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Secretion of *Escherichia coli* β-lactamase from *Bacillus subtilis* with the Aid of Usefully Constructed Secretion Vector

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The secretion vector with promoter and signal sequence region of neutral protease gene (*npr*) from *Bacillus amyloliquefaciens* was constructed by the technique of polymerase chain reaction (PCR). A unique restriction site was introduced into the 3′ of the signal coding region by the synthesis of PCR primer. To demonstrate the function of cloned promoter and signal sequence, we used the *E. coli* β-lactamase structural gene as a foreign gene. The signal sequence of β-lactamase gene was deleted by Bal31 exonuclease and only mature region was introduced into the secretion vector. *Bacillus subtilis* cells transformed by the recombinant vector synthesized the fusion protein and were also capable of removing the signal peptide from the original fusion protein, as judged by the assay of β-lactamase activity and secretion into the growth medium by western blotting.

KEY WORDS □ **Secretion vector, *npr*, *E. coli* β-lactamase, PCR, *Bacillus*.**

Bacilli are potential hosts for the biosynthesis of foreign gene products in microorganisms by recombinant DNA technology. Compared with *E. coli*, *B. subtilis* is able to secrete a wide variety of enzymes, such as proteases, α-amylase, β-glucanase, and nucleases (8). These characteristics were acquired by membrane structure and signal peptide. There have been reports of the cloning of several secreted enzymes from *Bacillus* species. All the genes in which the nucleotide sequences have been determined have a region coding for a signal peptide at the amino terminus (14, 15).

A large number of both prokaryotic and eukaryotic genes have been sucessfully fused to the promoter region and signal peptide sequence derived from the Bacillus system (6, 8, 11, 13). However, efficient secretion vector development and expression of foreign gene and secretion of the gene product in *Bacilli* were hampered by several problems, such as unknown mechanism of protein secretion in gram positive bacteria. Therefore, the development of secretion vector, which was constructed by promoter and signal peptide of secretory protein, contributed to the study of secretion mechanism and secretion of foreign gene products.

In most of the reported cases, because of the lack of appropriate restriction enzyme site, the fusion of foreign gene into the promoter and signal peptide region was difficult procedure. In addition to, the correct reading frame was considerable interest. Here, we report the construction strategy of secretion vector based on the gene for neutral protease of *B. amyloliquefaciens* by polymerase chain reaction was simple and advantageous (this secretion vector had the multiple cloning sites of pUC19, which could be used for the fusion of various mature foreign gene), and the resulted secretion vector had the ability of expression and secretion of β-lactamase gene product from *B. subtilis*.

MATERIALS AND METHODS

Chemicals and enzymes

The enzymes used in this study were purchased from suppliers as follows: Restriction endonucleases, Taq DNA polymerase from New England Biolab. (NEB), KOSKO, and Boehringer Mannheim.

Plasmids

The plasmid pUC19 was used for *E. coli* cloning vector and pC194 was used for *Bacillus* plasmid. The plasmid pUCBLAΔB containing intact mature β-lactamase gene was gifted from Dr. Se Yong Lee, University of Korea.

Bacterial strains and growth media

E. coli strains HB101 (F-, r-, m-, recAB, lacY1) was used for the isolation of plasmid and as a recipient of transformation. JM83 (ara-, Δ(lac-proAB), rpsL, thi, ψ80, lacZ DM15) was used for transformation with plasmid pUC19 and its derivative. Bacillus subtilis ATCC 33608 was used for transformation with plasmid containing gram

positive replication origin. *Bacillus subtilis* DB104 (apr-, npr-), which was protease negative mutant, was used for secretion of foreign gene product. *Bacillus amyloliquefaciens* ATCC 23844 (L. L. Cambell strain P) was the source of DNA from which neutral protease gene was contained. LB media was used for growth media of *E. coli* and *B. subilis*. 2X SG sporulation medium was used for expression and secretion of β-lactamase in DB 104.

Polymerase chain reaction

The PCR was performed by a modification of the originally described by Saiki *et al.* (1988). Size-fractionated chromosomal DNA was mixed with 100 μl of a solution containing 200 μM (each) dATP, dCTP, dGTP, and TTP, 1 μM of each primers, 50 mM KCl, 10 mM Tris-Cl(pH 8.4), 2.5 mM $MgCl_2$, 0.02% gelatin (w/v). The mixture was heated at 94℃ for 3 min, then primer annealed at 49℃ for 3 min. Subsequently, PCR reaction was carried out 30 cycles under the condition of primer extension at 72℃ for 5 min, heat denature at 94℃ for 1.5 min, and primer annealing at 49℃ for 3 min, respectively.

Western blotting

After electrophoresis in SDS 12% polyacrylamide, western transfer was carried out as described by Maniatis *et. al.* (1982). β-lactamase was visualized by the western blotting method using rabbit antiserum against *E. coli* β-lactamase and ECL kit (Amersham). Transferred nitrocellulose filter was blocked by 10% dried milk in PBS-T (0.15% Tween 20 in PBS) for 1 hr, and then washing with PBS-T several times. Primary antibody reaction was performed with gentle agitation in 1:3000 diluted PBS-T. Second antibody, HRP labelled antibody, reaction was performed with 1:2500 diluted PBS-T. After this reaction, washing was hardly performed. Detection was done by mixing the two reagents 1:1, and then drained excess reagents. Exposure was performed immediately by exposing the nitrocellulose filter to film for 30 seconds.

RESULTS AND DISCUSSION

Construction of secretion vector

To identify the neutral protease gene, Southern blot analysis was performed. This result showed that the strain contained chromosomal neutral protease gene (data not shown). Therefore, these fragments were eluted by electroelution method and used for the template DNA of polymerase chain reaction (PCR). This PCR product cloned into the XbaI and BamHI sites of pUC19, resulted in pUXB214, because it designed to have recog-

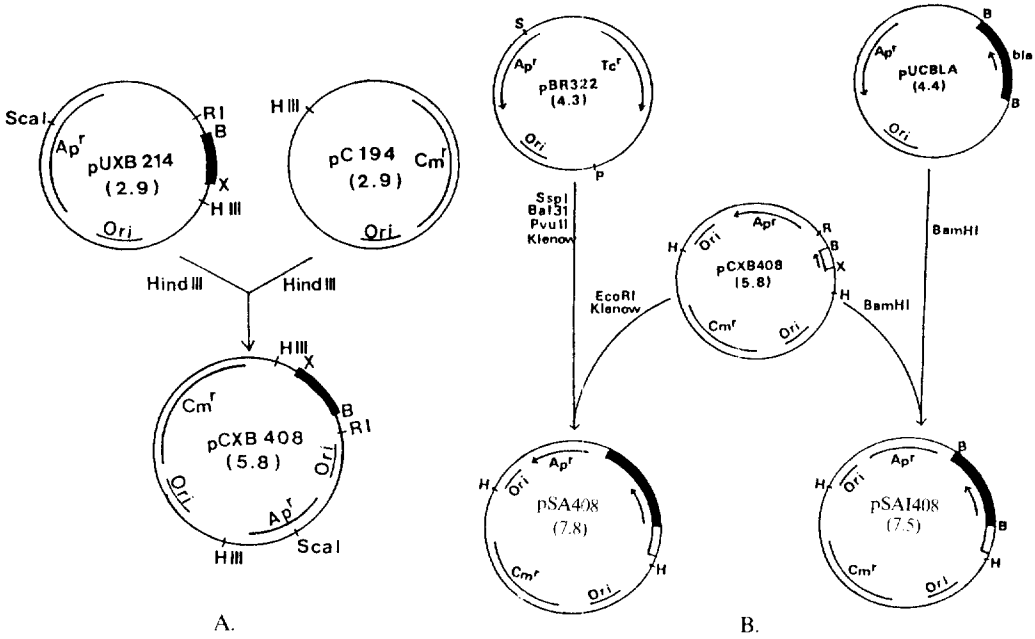

Fig. 1. *Strategy for the Construction of secretion vector, pSA408 and pSAI408.*
(A) Secretion vector pCXB408 was constructed by fusing the pUXB214 and pC194. Black region was promoter and signal sequence of neutral protease gene. (B) Each plasmid has the same β-lactamase gene, but elongated, intact mature region, respectively. R:EcoRI, S:SspI, B:BamHI, H: HindIII, X:XbaI, P:PvuII.

nition sites XbaI, BamHI at its 5′ and 3′ termini, respectively by synthetic primers. The PCR product was confirmed by nucleotide sequencing. This result showed that this fragment contained promoter and signal sequences of neutral protease gene (data not shown). The secretion vector pCXB 408 was constructed by fusion of pUXB214 with pC194, which was *Bacillus* cloning vector (Fig. 1. A).

Introduction of truncated β-lactamase gene into the pCXB408

The signal sequence of β-lactamase gene from pBR322 was deleted by Bal31 exonuclease, and then mature region was ligated with the secretion vector (Fig. 1.B). The hybrid plasmid which was fused in frame was selected by the assay of secreted β-lactamase activity from transformed *B. subtilis*. Unsucessfully, one kind of hybrid plasmids was obtained. It was proved that this plasmid pSA 408 contained incompletely deleted signal sequence of β-lactamase gene by the restriction enzyme mapping (almost one half of signal sequence). The pUCBLA contained mature β-lactamase gene had the BamHI linker attached to the +1 position in the β-lactamase mature amino acid sequence of the pUCBLAΔB. It was also ligated with BamHI site of secretion vector. This plasmid pSAI408 which was fused in frame contained nearly intact mature region of β-lactamase (Fig. 1.B).

Expression and secretion of the β-lactamase in *Bacillus subtilis*

B. subtilis carrying the pSA408, pSAI408 were grown in 2X SG sporulation medium, and the β-lactamase activity was determined following growth by using the PADAC as the β-lactamase substrate (Fig. 2). As shown in Fig. 2, the peak level of secreted β-lactamase activity from pSAI 408 was very higher than that from pSA408. The maximal activity, about 4,000 units/ml, 200 units/ml, respectively, was obtained at 6-8 hr after the logarithmic growth phase. The low yield of β-lactamse by pSA408 could be due to either a lower specific activity of the enzyme with an amino-terminal extension or lower late of synthesis and secretion. According to the quantitation from immunoblotting analysis (Fig. 3), the former possibility would exclude. Therefore, these results showed that the length of signal peptide-mature region junction affected on the expression and secretion efficiency. Palva *et al.* (1982) described that the amino acid sequence after the signal peptide clevage site may not be critical for the specificity of the signal peptide, but the fact that having more than 14 amino acids sequence after cleavage site was the cause of low yield of foreign gene product. Therefore, very low activity of pSA408 may be result from possessing about 20 amino acids sequence in junction region.

The appearance of the β-lactamase was estimated by SDS polyacrylamide gel electrophoresis in combination with an immunoblotting technique (Fig. 3). The β-lactamase secreted by the plasmid pSA408 was somewhat larger than

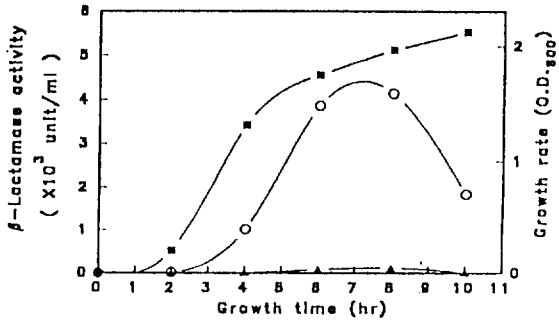

Fig. 2. *Expression and secretion of β-lactamase.* Cells were grown in 2XSG sporulation medium with chloroamphenicol (5 μg/ml). At different development stages, 500 μl of cells were harvested and pelleted by centrifugation; 300 μl, 10 μl, respectively, of supernatant was used for the β-lactamase assay (O'callaghan *et al.*, 1972). One unit of β-lactamase activity is defined as the hydrolysis of 1 μmol of substrate per min. Symbols: ■——■, growth rate of DB104; ▲——▲, β-lactamase activity of PB104 containing pSA408; ○——○, β-lactamase activity of PB104 containing pSAI 408.

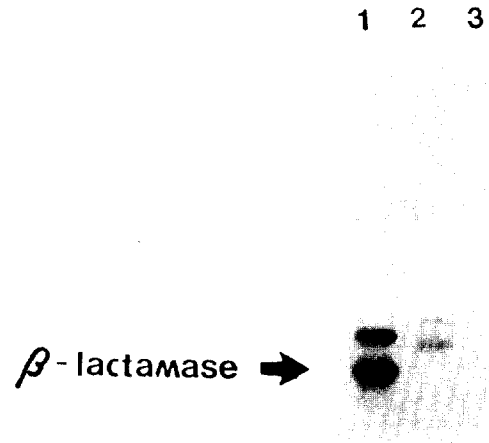

Fig. 3. *Immunoblotting analysis of β-lactamase.* The β-lactamase synthesized by secretion vector was indicated by western blotting with anti-β-lactamase antibody. Lane 1: supernatant of pSAI408, lane 2: supernatant of pSA408, lane 3: control supernatant.

by the pSAI408. This can be understood on the basis of the restriction enzyme mapping data, which indicates that the mature β-lactamase may be preceded by about 20 extra amino acids in the construction. Apparently the signal sequence of neutral protease is processed at or near the correct cleavage site. As shown in Fig. 2, at 5-7 hour after the cell enters the stationary phase, β-lactamase activity dropped significantly, almost to the background level. Generally, the rapid decrease in secreted heterologous gene product activity was due to the excretion of extracellular proteases into the medium. However, as DB104 was proteases defficient cell line, this result might be mainly caused by cell lysis, resulting in the release of intracellular proteases into the medium. This suggest was supported by immunoblotting data, which showed that the considerable amount of presumed precursor form of β-lactamase was appeared in upper site. This meant that cell lysis was happened during cell growth.

This approach to construction of the secretion vector had following advantages: 1) the cloning method (PCR) was simple and powerful, 2) restriction endonuclease site, which was useful in application to heterogeneous foreign gene, was able to make by synthesized primers. Furthermore, using the constructed secretion vector, other foreign genes will be expressed and secreted, and then the parameters that affect the secretion mechanism which focus on the signal peptide cleavage site will be studied.

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초 록: 고초균에서 유용하게 만들어진 분비성 벡터에 의한 대장균 β-lactamase의 분비

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*Bacillus amyloliquefaciens*의 neutral protease gene의 promoter 와 signal sequence region을 중합 효소 연쇄 반응 (PCR) 방법에 의해 클로닝하여 분비성 벡터를 만들었다. 제한 효소 인식 부위를 primer 합성에 의해 signal coding region의 3′ 위치에 도입하여 외래유전자의 발현과 분비를 위한 융합이 용이하게 이루어질 수 있도록 고안하였다. 클로닝된 promoter와 signal sequence의 기능을 알아보기 위해 signal region을 제거한 대장균의 β-lactamase 구조 유전자를 외래 유전자로서 이용하였다. 형질 전환된 고초균이 융합 단백질을 합성하고 이 단백질에서 signal peptide를 제거한 단백질을 배양액 밖으로 분비하는 사실을 β-lactamase의 효소 활성 측정과 western blotting에 의해 확인 하였다. 이 분비성 벡터는 여러 유용한 유전자 산물의 분비에 관련된 기작을 연구하는데 이용될 수 있을 것이다.