIVITY OF DNA FRAGMENTS GENERATED ON DIGESTION WITH RESTRICTION ENZYMES FROM pTUB4

DNA fragments were extracted from DNA bands in an agarose gel which was similar to that in Fig. 2A, and were added with pTUE1 to the competent culture of strain 207-21. About 10⁴ Aro⁺ transformants appeared on a selective plate with the addition of pTUE1 DNA (0.01 μ g/0.1 ml of the culture). Amy⁺ transformants were detected by halos around the colonies after KI-I₂ solution was sprayed onto the selective plates. Fragment A, large molecular size fragment; fragment B, small molecular size fragment.

DNA fragments	No. of Amy ⁺ transformants
<i>Eco</i> RI fragment A	0
<i>Eco</i> RI fragment B	0
<i>Bgl</i> II fragment	22
<i>Bgl</i> II- <i>Sal</i> I fragment A	0
<i>Bgl</i> II- <i>Sal</i> I fragment B	0
<i>Bgl</i> II- <i>Pst</i> I fragment A	0
<i>Bgl</i> II- <i>Pst</i> I fragment B	0
<i>Bgl</i> II- <i>Cla</i> I fragment A	0
<i>Bgl</i> II- <i>Cla</i> I fragment B	30
pTUB4 untreated	85

To determine the location of the α -amylase structural gene in pTUB4, the plasmid was digested with either *Eco*RI, or *Bgl*II, or both together, *Bgl*II and *Sal*I, *Bgl*II and *Pst*I, or *Bgl*II and *Cla*I. The digest was analysed by agarose gel electrophoresis (Fig. 2A). DNA bands in the gel were transferred onto a nitrocellulose filter by the method of Southern¹³⁾ and hybridized with [³²P]-labeled α -amylase probe DNA which was purified from ρ 11daroI⁺*amyE*⁺ DNA after digestion with restriction enzymes. An autoradiograph is shown in Fig. 2B. Every fragment generated was hybridized with the probe.

Subsequently each DNA band in Fig. 2A in another gel was sliced out under 365nm ultra-violet light. The Amy⁺ transforming activity of the DNA fragments extracted was tested by competent cell transformation experiments using 207-21 as the recipient strain. pTUE1,¹⁶⁾ an *E. coli* recombinant plasmid of pBR322 containing the *B. subtilis* *aro*I⁺ gene which encoded shikimate kinase¹⁷⁾ and the *tmrB* gene

which shows a tunicamycin-resistant character,¹⁸⁾ was added to the competent culture at the same time, for selection of transformed cells. Amy⁺ transformants were detected when pTUB4, the *Bgl*II-digest of pTUB4, or *Bgl*II-*Cla*I fragment B, the smaller molecular weight DNA fragment of the two fragments generated on digestion with *Bgl*II and *Cla*I from pTUB4 was added (Table I). Since the transformation activity from an α -amylase deficient mutation (*amyE07*) to AmyE⁺ of pTUB4 was destroyed on digestion with *Eco*RI, *Pst*I and *Sal*I, the restriction sites of these enzymes in the inserted DNA were located close to the mutational site, *amyE07*, that occurred in the *B. subtilis* α -amylase structural gene. α -Amylase activity was lost on the mutation but an immunological crossreacting material was produced.¹⁹⁾ The distance from the *Cla*I site to the *Sal*I site was approximately 0.5 Kb.

These results showed that the *B. subtilis* α -amylase structural gene was cloned in plasmid pUB110. α -Amylase expressed from the recombinant plasmid pTUB4 was secreted into the culture medium, and the enzyme activity in the medium was neutralized by a rabbit antiserum against *B. subtilis* α -amylase.

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