Gene Expression and Production of Bacillus No. 170 Penicillinase in Escherichia coli and Bacillus subtilis

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Received July 20, 1983

To compare the gene expression of the alkalophilic Bacillus penicillinase in Escherichia coli and Bacillus subtilis, we constructed a shuttle plasmid, pSAP21, from pUB110 and pEAP1 containing pMB9 and the penicillinase gene of alkalophilic Bacillus No. 170. When pSAP21 was transformed in E. coli, ampicillin resistant transformants (20 μg/ml) could be obtained by direct selection. The penicillinase gene was also expressed in B. subtilis carrying pSAP21. Although an ampicillin resistant transformant could not be obtained directly in B. subtilis, kanamycin resistant transformants carrying pSAP21 exhibited ampicillin resistance (10 μg/ml) with the replica method. Most of the penicillinase produced was found in the culture media of E. coli and B. subtilis. The penicillinase produced by E. coli carrying pSAP21 did not decrease on prolonged cultivation. On the other hand, the penicillinase produced by B. subtilis carrying pSAP21 became maximum after 20 hours cultivation and then decreased rapidly.

In our laboratory, many alkalophilic bacteria which grow well in a high pH range (pH 10 ~ 11) have been isolated.1 These bacteria produce many kinds of extracellular enzymes which have optimum pH in the alkaline region or are stable at alkaline pH. One of them, alkalophilic Bacillus No. 170, produced penicillinase in an alkaline medium.2 We cloned the penicillinase gene of alkalophilic Bacillus No. 170, and investigated the expression of the penicillinase gene in E. coli. Most penicillinase produced in E. coli carrying plasmid pEAP1 or pEAP2 was detected in the culture fluid.3

This paper deals with the construction of a shuttle plasmid, and the expression of the Bacillus No. 170 penicillinase gene in E. coli and B. subtilis will also be described.

MATERIALS AND METHODS

Bacterial strains, plasmids and medium. Bacterial strains and plasmids used are listed in Table I. Alkalophilic Bacillus No. 170, which produced penicillinase under alkaline culture conditions, was isolated from soil. The best growth and maximum enzyme production were observed at pH 9.0.2

LB-medium contained 10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, 10 g of NaCl and 1 g of glucose (autoclaved separately) in one liter of deionized water (pH 7.0), it was solidified with 20 g of agar, if necessary.

Preparation of plasmids. Plasmids pMB9, pEAP1 and pSAP21 were purified by the method of Bolivar et al.6 and plasmid pUB110 was isolated by the method of Gryczan et al.11 The plasmids were detected by miniscreening methods.4,8

Transformation. Transformation of E. coli with the plasmid was carried out by the calcium chloride treatment method of Lederberg and Cohen.9 Transformation of B. subtilis with the plasmid was done by the method of Anagnostopoulos and Spizizen.10

Gel electrophoresis. Electrophoresis of the plasmids was performed on 1% agarose slab gel with 0.09 M Tris-borate buffer, pH 8.3, at 14.5 V/cm for 1.5 ~ 5 hr.11 After the electrophoresis, the gel was stained with ethidium bromide solution (0.5 μg/ml). The DNA separated was detected with short wavelength UV transilluminator (Ultra-Violet Products, Inc.) and the molecular weight was estimated. As molecular size references, the DNA fragments of lambda phage digested with HindIII were used.

Fractionation of extracellular and intracellular penicillinase. The extracellular penicillinase was the activity
Table I. Strains and Vectors Used

<table>
<thead>
<tr>
<th>Bacterial strains and plasmid vectors</th>
<th>Genotypes and properties</th>
<th>Origins or references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkalophilic Bacillus No. 170</td>
<td>Produces a penicillinase</td>
<td>Our laboratory²,³</td>
</tr>
<tr>
<td>E. coli HB101</td>
<td>pro, leuB, B1, lacY, hsdR, hsdM, ara14, galKz, xyl5, mtl1, supE44, F⁻, endoI², recA⁻, str⁴</td>
<td>Goldfarb et al.⁴</td>
</tr>
<tr>
<td>B. subtilis CU741</td>
<td>leuC7, trpC²</td>
<td>Ward and Zahler⁵</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td>Bolivar et al.⁶</td>
</tr>
<tr>
<td>pMB 9</td>
<td>Tc²</td>
<td>Gryczan et al.⁷</td>
</tr>
<tr>
<td>pUB 110</td>
<td>Km¹</td>
<td>Our laboratory³</td>
</tr>
<tr>
<td>pEAP 1</td>
<td>The plasmid consists of pMB 9 and a 4.5 kb EcoRI fragment of No. 170 DNA, Tc², Ap¹ (penicillinase⁺)</td>
<td></td>
</tr>
<tr>
<td>pSAP 21</td>
<td>The shuttle plasmid consists of pUB 110 and pEAP 1, (Km¹, Tc² and Ap¹ in E. coli, Km¹ and penicillinase⁺ in B. subtilis)</td>
<td>Our laboratory</td>
</tr>
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found in the supernatant fluid of the culture. The total enzyme was the activity found in the supernatant fluid of the sonicated cell culture, and the intracellular enzyme activity was the total activity minus the extracellular activity.

**Assay of penicillinase.** Penicillinase was assayed by the method of Sawai et al.¹². One unit of penicillinase activity is defined as that amount of enzyme which hydrolyzes one μmol of benzylpenicillin per minute at 30°C. To isolate penicillinase positive (penicillinase⁺) colonies, the nitrocefin assay system was also used.¹³

**Enzymes and chemicals.** Restriction endonucleases, T₄ ligase and agarase were purchased from Bethesda Research Laboratories, U.S.A., and used according to the methods suggested by the manufacturer. Ampicillin (Ap), tetracycline (Tc), chloramphenicol and kanamycin (Km) were purchased from Sigma Chemicals, U.S.A. Nitrocefin was a gift from Glaxo Research Ltd., England.

**RESULTS**

**Construction of shuttle plasmid pSAP21**

As shown in Fig. 1, DNA of alkalophilic Bacillus No. 170 was digested with restriction endonuclease EcoRI and joined to plasmid pMB9. Competent E. coli cells were transformed with the hybrid plasmid, and Tc² Ap¹ transformants were selected. Plasmid pEAP1 was isolated from the transformants producing a penicillinase (penicillinase⁺) and the penicillinase gene was in a 4.5 kb EcoRI DNA fragment. By electrophoretic analysis, the restriction map of pEAP1 was determined.³

Partially digested pEAP1 DNA (1 μg) with EcoRI was mixed with an EcoRI digest of pUB110 (1 μg), followed by ligation. The ligated mixture was used to transform E. coli HB101, and some Km¹ Tc² Ap¹ (penicillinase⁺) transformants were obtained. A shuttle plasmid between E. coli and B. subtilis, designated as pSAP21, was isolated from the

![Fig. 1. Construction of Recombinant Plasmids.](image-url)

The thin line represents alkalophilic Bacillus No. 170 DNA and the thick line pUB110 DNA.
Gene Expression of *Bacillus* No. 170 Penicillinase in *E. coli* and *B. subtilis*

**Fig. 2.** Restriction Endonuclease Analysis of Plasmids pMB9, pUB110, pEAPl and pSAP21.
Lane 1, λDNA digested with HindIII; lane 2, pEAPl with EcoRI; lane 3, pMB9 with EcoRI; lane 4, pSAP21 with EcoRI; lane 5, pUB110 with EcoRI.

**Fig. 3.** Electrophoretic Patterns of pSAP21 DNA Digested with Restriction Endonucleases.
Lanes 1, 6, λDNA digested with HindIII; lanes 2~5, pSAP21 digested with endonucleases (lane 2, EcoRI; lane 3, HindIII; lane 4, BamHI; lane 5, HindII).

*E. coli* HB101 transformants. Results of agarose gel electrophoresis are shown in Fig. 2.

**Restriction map of pSAP21**
To determine the direction of inserted DNA, pSAP21 was digested with several restriction endonucleases (Fig. 3). The restriction map obtained on electrophoretic analysis is shown in Fig. 4.

**Expression of the cloned penicillinase gene**
Plasmid pSAP21 was introduced into *B. subtilis* CU741 by the method of Anagnostopoulos and Spizizen. No Ap’ (10 μg/ml) transformants could be obtained by direct selection, but Km’ transformants obtained exhibited Ap’ (10 μg/ml) with the replica method. Plasmid pSAP21 was detected in the cleared lyzate of the transformants (Km’, Ap’). But it was found that plasmid pSAP21 was unstable for production of penicillinase because almost all cells lost the plasmid during cultivation in the absence of kanamycin.

**Distribution of penicillinase during cultivation**
*E. coli* HB101 (pSAP21) was inoculated into a 500 ml flask containing 100 ml of LB-broth containing 0.2% glycerol and cultured at 37°C on a rotary shaker. A 2 ml aliquot of the broth was withdrawn from the culture at 4 hr-intervals. Cell growth was measured at 660 nm, and extracellular and intracellular penicillinases were assayed. As shown in Fig. 5, the extracellular penicillinase activity reached a
E. coli HB101 (pSAP21) was inoculated into LB-broth containing 0.2% glycerol and cultured at 37°C on a rotary shaker. Bacterial growth (absorbancy at 660 nm, □) and penicillinase activities (extracellular, ○; intracellular, △; and total, ▲) were determined.

B. subtilis CU741 (pSAP21) was aerobically cultured in LB-broth containing kanamycin 20 μg/ml at 37°C. Bacterial growth (absorbancy at 660 nm, □) and penicillinase activities (○) were determined. In the absence of kanamycin, penicillinase activities (□) were determined.

maximum after about 20 hr cultivation. The extracellular penicillinase was very stable and no significant decrease was observed on prolonged cultivation. Intracellular penicillinase activity was detected only at an early stage of cultivation.

B. subtilis CU741 (pSAP21) secreted all the penicillinase into the culture fluid but the enzyme activity, however, was about 1/3 that of E. coli HB101 (pSAP21). As shown in Fig. 6, the penicillinase produced in B. subtilis (pSAP21) reached a maximum at 20 hr cultivation in the presence of kanamycin, and then the activity decreased rapidly. In the absence of kanamycin, very weak penicillinase activity was observed at an early stage of cultivation and no activity was detected after 12 hr cultivation. Intracellular penicillinase activity was not detected at all throughout the cultivation time.

DISCUSSION

The penicillinase gene from alkalophilic Bacillus No. 170 was cloned and expressed in E. coli and B. subtilis using the shuttle plasmid, pSAP21.

From the results in Figs. 5 and 6, E. coli (pSAP21) was found to produce 3 times more penicillinase than B. subtilis (pSAP21). Extracellular penicillinase production by E. coli (pSAP21) continued for a long time without any significant decrease. But the activity of the penicillinase produced by B. subtilis (pSAP21) decreased rapidly. Two possibilities were considered: 1) Instability of pSAP21 in B. subtilis. In fact, in the absence of kanamycin, very weak penicillinase activity was observed at the early stage of cultivation and no activity was detected after 12 hr cultivation.

2) The effect of B. subtilis protease. Although no specific experiment has been done, it is highly possible that penicillinase produced may be hydrolyzed by a protease of B. subtilis.

It has been reported that transformants of B. subtilis and B. licheniformis carrying plasmids containing the penicillinase gene are sensitive to ampicillin, even those with high penicillinase activities. However, by the replica plate method, B. subtilis transformants expressed ampicillin resistance.14) We observed the same phenomenon in B. subtilis carrying pSAP21. This phenomenon may be due to the difference of surface structures between E. coli and B. subtilis.
These results indicate that the *E. coli* system is better than the *B. subtilis* system for the production of alkalophilic Bacillus No. 170 penicillinase.

Acknowledgment. This work was partially supported by a Grant for Life Science Promotion from the Institute of Physical and Chemical Research.

REFERENCES