Molecular Cloning and Expression of an α -Amylase Gene from an α -Amylase Extrahyper Producing *Bacillus subtilis*

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Bacillus subtilis 2633 hyperproduced α -amylase (about 3000-fold amount of the parental strain, *B. subtilis* 6160). An α -amylase gene of *B. subtilis* 2633 was cloned into *Escherichia coli* using a *B. subtilis*-*E. coli* shuttle vector, pHY300PLK. When the recombinant plasmid pAM26 containing the α -amylase gene was introduced into *B. subtilis* 6160, a transformant produced about 40,000 U/ml of α -amylase, which is 4000 times that of *B. subtilis* 6160. This indicates that the α -amylase gene (*amyR-amyE*) of 2633 has hyperproducing activity in itself. The nucleotide sequencing of the promoter region and comparison with those of other *B. subtilis* strains suggested that several differences in the nucleotide sequences upstream from the transcription initiation site could be responsible for the α -amylase production. Southern blot analysis showed that *B. subtilis* 2633 contains several copies of the α -amylase gene, which should also contribute to the hyperproductivity.

α -Amylase is one of several extracellular enzymes of *Bacillus subtilis*. In the production of α -amylase, at least three major regulation systems can be considered. 1) *amyR* is a specific regulatory gene for the production which is linked to the structural gene (*amyE*) and now considered as the promoter region.^{1~3)} Three types of *amyR* were known; *amyR1* originated from *B. subtilis* 168 (or 6160). *amyR2* from *B. natto* and *amyR3* from *B. subtilis* var. *amylo-sacchariticus*. The latter two types are considered to be hyperproducing promoters. 2) *pap* regulates the production of several extracellular enzymes including α -amylase, neutral protease, and alkaline protease.^{4~6)} This genetic locus is the same as *sacU* or *amyB*.⁷⁾ 3) *tmrA7* shows both tunicamycin resistance and α -amylase hyperproduction.^{8,9)} This mutation is mapped to a position just beside the *amy* gene and arises from gene amplification of the *tmr* region and the *amy* region simultaneously,¹⁰⁾ which results both in tunicamycin resistance and α -amylase hyperproduction by a

gene dosage effect.

During the past many years we have been engaged in genetic studies on the productivity of α -amylase in *B. subtilis*. We have succeeded in increasing the productivity of α -amylase by mutagenesis with NTG and DNA-mediated transformation, and obtained the final strain *B. subtilis* 2633, which showed a 3000-fold increase of α -amylase production.¹¹⁾ In this paper we isolated the α -amylase gene of *B. subtilis* 2633 and discuss its extrahyper α -amylase productivity in terms of its strong promoter activity and gene amplification.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this work are listed in Table I. The compositions of LB medium, BY medium, and ABY medium were described previously.^{11,16)}

Enzymes. Restriction endonucleases, T₄ DNA ligase, and the bacterial alkaline phosphatase of *Escherichia coli* were purchased from Takara Shuzo Co.

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TABLE I. LIST OF STRAINS AND PLASMID VECTORS USED IN THIS STUDY

Strains	Genotype
<i>B. subtilis</i>	
6160 ⁽¹²⁾	<i>purB6, trpB3, metB5, amyR1</i>
2633 ⁽¹¹⁾	<i>purB6, trpB3, metB5, amyR3, papS, amyS, tnrA7</i>
M15 ⁽¹³⁾	<i>amyE⁻, purB6, metB5</i>
<i>E. coli</i>	
HB101	<i>hsdS, leu, pro, lac, gal, strA, thi, recA</i>
MM294	<i>F⁻, supE44, endA1, thi1, hsdR4, (recA⁺)</i>
Plasmid vectors	
pHY300PLK ⁽¹⁴⁾	Ap ^r , Tc ^r
pUC18 ⁽¹⁵⁾	Ap ^r

DNA isolation. *B. subtilis* 2633 was grown in LB medium at 37°C for 15 h. After the harvest of the cells, chromosomal DNA was isolated essentially by the procedure of Saito and Miura.⁽¹⁷⁾ Plasmid DNAs were prepared essentially as described.⁽¹⁶⁾

Cloning procedures. A *B. subtilis*-*E. coli* shuttle vector, pHY300PLK (Yakult Co.),⁽¹⁴⁾ was cleaved with a restriction endonuclease, BamHI, and then treated with bacterial alkaline phosphatase. Chromosomal DNA isolated from *B. subtilis* 2633 was partially digested with *Sau*3AI. Both digests were ligated with T₄ DNA ligase using the conditions recommended by the supplier. The ligated DNA was introduced into *E. coli* MM294 by the method of Hanahan.⁽¹⁸⁾ Transformants (Ap^r, Tc^r) were replicated onto LB medium plates containing 0.5% soluble starch. Colonies producing haloes detected by I₂-KI were picked up and further analyzed.

Transformation of *B. subtilis*. Transformation of *B. subtilis* 2633, 6160, and M15 by a pHY300PLK recombinant plasmid prepared from *E. coli* MM294 (*recA*⁺) was done as described.⁽¹⁹⁾

α -Amylase activity. α -Amylase activity was assayed by a modification of the blue value method of Fuwa.⁽²⁰⁾ Two milliliters of 0.5% soluble starch in 50 mM phosphate buffer (pH 6.2) was mixed with 1 ml of an enzyme solution. After an appropriate period of incubation at 40°C, a 0.2-ml sample of the reaction mixture was added to 5 ml of M/3000 I₂-KI solution. The optical density at 700 nm was measured by a spectrophotometer (Shimadzu UV-100-01).

Localization of α -amylase in *E. coli*. Extracellular, periplasmic, and intracellular fractions were prepared as described by Neu and Heppel,⁽²¹⁾ and their α -amylase activities were measured as described above.

Thin layer chromatography analysis. Crude enzyme preparations of culture fluids of *B. subtilis* strains were

incubated with 1% soluble starch at 37°C for 18 hr. Hydrolysis products were analyzed by thin layer chromatography (TLC) on Kieselguhr as described by Edwin and Hanke⁽²²⁾ using a solvent system of butanol:pyridine:water = 75:15:10 by volume.

SDS-polyacrylamide gel electrophoresis. Slab gel electrophoresis was done under denaturing conditions as described by Laemmli.⁽²³⁾ Slab gels used were 1 mm thick and consisted of 8% polyacrylamide separating gels and 3% polyacrylamide stacking gels.

DNA sequencing. Appropriate restriction fragments of the recombinant plasmid pAM26 were subcloned into a plasmid vector pUC18,⁽¹⁵⁾ and the nucleotides were sequenced by the dideoxy chain termination method of Hattori and Sakaki.⁽²⁴⁾

Southern hybridization. Chromosomal DNA was digested with an excess of the indicated restriction enzymes. The digested DNA (1 μ g) was electrophoresed on 0.8% agarose gels and transferred to Gene Screen Plus membranes (New England Nuclear) by the method of Southern.⁽²⁵⁾ Membranes were prehybridized at 65°C overnight in a solution containing 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 0.2% (wt/vol) SDS, 250 μ g/ml of heat-denatured salmon sperm DNA, 0.2% Ficoll 400, 0.2% bovine serum albumin, and 0.2% polyvinylpyrrolidone. ³²P-labeled probe DNA was prepared with a nick translation kit (Amersham) using [³²P]- α -dCTP (3000 Ci/mmol, New England Nuclear). Hybridization was done in the same solution as the prehybridization containing 10⁶ cpm/ml of ³²P-labeled probe at 65°C for 24 hr. Membranes were finally washed at 65°C in 0.1 $\times$ SSC (SSC in 0.15 M NaCl, 0.015 M NaCl, 0.015 M sodium citrate) containing 0.1% SDS.

RESULTS

Cloning of the α -amylase gene

Among about 10^4 ampicillin- and tetracycline-resistant transformants tested, two colonies showed haloes detected by I_2 -KI. Since both of the plasmids prepared from the two positive transformants contained 2.3 kb.p. inserts and had the same restriction maps (Fig. 1), one of the two plasmids, named pAM26, was used for further analyses. When the plasmid pAM26 was re-introduced into *E. coli* MM294 and HB101, all of the transformants (Ap^r , Tc^r) produced haloes around the colonies in starch-containing plates detected by I_2 -KI. Then, we concluded that the plasmid pAM26 really contains the α -amylase gene. The restriction map of pAM26 (Fig. 1) was similar to those of the α -amylase gene of *B. subtilis* 168,²⁶⁾ NA64,²⁷⁾ and N7.²⁸⁾

*Expression and localization of α -amylase in *E. coli**

On plates α -amylase positive colonies were detected by the iodine technique, but a little activity was found in the supernatant in liquid growth (Table II). When the extracellular, periplasmic, and cellular fractions were prepared as described in MATERIALS AND METHODS and measured for their α -amylase activities, the main activity of α -amylase was detected in the periplasmic space (Table II).

 *α -Amylase activity of the cloned α -amylase gene in *B. subtilis**

Next, we introduced the cloned α -amylase gene into various *B. subtilis* strains by plasmid transformation.¹⁹⁾ *B. subtilis* 2633 carrying pAM26 produced 28900 U/ml of α -amylase (Table II). *B. subtilis* 6160 carrying pAM26 produced 39,600 U/ml (Table II), which was a great increase (about 4000 times) of α -amylase productivity upon the introduction of pAM26.

Identification of α -amylase encoded by pAM26 in culture fluids

As *B. subtilis* strains carrying pAM26 and *B. subtilis* 2633 showed extrahyper productivity

FIG. 1. Physical Map of pAM26 Constructed with pHY300PLK and *B. subtilis* 2633 DNA.

A circle indicates the vector pHY300PLK. The above open box indicates the insert encoding α -amylase of *B. subtilis* 2633.

TABLE II. α -AMYLASE PRODUCTION BY VARIOUS STRAINS OF *B. subtilis* AND *E. coli*

Strains	α -Amylase activity (U/ml)	
<i>B. subtilis</i>	6160	10
	2633	28,900
	6160 carrying pAM26	39,600
	2633 carrying pAM26	28,700
<i>E. coli</i>	MM294	0
	MM294 carrying pHY300PLK	0
	MM294 carrying pAM26	334
	(extracellular)	(59)
	(periplasmic)	(261)
	(intracellular)	(14)

Each of *B. subtilis* strains was cultured in ABY medium at 30°C for 72 hr except for *B. subtilis* 6160 which was cultured in BY medium.

E. coli MM294 strains were cultured in LB medium at 37°C and fractionated as described in MATERIALS AND METHODS.

of α -amylase, we could easily identify the α -amylase protein in the culture fluid (Fig. 2). From the mobility on SDS-polyacrylamide gel electrophoresis, we could estimate the molecular weight of the α -amylase. All of the extracellular α -amylase of *B. subtilis* 2633 carrying pAM26 and *B. subtilis* 6160 carrying pAM26 (lanes 3 and 4) have the molecular weight of 45000 which coincided with that of *B. subtilis* 2633 (lane 2).¹¹⁾ But *B. subtilis* 6160 alone showed no clear band of the α -amylase (lane 1).

Hydrolysis products of soluble starch by α -amylases

The thin-layer chromatogram in Fig. 3

FIG. 2. Analysis of the culture Fluid Proteins of Various *B. subtilis* Strains by SDS-Polyacrylamide Gel Electrophoresis.

Culture fluids of various *B. subtilis* strains were precipitated with acetone¹¹⁾ and electrophoresed on 8% polyacrylamide gels. Lane 1, *B. subtilis* 6160; lane 2, *B. subtilis* 2633; lane 3, *B. subtilis* 6160 carrying pAM26; lane 4, *B. subtilis* 2633 carrying pAM26.

shows the hydrolysis products released from soluble starch by various α -amylases. The α -amylases of *B. subtilis* 2633,¹¹⁾ *B. subtilis* 6160,²⁹⁾ *B. subtilis* 2633 carrying pAM26, *B. subtilis* 6160 carrying pAM26 and *B. subtilis* M15 carrying pAM26 produced glucose and maltose (lanes 4~8). On the other hand, that of *B. natto* IAM1212 did not produce glucose, as previously described (lane 3).²⁹⁾

The nucleotide sequence of the amyR region of pAM26

Since the cloned α -amylase gene of *B. subtilis* 2633 (pAM26) had hyperproductivity in *B. subtilis* 6160 as described above, it should have strong promotor activity. So, we sequenced the nucleotides of pAM26 corresponding to the *amyR* region (*amyR3*) and the N-terminal region of the coding sequence (*amyE*). Comparison with those of *amyR1* (*B. subtilis* 168)²⁶⁾ and *amyR2* (*B. subtilis* NA64²⁷⁾ or N7²⁸⁾) (Fig. 4) revealed the following results. 1) Significant sequence homology was observed among the three types of *amyR* (about 98%). 2) No change was observed in the nucleotide and amino acid sequences between the Shine-Dalgarno sequence and the signal peptide sequence except one residue of

FIG. 3. Hydrolysis Products by α -Amylases from Various *B. subtilis* Strains.

Crude enzyme preparations of culture fluids of *B. subtilis* strains were reacted with 1% soluble starch at 37°C for 18 hr. Lanes 1 and 2, markers of glucose and maltose, respectively; lane 3, *B. natto* IAM1212; lane 4, *B. subtilis* 6160; lane 5, *B. subtilis* 2633; lane 6, *B. subtilis* 6160 carrying pAM26; lane 7, *B. subtilis* 2633 carrying pAM26; lane 8, *B. subtilis* M15 (*amy*⁻) carrying pAM26.

FIG. 4. Nucleotide Sequence of pAM26 Corresponding to *amyR3* and a Portion of *amyE*, and Comparison with Those of Other *B. subtilis* Strains (*amyR1* of *B. subtilis* 168²⁶) and *amyR2* of *B. subtilis* NA64²⁷).

Left numbers in parentheses indicate the nucleotide residue numbers of *B. subtilis* 2633, numbered from the first residues of initiation methionine, negatively upstream and positively down stream.

FIG. 5. Southern Blot Analysis of Chromosomal DNA from *B. subtilis* 6160 and 2633.

Chromosomal DNA was digested with each of indicated restriction enzymes (B, *Bam*HI; H, *Hind*III; P, *Pst*I; S, *Sal*I). The letter M denotes the marker of λ DNA digested with *Hind*III. Left and right panels denote the profiles of ethidium bromide staining and an autoradiogram of Southern blot analysis probed by pAM26, respectively.

B. subtilis 168 in the signal sequence. This shows that the three types have almost the same translational and secreting activities. 3) The sequences of the upstream regions of the transcription initiation sites were different at several points among the three types (especially at -128 to -115 and -217 to -184). 4) *amyR1* and *amyR3* lacked the stem and loop structure which was shown in *amyR2* (indicated by the two arrows above the sequences in Fig. 4).

Southern hybridization analysis

Since *B. subtilis* 2633 is tunicamycin-resistant, which might be caused by gene amplification of the *tmr-amy* region as previously reported for other *B. subtilis* strains,^{8~10} we examined by Southern hybridization whether the α -amylase gene of *B. subtilis* 2633 was amplified. When a ³²P-labeled insert of pAM26 was used as a probe, one or two bands were detected for each digestion of *B. subtilis* 2633 and 6160 chromosomal DNA (Fig. 5). Although the lengths of hybridized DNA fragments of *B. subtilis* 2633 and 6160 were similar, the strengths were distinct; those of 2633 were several times higher than those of 6160. This indicates that the α -amylase gene of *B. subtilis* 2633 is amplified.

DISCUSSION

In this study we have isolated one of the several copies of the gene for α -amylase of *B. subtilis* 2633, an extrahyper α -amylase producing strain. In the cloning procedure, we used a *B. subtilis*-*E. coli* shuttle vector, pHY300PLK,¹⁴ and therefore, we could easily introduce the plasmid pAM26 from *recA*⁺ *E. coli* strains into *B. subtilis* strains without further plasmid constructions such as combination with another replication origin functioning in *B. subtilis*.

In *E. coli*, pAM26 containing an α -amylase gene of *B. subtilis* 2633 created clear haloes around the colonies, but most of the α -amylase existed in the periplasmic space (Table II). A similar situation was also observed with other

extracellular enzymes of *B. subtilis* which are expressed in *E. coli*.³⁰

When pAM26 was introduced into *B. subtilis* 6160, the Tc^r transformants produced about 40,000 U/ml of extracellular α -amylase (Table II). This value was nearly same as that of *B. subtilis* 2633 and was 4000 times higher than that of *B. subtilis* 6160 harboring no pAM26. Since the copy number of pHY300PLK in *B. subtilis* was estimated as about 50 (supplier's comments), one copy of pAM26 was supposed to produce about 800 U/ml of α -amylase in 6160. This indicates that the α -amylase producing activity of pAM26 is about 80 times higher than the resident α -amylase gene of 6160 on the assumption that the specific activities of both α -amylases are the same.

Molecular basis for the α -amylase hyperproductivity of pAM26 was investigated by nucleotide sequencing analysis (Fig. 4). The stem and loop structure found in *amyR2*²⁷ did not exist in *amyR3* of 2633 irrespective of its strong activity. That is, this secondary structure may have no or little effect on the α -amylase production. On the other hand, several differences in the nucleotide sequences were detected in the upstream regions of the transcription initiation sites (Fig. 4), and these differences might be crucial for the α -amylase production. Such an example was recently reported; a point mutation in the -10 region of the *sacQ* gene results in ten times higher promoter activity.³¹ In fact, the sequence in the -10 regions of *B. subtilis* 168 (a low producer of α -amylase, *amyR1*) was TTAAAT, whereas those of *B. subtilis* NA64 and 2633 (high producers, *amyR2* and *amyR3*) were TAAAAT (Fig. 4). Further analyses such as *in vitro* mutagenesis will clarify the precise mechanisms for the α -amylase hyperproduction.

When pAM26 was introduced into the parental strain, *B. subtilis* 2633, however, no increase of α -amylase production was observed (Table II). This may be because no further production (protein synthesis and/or secretion) of α -amylase might be possible in this

strain. That is, extracellular proteins of *B. subtilis* 2633 carrying pAM26 and *B. subtilis* 6160 carrying pAM26 were almost the only α -amylase (Fig. 2) and further production of extracellular α -amylase may be impossible.

Previously, a tunicamycin-resistant phenotype of *B. subtilis* was known to arise simultaneously with the hyperproduction of α -amylase.^{8,9)} Recently, this phenomenon was explained by gene amplification of the *tmr-amy* region.¹⁰⁾ As shown in Fig. 5, *B. subtilis* 2633 has several copies of the α -amylase gene, which should be also responsible for the α -amylase hyperproductivity by the gene dosage effect.

The molecular weight of the α -amylase encoded by pAM26 was 45000 from SDS-polyacrylamide gel electrophoresis. This value coincided with that of *B. subtilis* 2633,¹¹⁾ was smaller than that of 6160,²⁹⁾ similar to that of *B. subtilis* N7²⁸⁾ and larger than that of *B. natto* IAM1212.²⁹⁾ The hydrolysis products of soluble starch were glucose and maltose for α -amylases of 6160,²⁹⁾ 2633¹¹⁾ and pAM26 (Fig. 3), and maltose and maltotriose for those of N7²⁸⁾ and *B. natto*.²⁹⁾ The α -amylases of *B. subtilis* 2633 and N7 have similar molecular weights, but their final products of starch hydrolysis seem to be different. The substrate specificity will be further discussed after the completion of sequencing analyses of all these various α -amylase genes.

In summary, we show here that the mechanisms for the extrahyper productivity of α -amylase in *B. subtilis* 2633 are mainly explained by the strong promotor activity of the 2633 α -amylase gene and by the gene dosage effect. Further, the hyperproducing activity of the 2633 α -amylase gene in itself will be useful for the production of other proteins in *B. subtilis*.

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