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Construction of Secretion Vectors Using the α-amylase Signal Sequence of *Bacillus subtilis* NA64

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Two secretion vectors, pUBA240 and pUBS340 were constructed by using the promoter and secretory signal region of the α-amylase gene from an α-amylase hyperproducing strain, *Bacillus subtilis* NA64. In this secretion vector system, various restriction enzyme sites are located immediately after the proregion of the α-amylase gene for easy replacement of various foreign structural genes. To evaluate this secretion vectors, the β-lactamase gene of pBR322 was used as a reporter gene. The expressed and biologically active β-lactamase was secreted into the culture broth from *B. subtilis* LKS86 transformants harboring each β-/lactamase secreting plasmid, pUBAbla and pUBSbla. In both cases, more than 92% of expressed β-lactamases were located in the culture medium. The amount of the secreted β-lactamase was about 80% of the total secreted proteins in the culture medium.

Key words: ***B. subtilis*, secretion vector, β-lactamase secretion**

Production of prokaryotic and eukaryotic proteins by genetically engineered bacteria has mainly focused on intracellular over-expressions in *Escherichia coli* with well-defined inducible expression systems. Although high yields of product can be obtained from this approach, it has some problems, particularly for the production of pharmaceutically interesting proteins. These problems include the frequent presence of an unnatural methionine residue at the first amino acid, the need to remove endotoxins, the often high cost for purification and the difficulty in renaturation of insoluble protein aggregates formed at high expression rates. To circumvent these problems, bacterial protein secretion systems have been developed.

Bacillus subtilis is an attractive host strain for protein production by the secretion system because of its apathogenicity, high secretion capacity, well-studied genetic system, highly developed recombinant DNA technology and extended knowledge about its fermentation technology. Various foreign proteins were examined with heterologous secretion vectors containing the regulatory and signal sequence region of extracellular enzyme genes of *B. subtilis* or *B. amyloliquefaciens* (6, 15, 17, 21, 23, 24) but only a few eukaryotic proteins have been secreted with reasonable yield. Problems that account for these failures include proteolytic degradation of secreted target proteins, lack of chaperons required for the secretion of target proteins and optimal joining of secretion signals and target proteins (13). The sequences beyond the processing site can have a remarkable effect on the secretion of the target proteins (4, 5). The cell wall of *B. subtilis* might be a severe barrier at least for some foreign proteins. Human serum albumin, for example, was translocated and processed in *B. subtilis*, but it could not be secreted to culture medium unless the peptidoglycan layer of the cell wall was removed (20). The secretion of *S. aureus* protein A (3) and Streptococcus protein G (2) were also affected by the cell wall.

Another possible suggestion is that each eukaryotic protein may have different secretion efficiency in *B. subtilis* because eukaryotic cells and *B. subtilis* have different cell membrane systems. However, this is difficult to determine definitively because the reported variations in the secretion efficiency could result from the altered processing efficiency caused by different surrounding sequences or different additional amino acids in each sec-

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Table 1. Bacterial strains and plasmids used in this study

Strain	Description	Source/Ref
E. coli		
HB101	*F⁻, hsd20(r⁻ m⁻), recA13, ara14, proA2, lacY1, galK2, rpsL20(Sm^r), xyl-5, mtl-1, supE44*	in this lab
JM103	*Δ(lac-proAB), thi, strA, supE, endA, sbcB, hsdR, F' traD36, proAB, lacI^q ZΔM15*	in this lab
B.csubtilis		
DB104	*his, nprR2, nprE18, aprA3,*	(24)
LKS86	*his, nprR2, nprE18, aprA3, amyE*	in this study
Plasmid	**Description (size: kb)**	**Source/Ref**
pUC8	Ap^r 2.7 kb	in this lab
pEAT-α25	Em^r *amyR₂-amyE⁺*, 11.3 kb	(10)
pEAT-α11	Em^r *amyR₁-amyE⁻*, 11.3 kb	(10)
pUB140	Km^r pUB110 derivatives 3.7 kb	(10)
pSL4	Km^r promoter probe vector, 5.1 kb	(24)
pUBA240	Km^r secretion vector, 4.7 kb	in this work
pUBS340	Km^r secretion vector, 4.7 kb	in this work
pUBAbla	Km^r β-lactamase secretion vector 6.4 kb	in this work
pUBSbla	Km^r β-lactamase secretion vector 6.4 kb	in this work

retion vector. To investigate this possibility, therefore, a new secretion vector is required for the expression and secretion of various proteins within the same context.

We describe here the construction of two secretion vectors using the regulatory and secretory signal region of α-amylase gene from *B. subtilis* NA64 which enable further investigation into whether the intrinsic properties of eukaryotic proteins affect their secretion efficiency in *B. subtilis*. For easy replacement of various foreign genes retaining the same sequence and amino acid context at the processing site, the multi-cloning site from pUC8 was introduced into these secretion vectors. We used β-lactamase as a reporter protein to evaluate the efficiency of expression and secretion of target protein with these secretion vectors.

Materials and Methods

Chemicals and enzymes

Restriction enzymes, ligase and Klenow enzyme were purchased from New England Biolabs. The polyclonal antibody against β-lactamase was purchased from 5′→3′ Inc. (Cat. No. 5307-6211) and horseradish peroxidase-conjugated anti-rabbit IgG was from Cappel. PADAC {7-(thienyl-2-acetamide)-3-[2-(4-N,N-dimethylaminophenylazo)-pyrimidinemethyl]-3-cephem-4-carboxyric acid}, a substrate for β-lactamase, was purchased from Calbiochem. Skim milk and other bacterial culture media were from Difco Laboratories.

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are shown in Table 1.

Transformation of *E. coli* and *B. subtilis*

Transformation of *E. coli* strains with recombinant plasmids was done by the $CaCl_2$ method (12) and the method of Sadaie and Kada (18) was used for competent cell transformation of *B. subtilis*.

Plasmid DNA isolation

The rapid preparation of recombinant plasmids from *E. coli* and *B. subtilis* was performed as described by Birnboim and Doly (1).

Introduction of *amyE* mutation

To select β-lactamase-secreting transformant using the decolorization of starch-iodine reaction by degraded penicillin, an α-amylase deficient mutation was introduced into *B. subtilis* DB104 (24) as follows: pEAT-α1 plasmid, pEAT-A63 plasmid containing *amyR1-E⁺* gene, was transformed into *B. subtilis* WLN7 (*amyR1-E⁻*) (14) and erythromycin resistant colonies were selected. The α-amylase producing property of the transformants was checked on a LB agar plate containing 10 μg/ml erythromycin and 2% soluble starch by an starch-iodine reaction. Erythromycin resistant colonies that did not show clear halos were selected and the plasmid was named as pEAT-α11 (pEAT-A63 *amyR1-E⁻*). DB104 (*amyR1-E⁺*) was transformed with pEAT-α11 and transformants which could not produce the α-amylase were selected. The plasmids in the selected α-amylase deficient transformants were cured and LKS86 strain was obtained.

Screening of β-lactamase secreting transformants and assay of β-lactamase

β-lactamase secreting transformants were selected by the iodometric agar plate method of Jorgensen *et al.*, (8) with slight modifications. Transformants were grown on LB agar plates containing 10 μg/ml kanamycin and 2% soluble starch at 37°C for 18 hours and 3 ml of penicillin-iodine solution was poured carefully onto the agar. One minute later, the penicillin-iodine solution on the agar was removed with a Pasteur pipette. The medium would immediately turn deep purple. The presence of β-lactamase producer was indicated by the formation of a colorless surrounding zone. Penicillin-iodine solution was prepared by addition of 10 ml penicillin solution (6 mg/ml penicillin G in 0.1 M phosphate buffer, pH

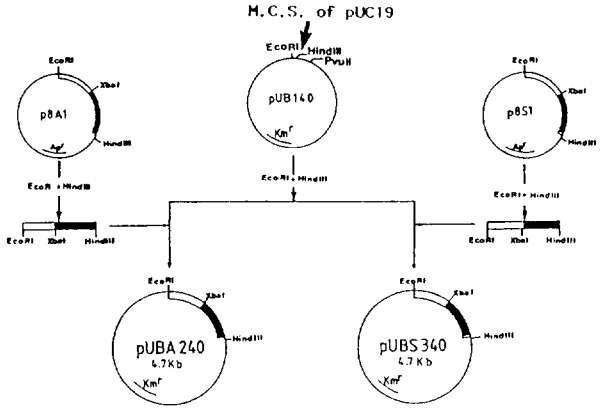

Fig. 1. Scheme for the construction of secretion vectors, pUBA240 and pUBS340.

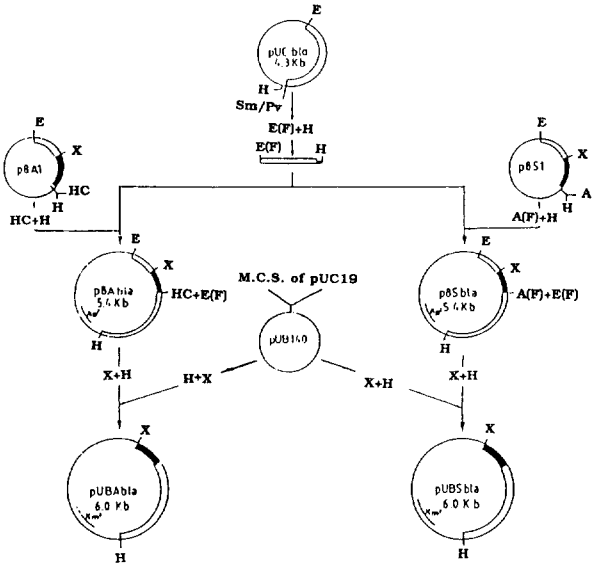

Fig. 2. Scheme for the construction of β-lactamase secreting plasmids, pUBAbla and pUBSbla. E: *Eco*RI, H: *Hin*dIII, A: *Acc*I, X: *Xba*I, HC: *Hin*CII, Sm: *Sma*I, Pv: *Pvu*II, (F): blunt ended by Fill-in reaction with Klenow.

6.0) to 1.7 ml iodine stock solution (2.03 g iodine and 52.22 g potassium iodine in 100 ml of distilled water). This penicillin-iodine solution was freshly prepared before use.

β-lactamase activity in the culture broth was measured by the method of Wong and Doi (24).

Culture media and growth conditions

E. coli strains were grown in Luria-Bertani (LB) broth and transformed *E. coli* strains and *B. subtilis* were selected on LB medium containing appropriate antibiotics. Spizizen's minimal media, SPMM-I and SPMM-II, were used for transformation of *B. subtilis* (18). For the secretion of the β-lactamase by the transformants, the selected transformants were cultured in Medium A (3) containing 10 μg/ml of kanamycin at 37°C overnight with agitation, diluted 100-fold in the same fresh medium, and cultured under the same condition for 16 hours. To measure the β-lactamase activity in the culture, the supernatant was collected from the culture at hourly intervals.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

Proteins in the culture supernatant were precipitated by the addition of trichloroacetic acid and washed twice with cold acetone. For SDS-polyacrylamide gel eletrophoresis (SDS-PAGE), the precipitates were resuspended in 1/10 volume of Laemmli sample buffer and boiled for 3 minutes. Samples were applied to 10% SDS-polyacrylamide gel and subjected to the electrophoresis as described by Laemmli (11). Western blot analysis was performed by the method of Hudson and Hay (7).

Results and Discussion

Construction of secretion vectors based on the secretory region of α-amylase gene

pEAT-α25, a source of the complete α-amylase gene (*amyR*$_2$-E$^+$) of *B. subtilis* NA64 was obtained by a gene conversion with pEAT-α1 (14) in previous work (10). The 1.38 kb *Eco*RI- fragment of pEAT-α25 was digested with two different restriction enzymes. In one case, the fragment was partially digested with *Alu*I and an approximately 1 kb-DNA fragment was eluted. This DNA fragment was ligated with pUC8 which was previously digested with *Eco*RI and *Sma*I, and p8A-1 plasmid was obtained. In the other case, the 1.38 kb-DNA fragment was digested with *Sau3*AI and ligated with pUC8 which had been digested with *Eco*RI and *Bam*HI. The resulting plasmid was named p8S-1. Secretion vectors pUBA240 and pUBS340 were constructed by transferring the 1 kb *Eco*RI-*Hin*dIII DNA fragments from p8A-1 and p8S-1 respectively onto plasmid pUB140 (Fig. 1). The p8A-1 or pUBA240 plasmid contains the promoter, signal sequence and proregion of α-amylase gene, whereas the p8S-1 or pUBS340 plasmid possesses 6 more amino acids of NH_2-terminal of α-amylase than p8A-1.

Construction of β-lactamase secretion vectors

From the promoter probe vector, pSL4 (24), β-lactamase structural gene (*bla*) was transferred to pUC8 and pUCbla was obtained. The *bla* gene fragment from pUCbla plasmid was joined with the p8A-1 and p8S-1 as shown in Fig. 2. Plasmids p8A-1 and p8S-1 were used for the construction of β-lactamase secreting plasmids since *Acc*I and *Hin*cII restriction enzyme sites were not unique sites in pUBA240 and pUBS340 but were unique

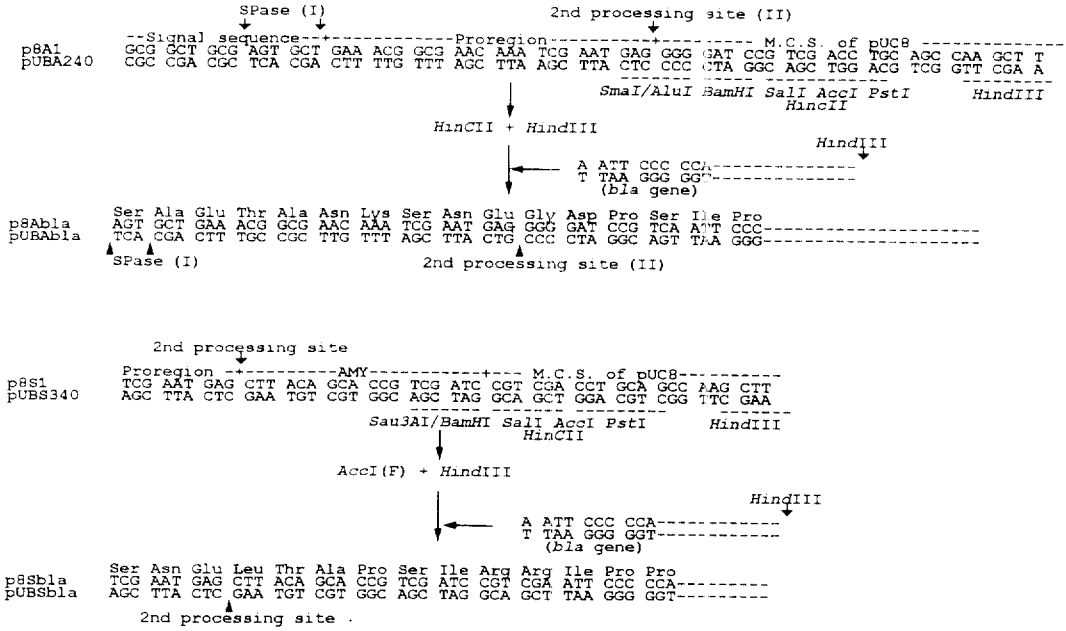

Fig. 3. DNA and corresponding amino acid sequences at the amy-bla fusion boundary of β-lactamase secreting plasmids pUBAbla and pUBSbla. SPase (I): signal peptide processing site by signal peptidase. 2nd processing site (II): processing site for the maturation of α-amylase.

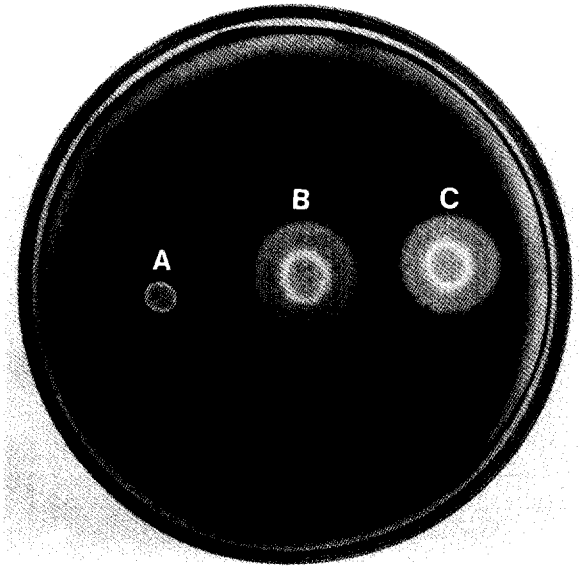

Fig. 4. Clear halo formation by β-lactamase secreting transformants with iodometric agar plate method. A; LKS86/pUB140 , B; LKS86 /pUBAbla , C; LKS86/pUBSbla.

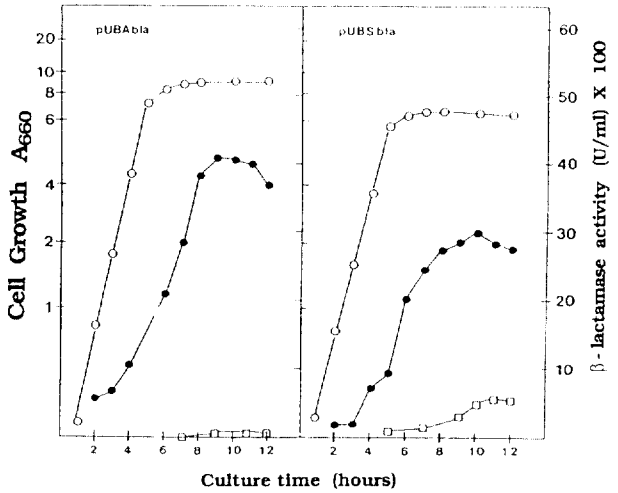

Fig. 5. Expression and secretion of β-lactamase by secreting plasmid during growth of LKS86/pUBAbla and LKS/pUBSbla. All cells were grown in Medium A containing 5 μg/ml of kanamycin at 37°C. Open circles show the cell growth, closed circles represent the β-lactamase activity in the culture broth and open squares demonstrate the intracellular β-lactamase activity. Crude intracellular enzyme was prepared according to the method of Table 4.

sites in p8A-1 and p8S-1. The constructed plasmids were named p8Abla and p8Sbla, respectively. The *Xba*I-*Hin*dIII DNA fragment from p8Abla and p8Sbla were transferred to pUB140 (10) which had been digested with the same enzymes. As a result, pUBAbla and pUBSbla were obtained (Fig. 2). The DNAs and their corresponding amino acid sequences in the *amy-bla* fusion boundary of the β-lactamase secreting plasmids are shown in Fig. 3.

Secretion of β-lactamase

β-lactamase secreting transformants harboring the pUBAbla or pUBSbla were selected by the iodometric agar plate method. The β-lactamase secreting transformants showed large clear halos around colonies by secreted β-lactamase (Fig. 4). Isolated transformants LKS86/pU-

Table 2. The secreted β-lactamase activities in different culture media

Strain and culture time	Activity of β-lactamase (U/ml)		
	Medium A	Medium A+3% glucose	Medium A+3% starch
LKS86/pUBA-bla			
4 hrs	1150	350	1600
7 hrs	2810	2070	3820
9 hrs	4030	2990	7060
LKS86/pUBSbla			
4 hrs	260	120	410
7 hrs	2180	1200	2320
9 hrs	2800	2290	3430

All cells were grown in medium A at 37℃ with or without carbohydrate. Supernatant was collected by centrifugation at the indicated time after inoculation. 1U of β-lactamase activity was defined as the hydrolysis of 1 mol of PADAC {7-(thienyl-2-acetamido)-3-[2-(4-N,N-dimethyl-aminophenylazo)-pyridiniummethyl]-3-cephem-4-carboxylic acid} per minute by measuring the decrease of absorbance at 572 nm.

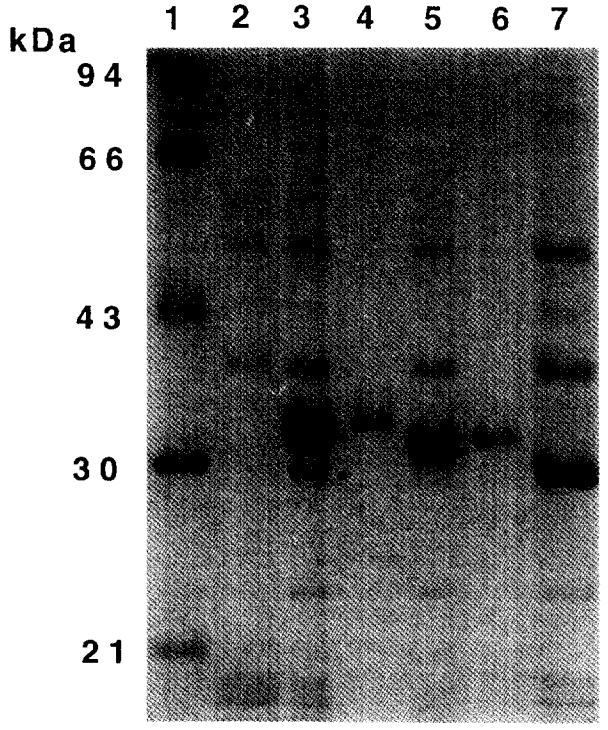

Fig. 6. SDS-polyacrylamide gel patterns of the secreted β-lactamases. Lane 1: size marker, 2: culture supernatant of LKS86/pUB140, 3: culture supernatant of LKS86/pUBAbla, 4: partial purified β-lactamase of LKS86/pUBAbla, 5: culture supernatant of LKS86/pUBSbla, 6: partial purified β-lactamase of LKS86/pUBSbla, 7: culture supernatant of LKS86/pSRbla, Expected β-lactamases are indicated with dots. pSRbla plasmid was constructed by the insertion of the *sacR*'47 promoter in the pSL4 (7). Size marker; 97 kDa: phosphorylas b, 66 kDa: bovine serum albumin, 43 kDa: egg white ovalbumin, 30 kDa: carbonic anhydrase, 21 kDa: soybean trypsin inhibitor.

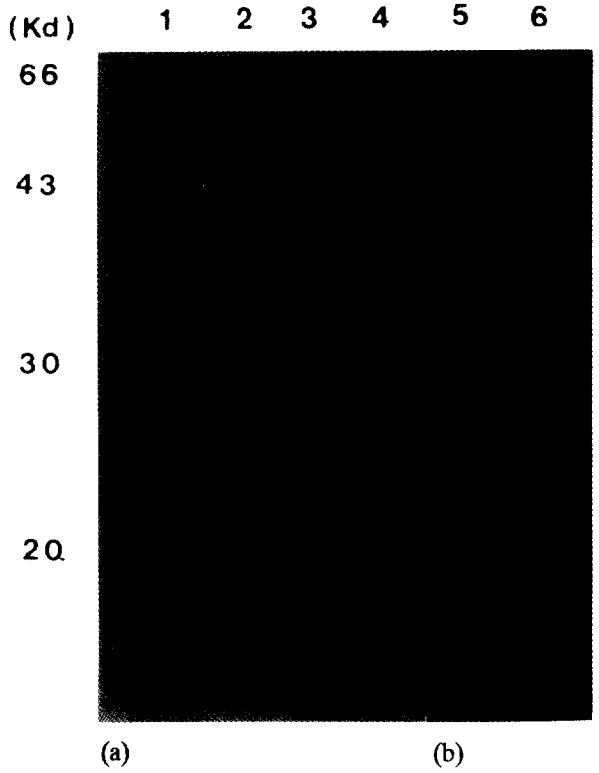

Fig. 7. Western blot analysis of the secreted β-lactamases. Panel A is the Coomassie blue R250 stained gel and panel B is the western blot analysis. Lane 1: Size standard protein, 2: LKS86/pUB 140; 3, 5: LKS86/pUBAbla; 4, 6: LKS86/pUBSbla.

BAbla and LKS86/pUBSbla were cultured and secreted β-lactamase activities were assayed during growth. The maximum β-lactamase activities in the culture broth were obtained at 8-10 hours after inoculation (Fig. 5). When 3% of starch which was known to elevate the transcription level of *amyR*$_2$ promoter (22) was added into the culture broth, the β-lactamase activities in the culture broth were increased about 1.7 fold. Although the addition of 3% glucose increased cell mass about 3-fold (data not shown), the secreted β-lactamase activities were reduced because of catabolite repression. Under all conditions, the amount of β-lactamase in the culture supernatant of LKS86/pUBAbla was higher than that of LKS86/pUBSbla (Table 2).

Analysis of the secreted β-lactamase by SDS-PAGE and Western blotting

Ten-fold concentrated protein samples were analyzed by the electrophoresis on 10% SDS-polyacrylamide gel. The expected β-lactamase bands appeared at a position around 30 kDa (indicated with a dot in lane 3, 5 and 7 of Fig. 6). When β-lactamase was expressed by LKS86 /pUBAbla, there were two expected β-lactamase bands.

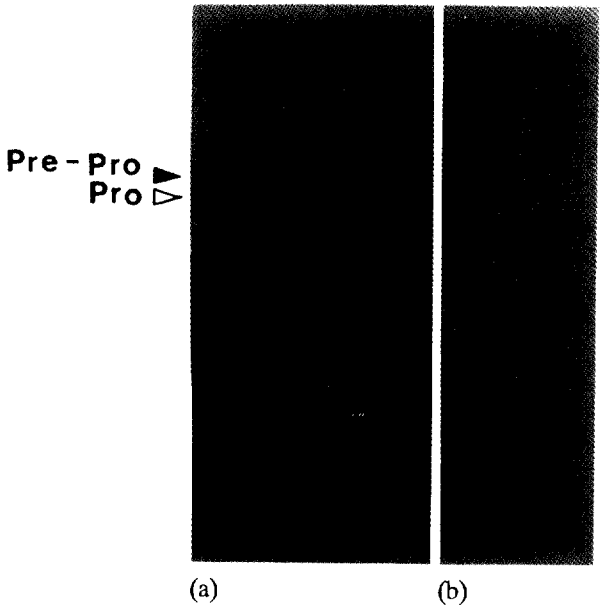

Fig. 8. Western blot analysis of the intracellular β-lactamase in LKS86/pUBAbla and LKS86/pUBSbla. Lane 1: partially purified β-lactamase secreted by LKS86/pUBSbla, 2: intracellular fraction of LKS86/pUBSbla, 3: intracellular fraction of LKS86/pUBAbla, 4: partially purified β-lactamase secreted by LKS86/pUBAbla, Pre-Pro: β-lactamase containing signal sequence and pro region, Pro: β-lactamase containing only pro region.

Table 3. Calculated molecular weights of additional amino acids in the NH_2-termini of secreted β-lactamase

used plasmids	Molecular weights of additional amino acids (dalton)	
	Processed at I*	Processed at II (2nd processing site)*
pUBAbla	1691	519
pUBSbla	2261	1244

*Represents the processing sites illustrated in Fig. 3.

By the comparison with β-lactamase expressed by plasmid pSL4 with the *sacR*-47 promoter (pSRbla), it seemed that the large one was processed only at the signal sequence processing site (processing site I in Fig. 3) and the small one was processed at the 2nd processing site in Fig. 3. Therefore, the major part of secreted β-lactamase by pUBAbla was processed at the signal sequence processing site. In the case of β-lactamase expressed by pUBSbla, there was one expected band and its size indicates that almost all of the expressed β-lactamase were processed not only at the signal sequence processing site but also the 2nd processing site. To confirm that the the suggested proteins were β-lactamase, Western blot analysis was carried out and all the suggested protein bands in Fig. 5 were reacted with the antibody against β-lactamase. (Fig. 7). Also, the apparent molecular weights of β-lactamase expressed by pUBAbla and pUBSbla were larger than that by the parent plasmid, pSL4 (lane 7 in Fig. 6). This might be caused by the presence of additional amino acids in the NH_2-terminal region. The calculated molecular weight of additional amino acids are shown in Table 3. When the level of secreted β-lactamases were estimated using densitometry (Shimadzu, Model No. CS-9000), in both cases they comprised about 70% to 80% of the total secreted proteins in the culture broth (data not shown).

Table 4. Secretion efficiency of β-lactamase in LKS86/pUBAbla and LKS86/pUBSbla

Strain and media	Activities of β-lactamase (U/ml)		
	Extracellular[a] (E/T)	Intracellular[b] (I/T)	Total
LKS86/pUBAbla			
Medium A	4074 (98.9%)	45 (1.1%)	4119
+ 3% glucose	2990 (99.2%)	25 (0.8%)	3015
+ 3% starch	7060 (94.9%)	372 (5.1%)	7432
LKS86/pUBSbla			
Medium A	2687 (92.1%)	230 (7.9%)	2917
+ 3% glucose	2290 (98.4%)	38 (1.6%)	2328
+ 3% starch	3430 (72.5%)	300 (27.5%)	4730

[a] Cells were cultured and supernatant was obtained at 9 hours by the same methods in Table 3.
[b] Harvested cells from 5 ml of culture were washed and resuspended in 0.5 ml of 50 mM. Potassium phosphate buffer (pH 7.0) containing 5 mg/ml of lysozyme. The treated cells were incubated for 10 min and lysed by vigorous vortexing with glass beads. The lysed cells were centrifuged and collected supernatants were used as crude intracellular extracts. E: β-lactamase activity in culture broth. I: β-lactamase activity in intracellular extract. T: Total β-lactamase activity (T = E + I).

Secretion efficiency of the produced β-lactamases

In order to compare the secretion efficiency of β-lactamases expressed by the two different transformants, proteins in intracellular fraction were analyzed by a Western blotting (Fig. 8). While the high amount of prepro-form β-lactamase was detected in the intracellular fraction of LKS86/pUBSbla, little prepro-form was observed in that of pUBAbla. This result, therefore, suggests that the additional amino acids at the fusion boundary might affect the secretion efficiency of the expressed β-lactamase even though they used the same promoter and signal sequence. The accumulation of the prepro-form β-lactamase in the cell was affected by its expression level. Under the high expression condition resulting from starch addition, the accumulation of prepro-form β-lactamase was increased in both cases. Particularly in LKS86/pUBSbla case, the prepro-form β-lactamase increased

to 27.5% of total expressed β-lactamase (Table 4). By the addition of glucose in the culture, in both cases, the level of prepro-form β-lactamase by catabolite repression. It seemed that the increase of the prepro-form β-lactamase might be caused by saturation of secretory machinery with unfavorable precursor form of β-lactamase which has slow processing kinetics. The total amounts of β-lactamases expressed by LKS86/pUBAbla and LKS86/pUBSbla were different (Table 4). This is similar to the results of Sasamoto *et al.* (19), and Nakamura *et al.* (13), who suggested that the secretion efficiency of the expressed protein might affect the transcriptional and translational level of the target gene.

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