MOLECULAR CLONING OF α-AMYLASE GENE FROM A MUTANT OF BACILLUS LICHENIFORMIS AND ITS EXPRESSION IN VARIOUS STRAINS

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The amylase gene of a mutant of Bacillus licheniformis was cloned in a plasmid pBR322 by a shotgun method and transferred into Escherichia coli HB101. The transformants produced amylase in the medium. A restriction map of the plasmid carrying amylase gene (pNTK1) was constructed and the smaller plasmids pNTK2 and pNTK3 were made. Ninety-three percent of the amylase activity in the E. coli carrying pNTK3 was found in the periplasmic region. A shuttle vector (pSNK1) was constructed from pNTK3 and pUB110 and it is maintained in both E. coli and Bacillus. The amylase productivity of various Bacillus cells carrying pSNK1 were found to be different from strain to strain. The causes of these differences are discussed.

In a previous paper (1) we reported that in Bacillus licheniformis the productivity of thermostable α-amylase was enhanced about 2,000 times by a series of mutations and mono cell selection in D-cycloserine-containing media in increasing concentrations. The highest-amylase-producing strain SMN11 reported in that paper was further treated by N-methyl-N’-nitro-N-nitrosoguanidine and a mutant (TY002) was obtained which produced about 4,000 units/ml of α-amylase (i.e. two times as much as SMN11). In this experiment, we used this strain (TY002) as a source of amylase gene and have cloned it in pBR322. The resultant plasmid, named pNTK1, was made smaller by restriction endonuclease digestion and self ligation. The smallest plasmid pNTK3 was combined with Bacillus plasmid pUB101. A plasmid thus obtained (named pSNK1) was maintained in both Escherichia coli and Bacillus subtilis, showing the nature of the shuttle vector. We examined the amount and the heat stability of amylase produced by various strains which were introduced these plasmids.

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MATERIALS AND METHODS

**Bacterial strains and media.** The bacterial strains used in this study are listed in Table 1. Plasmids pBR322 and pUB110 were used as vectors. Medium BY containing meat extract 0.5%, polypeptone 1.0%, NaCl 0.2%, and yeast extract 0.2% (pH 7.2) was used for general cultures. Medium DM3, containing sodium succinate 13.5%, casamino acids 0.5%, yeast extract 0.5%, glucose 0.5%, K₂HPO₄ 0.35%, KH₂PO₄ 0.15%, MgCl₂ 40 mM, bovine serum albumin 0.01% (pH 7.2), was used to regenerate *Bacillus* protoplasts. BY medium supplemented with 0.5% soluble starch was used to detect transformants carrying amylase gene.

**Preparation of DNA and gel electrophoresis.** Chromosomal DNA was prepared from *Bacillus licheniformis* TY002 by the method described by Okanishi and Gregory (5). Plasmid DNAs were prepared by the method described by Maruo and Tojo (6). DNAs were digested at 37°C for 1 hr in 10 µl of reaction mixture containing 0.5–1.2 µg DNA, 3–10 units of restriction endonuclease and 1 µl of buffer, as recommended by the authors (7). After the incubation, restriction mixtures were treated at 70°C for 7 min. Samples containing 0.1 µg DNA were added with 1/4 volume of pigment solution (50% sucrose, 100 mM EDTA, 3 Na, 0.14% bromocresol purple) and applied to agarose gel. Agarose gel electrophoresis (AGE) was carried out in 0.8% agarose gel in Tris phosphate buffer at 100 V for 1 hr. In AGE, Hind III fragments of λ-DNA and Hae III digests of φX174 phage DNA were used as molecular weight standards.

**Transformation.** *Escherichia coli* were transformed with plasmid DNA by calcium chloride treatment according to the method of Mandel and Higa (8). Transformants carrying amylase genes were grown on 0.5% BY-starch plate containing 20 µg/ml of ampicillin and detected by starch-iodine reaction. The protoplast transformation was performed as described by Chang and Cohen (9). Transformants were selected on DM3 regeneration medium containing 150 µg/ml of kanamycin.

**Detection of plasmids in transformants.** Plasmids in transformants were

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amylase activity</th>
<th>Origin</th>
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<tbody>
<tr>
<td><em>E. coli</em> HB101</td>
<td>ND</td>
<td>laboratory stock</td>
</tr>
<tr>
<td><em>E. coli</em> C600</td>
<td>ND</td>
<td>laboratory stock</td>
</tr>
<tr>
<td><em>B. licheniformis</em> NYK74</td>
<td>1 U*</td>
<td>laboratory stock</td>
</tr>
<tr>
<td><em>B. licheniformis</em> TY002</td>
<td>4,000 U*</td>
<td>see text</td>
</tr>
<tr>
<td><em>B. subtilis</em> M15</td>
<td>ND</td>
<td>ref. 2</td>
</tr>
<tr>
<td><em>B. subtilis</em> 6160</td>
<td>10 U</td>
<td>ref. 3</td>
</tr>
<tr>
<td><em>B. subtilis</em> 2633</td>
<td>30,000 U</td>
<td>ref. 4</td>
</tr>
</tbody>
</table>

ND: not detected.

*Thermostable.*
detected by a modification of the alkaline lysis method reported by KADO and LIU (10).

Measurement of amylase activity. Amylase was assayed by the modified method of FUWA (11): 2 ml of 0.5% soluble starch in 0.05 M phosphate buffer (pH 6.2) was mixed with 1 ml of an enzyme solution diluted appropriately and incubated at 40°C.

A 0.2-ml aliquot of the reaction mixture was added to 5 ml of 1/3,000 N I$_2$-KI solution. The optical density at 700 nm was measured and the amylase units in 1 ml of original enzyme solution were calculated as follows: \( \frac{(OD_0 - OD_t) \times 100}{\text{incubation time (min) \times dilution ratio}} \), where \( OD_0 \) is the optical density at 0 min and \( OD_t \) is the optical density at \( t \) min. Reaction was stopped at the time when \( OD_t \) became 75 to 70% of \( OD_0 \).

Enzymes. Restriction endonucleases were purchased from Takara Co. except Cla I which was from New England Biolabs Inc. Alkaline phosphatase of calf intestine was purchased from Boehringer Mannheim.

RESULTS

Cloning of the amylase gene

Hind III-digested-chromosomal DNA of B. licheniformis TY002 was ligated with Hind III-digested and alkaline phosphatase treated pBR322. The ligated DNA was introduced into E. coli HB101 and the transformants were selected on BY plates containing 20 µg/ml of ampicillin. Twenty-four hundred ampicillin-resistant colonies were replicated onto BY plates containing 20 µg/ml of ampicillin and 0.5% of starch. Among these, four strains showed amylase activity. These strains were

![Fig. 1. A: Electrophoresis of pNTK1 and pNTK1 digested with Hind III restriction enzyme. B: Restriction map of pNTK1. Thin line indicates pBR322 (4.3 Kb) and solid bar indicates the fragment derived from B. licheniformis TY002 containing the amylase gene (3.7 Kb).](attachment:image.jpg)
tested to detect plasmids and the presence of plasmids larger than pBR322 was recorded. One plasmid, pNTK1 was extracted and re-introduced into E. coli HB101 and C600. All ampicillin-resistant transformants formed halos around colonies on BY-starch plates when I2–KI was added, indicating the formation of amylase.

*Restriction endonuclease map of pNTK1*

pNTK1 was digested with HindIII, ClaI, EcoR1, and PstI, and the
Expression of B. licheniformis Amylase

Endonuclease-digested products were analyzed by agarose gel electrophoresis. Figure 1 shows the resulting restriction map of pNTK1. The molecular weight of the insert was calculated to be 3.7 Kb or 2.4 Md.

Construction of small plasmids
To determine the location of the amylase gene in the insert, smaller plasmids were constructed from pNTK1 by restriction endonuclease digestion and self-ligation (Fig. 2). At first, the fragment between Eco R1 site of pBR322 and that of the insert was removed by Eco R1 digestion and self-ligation. The resulting plasmid, named pNTK2, showed by the transformation test described above that it contained an amylase gene. A smaller plasmid named pNTK3 was made from pNTK2 by Hind III digestion and self-ligation as shown in Fig. 2. The presence of an amylase gene in pNTK3 indicated that the essential region of the amylase gene was located in the Eco R1–Hind III fragment of 2.7 Kb in pNTK3.

Expression and localization of amylase in E. coli
E. coli HB101/pNTK3 was grown in 100 ml of BY medium containing 20 µg/ml of ampicillin at 30°C. Distribution of extracellular, periplasmic, and intracellular amylase activities were measured according to the method described by Tsukagoshi
et al. (12). The results are shown in Fig. 3. Most of the amylase activity, in this case, were present in the periplasmic region and only a little was seen in extra- and intracellular regions. All of these amylases showed the same heat stability as the original B. licheniformis amylase.

Construction of shuttle vector pSNK1

To express the amylase gene of pNTK3 in B. subtilis, a shuttle vector was constructed as follows: pNTK3 was digested by Eco R1 and was ligated by T4DNA ligase with pUB110 which had been digested with Eco R1 and treated with alkaline phosphatase (Fig. 2). The ligated DNA preparation was introduced by the protoplast method into B. subtilis M15 which lacks an amylase gene (9). Protoplasts were regenerated on DM3 medium containing 150 μg/ml of kanamycin. A plasmid named pSNK1 was obtained from a transformant which produced amylase and was resistant to kanamycin. As expected, when pSNK1 was introduced into E. coli HB101, the resultant transformants showed ampicillin and kanamycin resistance and synthesized amylase.

To test the stability of pSNK1 in E. coli and B. subtilis, E. coli HB101/pSNK1 and B. subtilis M15/pSNK1 were cultured in BY medium using an L-tube at 30°C in 150 strokes/min for 48 hr in the presence or absence of antibiotics. The results showed that antibiotics did not influence amylase syntheses and plasmid conservation in these strains. This indicates that pSNK1 is a stable shuttle vector carrying antibiotic markers between E. coli and B. subtilis and is able to express the amylase gene in both E. coli and B. subtilis.

Expression of the amylase gene of pSNK1 in B. subtilis

In a previous paper (4), we reported that the amylase productivity in Bacillus subtilis 6160 was increased step by step by transformation and mutation and the last strain, named B. subtilis 2633, produced about 4,000 times as much amylase as 6160.

Table 2. Amounts of amylase produced by various strains carrying pSNK1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>BY activity (U/ml)</th>
<th>ABY activity (U/ml)</th>
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<tbody>
<tr>
<td>B. subtilis M15/pSNK1</td>
<td>60 (60)</td>
<td>6.3 (5.7)</td>
</tr>
<tr>
<td>B. subtilis 6160/pSNK1</td>
<td>110 (95)</td>
<td>17.1 (7.9)</td>
</tr>
<tr>
<td>B. subtilis 2633/pSNK1</td>
<td>650 (170)</td>
<td>18,000 (330)</td>
</tr>
<tr>
<td>B. licheniformis NYK74/pSNK1</td>
<td>400 (400)</td>
<td>110 (110)</td>
</tr>
</tbody>
</table>

Units in ( ) indicate the residual activities after heating at 70°C for 10 min.

Strains were cultured in BY and ABY media containing kanamycin in L-tubes at 30°C in 150 strokes/min for 48 hr.

B. subtilis M15 deletes the amylase gene. B. subtilis 6160 and 2633 produce 10 U/ml and 30,000 U/ml of amylase respectively in ABY medium (72 hr). This amylase is not thermostable. B. licheniformis NYK74 produces 1 U/ml of thermostable amylase.
To examine the rate of expression of the amylase gene in pSNK1 in these strains, pSNK1 was introduced into both of these strains and into strain M15 (amy^-) and their total and heat-stable amylase productivities were compared (Table 2). As shown in Table 2, strain M15/pSNK1 produced only heat-stable and no heat-labile amylase. The former amylase belongs to B. licheniformis and the latter to B. subtilis. The amylase produced by B. subtilis 6160/pSNK1 contained 86% heat-stable amylase, while that produced by B. subtilis 2633/pSNK1 contained large amounts of both heat-labile and heat-stable amylase.

DISCUSSION

The mechanisms which control the productivity of extracellular enzymes of microorganisms have been a matter of much discussion. One way to approach this problem might be to insert one gene which controls specific extracellular enzyme into several strains producing different amounts of extracellular enzyme. For this purpose, we attempted to isolate an amylase gene from B. licheniformis TY002 which produces a large amount of heat-stable amylase outside the cells. As mentioned in the text, this gene was cloned by pBR322 and the new plasmid pNTK1 with an amylase gene in it was obtained. This was made smaller by elimination of nonessential parts. Thus the smallest one, pNTK3, was acquired. pNTK3 could be maintained in E. coli, but not in Bacillus because its vector belonged to pBR322. Therefore, pNTK3 was combined with Bacillus plasmid pUB110 and a shuttle vector named pSNK1 was constructed. This vector plasmid was maintained in both E. coli and Bacillus. When pNTK3 was introduced into E. coli HB101, the cells produced a small amount of amylase in the medium. However, when the cells were disrupted and fractionated, a large amount of amylase was found in the periplasmic region and only small amounts were found in and outside the cells (Table 1). This indicated that the amylase molecules were secreted at least into the periplasmic region. Therefore, it appeared that the amylase gene cloned in pNTK3 had enough leader part necessary for secretion.

When pSNK1 was introduced into various strains of B. subtilis, heat-stable amylase was produced in the medium. As shown in Table 2, however, the amounts of total amylase and heat-stable amylase produced differed from strain to strain. For example, B. subtilis M15/pSNK1 produced only heat-stable amylase and no heat-labile amylase. This is obviously due to the fact that M15 lacks an amylase structural gene. B. subtilis 6160/pSNK1 produced 110 U/ml of amylase in total and 86% of it was heat-stable. The 6160 strain itself produces about 10 U/ml of heat-labile amylase. This shows that heat-stable amylase gene in pSNK1 was expressed fairly well. On the other hand, B. subtilis 2633/pSNK1 produced a total of 18,000 U/ml of amylase, 330 U/ml of which was heat-stable, indicating that amylase gene in pSNK1 was expressed very well in this strain. When pSNK1 was introduced into B. licheniformis NYK74 which produced only 1 U/ml of heat-stable amylase, the transformant, B. licheniformis NYK74/pSNK1, produced 400 U/ml of heat-
stable amylase. In this case, note that the amylase genes may be different from each other, since the amylase gene in pSNK1 was taken from *B. licheniformis* TY002 which was derived from strain NYK74 by repeated mutations. Therefore, the amylase gene, including the leader portion, may be changed. The details of the structure of the amylase gene in pNTK3 is now under investigation.

In terms of gene expression, the relationship between host and a gene carried in it is of particular interest. Examining the expression of one gene in various mutant cells should give us much information about the matter.

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REFERENCES