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Molecular Cloning and Expression of Alkaline Amylase Gene of Alkalophilic *Bacillus* sp. in *Bacillus subtilis* and *Escherichia coli*

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알카리성 *Bacillus* sp.의 호알카리성 amylase 유전자의 *Bacillus subtilis* 와 *Escherichia coli* 로의 cloning 과 발현

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A 5.7Kb EcoRI fragment containing alkaline amylase gene of *Bacillus* sp. AL—8 obtained in the previous experiment (10) was transformed in *B. subtilis* via plasmid pUB110. The enzymatic properties of the amylase produced by the transformants were identical to those of the donor strain. Thus, the alkaline amylase activity from the transformant was maximum at pH 10 and 50℃. And the enzyme was very stable over the ranges of alkaline pH. In order to determine the location of the alkaline amylase gene within the 5.7Kb DNA fragment, the fragment was subcloned in *E. coli*. It was found that the alkaline amylase gene was located in EcoRI fragment of 3.7Kb.

Amylase is one of the most important industrial enzymes which are used in various processes. And there have been many reports on the genetic and biochemical characteristics of the enzyme (1-2).

And several studies on molecular cloning and its expression for the characterization of molecular genetic and biochemical properties (3-6) have been reported. Recently, DNA sequences of α-amylase from Genus *Bacillus* (*B. subtilis, B. amyloliquifacience, B. licheniformis* and *B. stearothermophilus*) were determined (7-9). But there have been few reports on the alkaline amylase gene of alkalophilic bacteria but those of above 4 species of Genus *Bacillus*.

We have introduced the alkaline amylase gene of alkalophilic *Bacillus* sp. AL-8 into *E. coli* by using recombinant plasmid pJW8 and studied the enzymatic properties of the transformants in the previous experiment (10).

In this paper, we have cloned the alkaline α-amylase gene from *E. coli* carrying pJW8 into *B. subtilis* (Amy^-) and studied its expression.

Then, the insert was subcloned for the localization of the alkaline amylase gene in EcoRI fragment of pJW8.

Material and Methods

Bacterial strains and media

Alkalophilic *Bacillus* sp. AL-8 isolated and described previously (10) was used as donor strain of the alkaline amylase gene. *E. coli* HB101 and *B. subtilis* sta-1 (KCTC 1332), mutants were not able to produce amylase, were used as host strains in transformation with recombinant DNA. *Bacillus* sp. AL-8 was cultured in alkaline medium II(11), and *B. subtilis* was cultured at 37℃ in nutrient broth. *E. coli* HB101 was cultured in LB medium at 37℃.

Assay of amylase

Activity of alkaline amylase was measured by the method described previously (12).

Key words: Alkalophilic *Bacillus* sp. AL-8, alkaline amylase gene, molecular cloning

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Cloning and subcloning

In order to clone for *B. subtilis*, the recombinant plasmid (pJW8) harvoring the alkaline amylase gene (10) and pUB110 were cleaved with EcoRI and ligated. Ligation was carried out at 12 °C for 14 hr in 66 mM Tris-HCl (pH 7.6), 6.6mM $MgCl_2$ and 10 mM Dithiothritol buffer with T4 DNA ligase. For subcloning into *E. coli*, pBR325 was used as vector. The insert (5.7 Kb) and the vector were digested with EcoRI and PstI. Ligation reaction was made as above.

Transformation

After ligation, the hybrid plasmid was transformed into *E. coli* using $CaCl_2$ method (13). Transformation into *B. subtilis* sta-1 was carried out as described by Young and Spizizen (14).

Stability of recombinant plasmids in host strain

In order to determine the genetical stability of the recombinant plasmids in host strain, *B. subtilis* sta-1, the methods described by Grandi *et al.* (15) and Watanabe (16) were used.

Results and Discussion

Cloning for alkaline amylase gene

To introduce the cloned alkaline amylase gene of pJW8 into *B. subtilis*, the EcoRI fragment of pJW8 was inserted into EcoRI site of pUB110 (Fig. 1). Transformants of *B. subtilis* were isolated on nutrient agar plate containing 5 ug/ml of kanamycin. A large number of kanamycin resistant colonies (613 colonies) were isolated. And among them, 297 colonies were selected as transformants showing clear zone around colonies by iodine staining of starch plate. About half of them produced large clear zones. These revealed that the alkaline amylase gene was inserted into pUB110 (17). *B. subtilis* MB802 and MB809 were obtained to produce alkaline amylase 1.8 fold to the original strain, *Bacillus* sp. AL-8 (Table 1).

Stability of recombinant plasmids

The stability of recombinant plasmids, pMB802 and pMB809, in *B. subtilis* sta-1 was studied by replica-plating method. The recombinant plasmids were genetically quite stable in *B. subtilis* sta-1 during the course of 20 successive subcultures in Panassay broth. Spontaneous loss of the recombinant plasmids was not observed (Fig. 2).

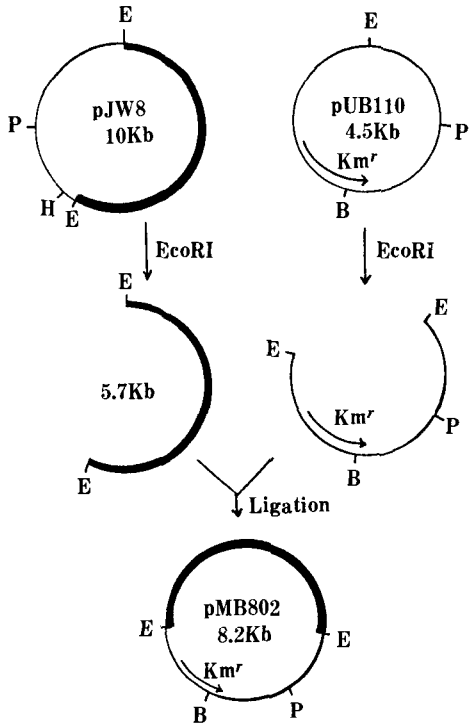

Fig. 1. Construction of recombinant plasmid (pMB802) for *B. subtilis*.
EcoRI fragment containing alkaline amylase gene fragment (thick line) was inserted into plasmid pUB110. Restriction sites, E; EcoRI, P: PvuII, B: BalII, H: HindIII, Km: Kanamycin

Table 1. Alkaline amylase activity of the transformants and donor strain.

Strains	Alkaline amylase activity (units/ml)
Bacillus sp. AL-8	240
B. subtilis MB802	268
B. subtilis MB809	366

Properties of amylase produced in *B. subtilis*

The pH and temperature dependence of the alkaline amylases produced by transformants were compared with that of the donor strain, *Bacillus* sp. AL-8. These enzymes exhibited similar pH- and temperature-profil. And they were optimally active at pH 10 and 50 °C (Table 2). Thus, enzymatic properties of the amylase from the transformants, *B.*

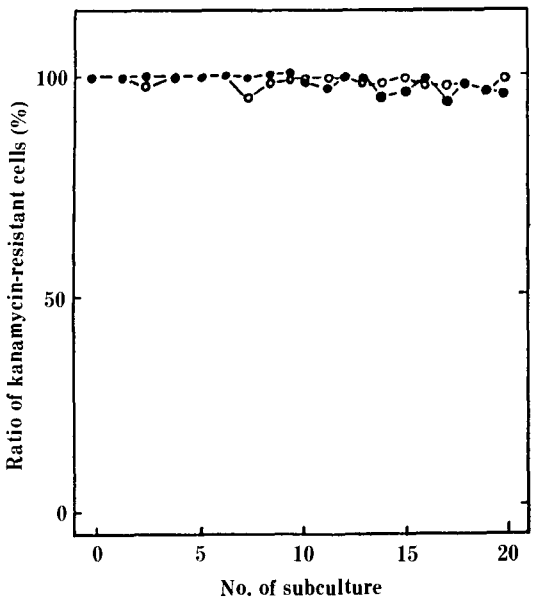

Fig. 2. Stability of recombinant plasmid in host strain *B. subtilis* sta-1.

Cells were incubated in Penassay broth at 37 °C for 24 hr without shaking. It was plated on kanamycin-free nutrient agar, and the developing colonies were studied for their drug-resistance markers by replica-plating. -○-, *B. subtilis* MB802; -●-, MB809

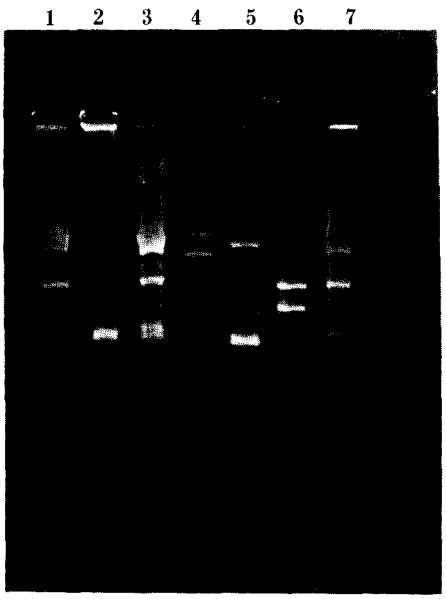

Fig. 3. Restriction enzyme digestion of pJW8.

lane 1; EcoRI digestion
lane 2; PvuII digestion
lane 3; PstI digestion
lane 4; molecular weight marker (λDNA digested with *Eco*RI)
lane 5; EcoRI, PvuII double digestion
lane 6; EcoRI, PstI digestion
lane 7; PstI, PvuII digestion

Table 2. Comparison of alkaline amylases of alkalophilic *Bacillus* sp. AL-8 and transformants.

Strains	Optimal activity		Stable range	
	pH	Temperature (°C)	pH	Temperature (°C)
Bacillus sp. AL-8	10	50	8-11	up to 60
B. subtilis MB802	10	50	8-11	up to 60
B. subtilis MB909	10	50	8-11	up to 60

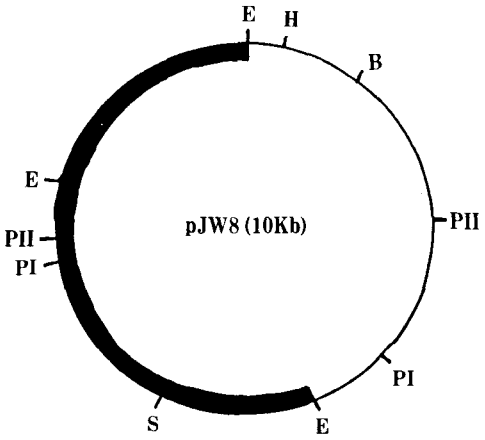

Fig. 4. Restriction map of pJW8.

The thin line is pBR322 and the thick line the inserted 5.7Kb DNA fragment. Restriction sites, E: EcoRI PI: PstI, PII: PvuII, S: SmaI, H: HindIII, B: BamHI

subtilis MB802 and MB809, were indistinguishable from those of the donor strain. Similar results were reported on the amylase of *B. stearothermophilus* (17).

Restriction endonuclease map of the 5.7Kb fragment

For mapping restriction sites of the insert, the recombinant plasmid (pJW8) was digested with EcoRI, PvuII, PstI, SalI, BamHI and SmaI. The fragments were electrophoresed on 1% agarose gel. The 5.7Kb fragment had a single site for PstI, PvuII, SmaI and EcoRI. But the site for BamHI and SalI were not found. (Fig. 3). The restriction map of the fragment is shown in Fig. 4.

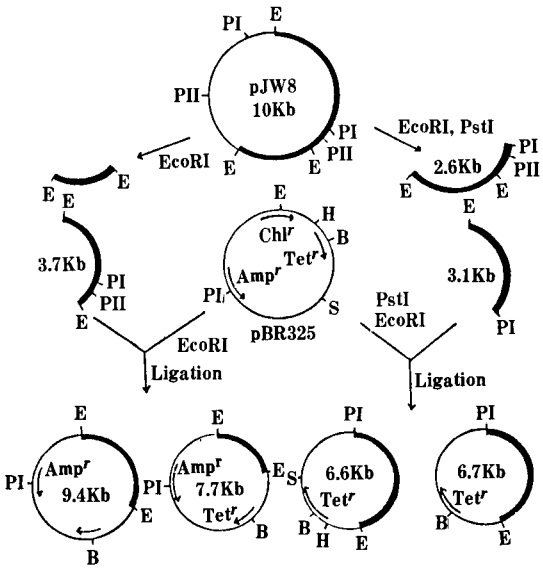

Fig. 5. Strategy for subcloning *E. coli*.
Thick lines are inserts and thin lines vectors. Restriction sites, E: EcoRI, PI: PstI, PII: PvuII, H: HindIII, S: SalII, B.: BamHI

Table 3. Detection of alkaline amylase gene by subcloning of each DNA fragment.

DNA fragment (Kb)	Restriction enzyme	Antibiotics resistance			Alkaline amylase production
		Tet	Chl	Amp	
2.0	EcoRI	+	−	+	−
2.6	EcoRI/PstI	+	−	−	−
3.1	EcoRI/PstI	+	−	−	−
3.7	EcoRI	+	−	+	+

Tet: tetracycline, Chl: Chloramphenicol, Amp: ampicillin

Subcloning in *E. coli* and localization of the alkaline amylase gene

For subcloning of 5.7Kb fragment, the fragment was digested with EcoRI and PstI. And each fragment was ligated with pBR325 and transformed into *E. coli* HB101. The schemetic illustration of the construction of recombinant plasmids is shown in Fig. 5. Tetracycline-resistant and ampicillin-resistant and chloramphenicol-sensitive clonies were isolated as transformants at EcoRI digestion. And tetracycline-resistant, chloramphenicol-sensitive and ampicillin-sensitive colonies were isolated as transformants using double digestion with EcoRI and PstI. Among transformants, alkaline amylase producing colonies were selected based on the formation of clear zone by iodine staining on starch plate. It was found that the amylase producing colonies contained 3.7Kb of EcoRI fragment (Table 3). From the results obtained, it seems that the alkaline EcoRI site through, PstI site. It was reported that the size of amylase gene from *B. subtilis* was 1.8Kb (8), and those of *B. licheniformis* and *B. stearothermophilus* were 1.9Kb and 1.6Kb (18, 19). Compararing with these reports, 3.7Kb is large enough to contain the alkaline amylase gene.

Acknowledgement

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요 약

알카리성 *Bacillus* sp. AL-8의 알카리성 amylase 유전자를 포함하는 5.7Kb의 EcoRI 단편을 pUB 110을 vector로 하여 amylase를 생산하지 못하는 *B. subtilis* sta-1에서 발현시켰다. 재조합 plasmid pMB 802와 pMB 809는 숙주세포인 *B. subtilis*에서 매우 안정하게 유지되었으며 amylase 생산이 공여균주에서 보다 1.8배 증가하였다. 형질전환주에서 생산된 amylase는 공여균주와 같은 효소적 성질을 나타내었다. 5.7Kb 단편을 *E. coli*에 subcloning한 결과 3.7Kb의 EcoRI 단편에 알카리성 amylase 유전자가 존재하였다.

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