Effect of *in vitro* DNA Rearrangement in the NH₂-terminal Region of the Penicillinase Gene from *Bacillus licheniformis* on the Mode of Expression in *Bacillus subtilis*

By TADAYUKI IMANAKA,* TAKESHI HIMENO AND SHUICHI AIBA

Department of Fermentation Technology, Faculty of Engineering, Osaka University,
Yamada-oka, Suita-shi, Osaka 565, Japan

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We have constructed secretion vector plasmids that have unique *Bg*/II sites within or near the signal sequence of *Bacillus licheniformis* penicillinase, and have also constructed penicillinase cartridges that lack either one, two or three of the processing sites for the membrane-bound, exo-large and exo-small enzymes. Each of these penicillinase cartridges was cloned on secretion vectors in *Bacillus subtilis*, and enzyme production was examined. The presence of both the signal sequence and the three host-specific processing sites on the secretion vector was required for an effective expression of the enzyme in *B. subtilis*. The presence of any of the processing sites on the cartridge reduced the accumulation of penicillinase in the culture medium. When a vector plasmid lacking part of the hydrophobic region of the signal sequence and lacking the three processing sites was used, total penicillinase production decreased and enzyme accumulation in the medium was extremely low, despite the complete or incomplete presence of the processing sites on the cartridge. Molecular mass determination of these extracellular penicillinases suggested the existence of a new cleavage site for the enzyme.

INTRODUCTION

Secretion of protein is an essential function of all living cells. It is well known that secretion of a protein usually requires the presence of a relevant signal sequence (Blobel, 1980; Kreil, 1981; Michaelis & Beckwith, 1982). In fact, many kinds of signal sequence have been discovered (Watson, 1984), and their structures and functions have been characterized (Inouye *et al.*, 1982; Emr & Silhavy, 1983; von Heijne, 1983; Perlman & Halvorson, 1983).

Escherichia coli has been the prokaryote most widely used for study of the basic features of protein secretion through the plasma membrane. The secreted protein of *E. coli* is located within the periplasmic space or in the outer membrane. In contrast, Gram-positive bacteria, such as *Bacillus subtilis*, have a simpler cell-envelope, composed of a single layer of membrane. When a secretory protein is transported across the cytoplasmic membrane of bacilli, the protein is processed and released directly into the culture medium. We were interested to know whether or not, and to what degree, all or part of the processing steps would affect the secretion efficiency. We have focused our attention on the penicillinase of *Bacillus licheniformis* as a model for studying the structural requirements for protein export, because the secretion and processing of penicillinase in the genus *Bacillus* have been studied extensively (Izui *et al.*, 1980; Nielsen *et al.*, 1981; Smith *et al.*, 1981; Chang *et al.*, 1982; Imanaka *et al.*, 1983).

Prepenicillinase is initially synthesized as a precursor molecule, and is then translocated through the cytoplasmic membrane with concomitant cleavage of the signal peptide. This membrane-bound penicillinase is converted to an extracellular enzyme, exo-large or exo-small, by two proteolytic cleavage steps. The structural gene of penicillinase, *penP*, has been cloned in *E. coli*, *B. subtilis*, *B. licheniformis* and *Bacillus stearothermophilus* (Brammer *et al.*, 1980; Gray & Chang, 1981; Imanaka *et al.*, 1981*b*; Fujii *et al.*, 1982), and has also been sequenced

(Neugebauer *et al.*, 1981). Gene expression was observed in these host strains, although the level of expression and of enzyme secretion and the site of processing depended on the species used as the host (Imanaka *et al.*, 1981*b*; Fujii *et al.*, 1982).

We have constructed various secretion vectors and *penP* cartridges to produce the fused protein in *B. subtilis*. This paper describes the structural requirements of both the vector and the cartridge that facilitate the secretion of the enzyme and the proteolytic cleavage that follows.

METHODS

Bacteria and plasmids. The bacterial strains and plasmids used are listed in Table 1. Penicillinase genes from a constitutive strain of *B. licheniformis* ATCC 9945A have been cloned in *E. coli*, with pMB9 as a vector plasmid. *penP* and *penI* are the structural gene and the repressor gene for penicillinase, respectively, and are coded on a 2.8 MDal *EcoRI* fragment (Imanaka *et al.*, 1981*b*). The NH₂-terminal amino acid sequence of penicillinase for *B. licheniformis* ATCC 9945A is identical with that for *B. licheniformis* 749/C (Imanaka *et al.*, 1983).

Transformation. The transformation of *E. coli* with plasmid DNA was done as described earlier (Imanaka *et al.*, 1981*b*). Transformants were selected on L agar (10 g Bacto-tryptone (Difco), 5 g yeast extract, 5 g NaCl and 15 g agar in 1 litre deionized water, pH 7.0) plus tetracycline (Tc, 20 µg ml⁻¹) or ampicillin (Ap, 20 µg ml⁻¹).

Transformation of competent *B. subtilis* cells was done as described previously (Imanaka *et al.*, 1981*a*). Transformants were selected on L agar plus Tc (25 µg ml⁻¹).

Preparation of plasmid DNA. Small-scale preparation of plasmid DNA from *E. coli* was done by the alkaline phenol procedure or the alkaline isopropanol procedure (Maniatis *et al.*, 1982); the latter procedure was used for large-scale extraction of plasmids. *B. subtilis* cells were treated with lysozyme at 37 °C for 20 min, and then the alkaline isopropanol procedure and the cleared lysate method (Imanaka *et al.*, 1981*a*) were used to extract plasmids in small- and large-scale preparations, respectively. Covalently closed circular plasmid DNA was separated by CsCl/ethidium bromide equilibrium density gradient centrifugation (Imanaka *et al.*, 1981*a*).

Cleavage of DNA with restriction enzymes, repair of cohesive ends and ligation of DNA and linker. Treatment of DNA with restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Pst*I and *Pvu*II), repair of cohesive ends with the Klenow fragment of DNA polymerase I, and ligation of DNA with T4 DNA ligase were done according to the manufacturer's recommendations. *Bgl*II and *Bam*HI linkers were treated with T4 polynucleotide kinase. All the enzymes and linkers used in these experiments were purchased from Takara Shuzo Co., Kyoto, Japan.

Digestion of DNA with exonuclease. DNA was digested with the double-stranded exonuclease Bal31 (Takara Shuzo Co., Kyoto, Japan); 5 µg DNA and 1 unit Bal31 were incubated at 25 °C, in 100 µl 20 mM-Tris/HCl (pH 8.0), 12 mM-CaCl₂, 12 mM-MgCl₂, 100 mM-NaCl and 1 mM-EDTA (Maniatis *et al.*, 1982). The reaction was stopped by addition of EGTA (final concentration 20 mM) and incubation at 68 °C for 10 min to inactivate Bal31.

Gel electrophoresis for DNA analysis and isolation. DNA was analysed by agarose gel electrophoresis, with horizontal slab gel apparatus in Tris/acetate buffer (40 mM-Tris, 20 mM-sodium acetate (pH 8.0), 1 mM-EDTA and 0.5 µg ethidium bromide ml⁻¹) (Imanaka *et al.*, 1981*a*).

DNA fragments were isolated by gel electrophoresis, with a low melting point (LMP) agarose gel (1%, w/v; Takara Shuzo Co., Kyoto, Japan) in Tris/acetate buffer (pH 7.4). After gel electrophoresis, slices of gel containing DNA fragments were melted at 68 °C, and two volumes of 50 mM-Tris/HCl buffer (pH 8.0) with 0.5 mM-EDTA were added. After 5 min at 38 °C, an equal volume of phenol saturated with the buffer was added. After a further 5 min, the mixture was centrifuged and the water layer was transferred to another tube. This phenol treatment was repeated three times. Diethylether was added to the water layer to remove phenol, and then sodium acetate was added to a final concentration of 0.3 M. DNA was collected by ethanol precipitation.

DNA fragments less than 1 kbp in size were analysed and isolated by PAGE, with Tris/acetate buffer (pH 7.4) lacking ethidium bromide. The concentration of acrylamide was 4%, 6% or 8% (w/v). Other experimental conditions were standard (Maniatis *et al.*, 1982). After gel electrophoresis, DNA bands were stained with ethidium bromide. For DNA isolation, the gel was sliced and broken up, and DNA was eluted in MG elution

Table 1. *Bacterial strains and plasmids*

Strain	Characteristics		Reference
<i>Escherichia coli</i> C600-1	<i>leu-6 thr-1 thi-1 supE44 lacY tonA21 hsdR hsdM Trp⁻</i>		Imanaka <i>et al.</i> (1980)
<i>Bacillus subtilis</i> M1113	<i>arg-15 trpC2 r_M m_M</i>		Imanaka <i>et al.</i> (1981 <i>a</i>)
Plasmid	Mol. mass (MDal)	Characteristics	Reference
pTTE11	6.3	Tc ^r <i>penP</i> ⁺ (Ap ^r) <i>penI10</i>	Imanaka <i>et al.</i> (1981 <i>b</i>)
pBR322	2.8	Tc ^r Ap ^r	Imanaka <i>et al.</i> (1981 <i>b</i>)
pTB52	7.0	Tc ^r	Imanaka <i>et al.</i> (1981 <i>a</i> , 1984)

buffer (0.5 M-ammonium acetate, 10 mM-magnesium acetate, 0.1 mM-EDTA, 0.1% SDS) (Maniatis *et al.*, 1982) at 37 °C for 12 h. The buffer solution was separated from the gel by centrifugation, and DNA was isolated by ethanol precipitation.

DNA sequencing. DNA fragments were treated with bacterial alkaline phosphatase (Takara Shuzo Co., Kyoto, Japan), and 5'-end labelled in the presence of [γ - 32 P]ATP (New England Nuclear) with T4 polynucleotide kinase (Takara Shuzo Co., Kyoto, Japan). Sequence analysis was done according to Maxam & Gilbert (1980).

Penicillinase production and enzyme assay. *B. subtilis* carrying the *penP* gene was cultivated overnight in CH/S medium (10 g Casamino acids (Difco), 2.8 g KH_2PO_4 and 1 ml Pollock's salt solution in 1 litre deionized water, pH 7.0) (Imanaka *et al.*, 1983) at 37 °C. A sample (1%) of the preculture was inoculated in 100 ml of the fresh medium in a 500 ml conical flask and cultivated on a shaker at 37 °C. The culture broth was used for the assay of total penicillinase. The supernatant of the culture broth that was obtained by centrifugation (8000 g, 10 min) was used for the assay of extracellular penicillinase. The enzyme activity was assayed as described earlier (Imanaka *et al.*, 1981*b*). We have verified that the different forms of the enzyme show similar specific activities when purified (unpublished). The penicillinase positive colonies on plates were detected as described previously (Imanaka *et al.*, 1981*b*).

Preparation of extracellular penicillinase and analysis by gel electrophoresis. The supernatant of a 12 h culture broth was used as the enzyme source. Penicillinase was partially purified by both phosphocellulose treatment and carboxymethylcellulose chromatography (Imanaka *et al.*, 1983). The discontinuous SDS-PAGE system of Laemmli was used (Laemmli, 1970; Imanaka *et al.*, 1983).

RESULTS

Construction of secretion vectors which carry the promoter and signal sequence region of the penicillinase gene

We altered the penicillinase gene, *penP*, to create a set of sites for cloning closer to and/or within the signal sequence region. A scheme for the construction of these secretion vectors is given in the left-hand half of Fig. 1. Plasmid pTTE11, carrying *penP*, was cleaved at a unique *Bgl*II site (in the middle of the *penP* gene), and was digested with *Bal*31 for 2 or 2.5 min. *Bal*31-treated DNA was used for the subsequent cloning. After repair with the Klenow fragment of *E. coli* DNA polymerase I, *Bgl*II linker was added to the DNA, which was then self-ligated with T4 DNA ligase. The ligation mixture was used to transform *E. coli* C600-1.

Plasmids carrying deletions and a *Bgl*II linker were screened amongst the transformants, and studied by DNA sequencing. Fig. 2 shows a deletion map for seven derivatives of pTTE11. Two of these plasmids, pPS202 and pPS221, code for 20 amino acids of the signal sequence and lack the MB site for the membrane-bound form, where the signal sequence is cut off by a signal peptidase (Chang *et al.*, 1982). Plasmid pPS221 unexpectedly contained a five base-pair (CAGAT) product of the *Bgl*II linker in addition to the standard sequence (CAGATCTG). Plasmids pPS312 and pPS321 contained the entire signal sequence region and MB site, but lacked the two downstream processing sites, L and S (S1, S2, S3), for exo-large and exo-small penicillinase, respectively. S2 and S3 are the cleavage sites of exo-small enzyme from *B. subtilis*, while S1 is that for *B. licheniformis* (Imanaka *et al.*, 1983). Plasmid pPS352 contains the MB and L sites, but not the S site. Plasmid pPS422 codes for 41 original amino acids from the NH_2 -terminal methionine, and contains the S3 but not the S2 site. Plasmid pPS451 carries all the processing sites, MB, L and S (S1, S2 and S3).

Construction of penP cartridges

To examine the role of the signal sequence and the processing sites in protein secretion by using the secretion vectors, we constructed *penP* cartridges. The penicillinase gene was used for the above-mentioned purpose, as it appeared unlikely that the remaining portion of the penicillinase lacking the signal sequence might cause a specific secretion barrier.

The right-hand half of Fig. 1 shows schematically the construction of *penP* cartridges. Plasmid pTTE11 was cleaved at a single unique *Pst*I site which is in the signal sequence of *penP*, and was digested with *Bal*31 for 70, 80 or 90 s. *Bal*31-treated DNA was cleaved with *Pvu*II, and analysed with low melting point agarose gel electrophoresis. After gel electrophoresis, a fragment of about 1 MDal, containing the COOH-terminal region of *penP*, was isolated from the gel. After repair

Fig. 1. Schematic representation of the construction of secretion vectors (pPS) and *penP* cartridges (pPC). Cleavage sites of *Bam*HI, *Bgl*II, *Eco*RI, *Pst*I and *Pvu*II are indicated by Ba, Bg, E, Ps and Pv, respectively. , Signal sequence region of penicillinase; , structural gene of penicillinase.

pTTE11	GCAGTGTGCTTTTCTCTTCGCGTCGGCTTGCAGGATGCGCTAACAAATCAAACGAATGCCTCGCAACCTGCCGAGAAGAATGAAAAGACGGAGATGAAA AlaValLeuLeuPheSerCysValAlaLeuAlaGlyCysAlaAsnAsnGlnThrAsnAlaSerGlnProAlaGluLysAsnGluLysThrGluMetLys 15 20 25 30 35 40 45
	MB L S3 S2 S1
pPS202	GCAGTGTGCTTTTCTCCAGATCTG AlaValLeuLeuPheSer
pPS221	GCAGTGTGCTTTTCTCCAGATCAGATCTG AlaValLeuLeuPheSerArgSer
pPS312	GCAGTGTGCTTTTCTCTTCGCGTCGGCTTGCAGGATGCGCTAACAAATCAGATCTG AlaValLeuLeuPheSerCysValAlaLeuAlaGlyCysAlaAsnAsnHis
pPS321	GCAGTGTGCTTTTCTCTTCGCGTCGGCTTGCAGGATGCGCTAACAGATCAGATCTG AlaValLeuLeuPheSerCysValAlaLeuAlaGlyCysAlaAsnThrAspPro
pPS352	GCAGTGTGCTTTTCTCTTCGCGTCGGCTTGCAGGATGCGCTAACAAATCAAACGAATGCCTCGAGATCTG AlaValLeuLeuPheSerCysValAlaLeuAlaGlyCysAlaAsnAsnGlnThrAsnAlaSer
pPS422	GCAGTGTGCTTTTCTCTTCGCGTCGGCTTGCAGGATGCGCTAACAAATCAAACGAATGCCTCGCAACCTGCCGAGAAGAATGACAGATCTG AlaValLeuLeuPheSerCysValAlaLeuAlaGlyCysAlaAsnAsnGlnThrAsnAlaSerGlnProAlaGluLysAsnAsp
pPS451	GCAGTGTGCTTTTCTCTTCGCGTCGGCTTGCAGGATGCGCTAACAAATCAAACGAATGCCTCGCAACCTGCCGAGAAGAATGAAAAGACGGAGATCTG AlaValLeuLeuPheSerCysValAlaLeuAlaGlyCysAlaAsnAsnGlnThrAsnAlaSerGlnProAlaGluLysAsnGluLysThrAla

Fig. 2. Sequences of the signal sequence region of secretion vectors. Part of the sequence of *penP* in pTTE11 is shown for reference. The sequence from 1st to 14th amino acid is common for all of the plasmids and is omitted. MB, L and S (S1, S2, S3) are the processing sites for the membrane-bound, exo-large and exo-small enzymes, respectively. The amino acids are numbered from the first amino acid (no. 1, methionine) of the signal sequence. The bases and amino acid residues donated by the linker are indicated in *italics*. The first two digits of the pPS plasmid refer to the number of NH₂-terminal amino acids from *penP*; the last digit is the frame number after cleavage of the *Bgl*II linker.

Fig. 3. Sequences of *penP* cartridges in the vicinity of the processing sites. The corresponding region of pTTE11 is shown for reference. MB, L and S (S1, S2, S3) are the processing sites for the membrane-bound, exo-large and exo-small enzymes, respectively.

Fig. 4. Construction of the fused gene from the secretion vector and the *penP* cartridge. The symbols are defined in the legend to Fig. 1.

with the Klenow fragment of DNA polymerase I, *Bam*HI linker was added to the DNA, which was then inserted into the *Bam*HI site of pBR322. The ligation mixture was used to transform *E. coli* C600-1. Ap^r and Tc^s transformants were isolated, from which recombinant plasmids were screened.

The *Bam*HI-ended gene fragment of *penP* was sequenced (Fig. 3). Plasmids pPC27L, pPC36S and pPC44X lacked one, two and three processing sites (MB, L and S), respectively.

Insertion of the *penP* cartridge into the secretion vectors

By using the protruding ends of the *Bam*HI and *Bgl*II sites, the *penP* cartridge from *Bam*HI digest of pPC plasmids could be inserted into the *Bgl*II site of pPS plasmids in *E. coli* C600-1 whenever the reading frames were matched (Fig. 4). The newly constructed plasmids were designated as pPSC plasmids; the predicted amino acid sequence of each hybrid protein is given

in Fig. 5. The number in the pPSC plasmid code refers to the number of amino acids originating from pPS plasmids, while the letters L, S or X indicate the existence of L, S or no processing sites in the *penP* cartridge, respectively. Joining the vector to the cartridge led to the appearance of two or three amino acids (in italics in Fig. 5) between the last amino acid of the NH₂-terminal sequence of penicillinase in pPS plasmids and the first amino acid of the conserved sequence in the *penP* cartridge. Some combinations of secretion vector and cartridge gave unexpected plasmids, such as deleted ones. The plasmids pPSC20X, pPSC20L, pPSC22S and pPSC32S are all putative.

To examine penicillinase production in *B. subtilis*, we transferred the fused gene fragment into a *B. subtilis* vector plasmid. Prior to the experiment, one of the two *Eco*RI sites of the vector plasmid was eliminated as follows (see the right-hand side of Fig. 4). Plasmid pTB52 (Tc^r) was digested with *Bgl*II, and was then partially digested with *Eco*RI. Phenol-treated DNA was repaired with the Klenow fragment of DNA polymerase I, and was self-ligated. The ligation mixture was used to transform *B. subtilis* MI113. A new vector plasmid pTB522, with one *Eco*RI site, was isolated from the Tc^r transformants. The *Eco*RI fragment containing the fused *penP* gene from plasmid pPSC was inserted into the *Eco*RI site of pTB522, resulting in the formation of the pTBS series of plasmids. Likewise, the *Eco*RI fragment carrying wild-type *penP* from pTTE11 was inserted into the *Eco*RI site of pTB522. The recombinant plasmid, pPTB50, was used as a control.

Since some pPSC plasmids (20X, 20L, 22S and 32S) could not be obtained in *E. coli*, the fused gene of penicillinase was constructed directly in *B. subtilis* as shown in Fig. 4. The *Eco*RI fragment containing the *Bgl*II site of the pPS plasmid was first cloned in pTB522. The plasmids were designated as pTS plasmids. The *penP* cartridge from the pPC plasmid was inserted into the pTS plasmids. Recombinant plasmids pTBS20X, pTBS20L and pTBS22S were constructed, although pTBS32S could not be obtained in *B. subtilis*. The amino acid sequence of the hybrid proteins are shown in Fig. 5.

Production of penicillinase by B. subtilis carrying recombinant plasmids

Growth curves of *B. subtilis* strains carrying *penP* plasmids are shown in Fig. 6. Maximum cell concentrations were nearly the same in all cases, with OD₆₆₀ values ranging from 2.0 to 2.4.

In the control experiment with pPTB50 (Fig. 6), penicillinase production occurred during cell growth. Therefore, enzyme production and secretion at the late exponential phase of growth were compared to avoid the influence of cell lysis and the degradation of protein (Table 2).

For pTBS20X, pTBS20L and pTBS22S, whose vector plasmids lacked a part of the hydrophobic region of the signal sequence and the three processing sites, total penicillinase activity was about one-fifth of that in the control experiment, and the enzyme accumulation in culture medium was extremely low, irrespective of the presence of processing sites on the cartridge. For pTBS42X, pTBS42L and pTBS45S, whose vector plasmid carried three processing sites, enzyme activity was at the same level as in the control. For other plasmids, whose vector lacked one or two processing sites, enzyme activity was in between the values obtained with pTBS20X and pPTB50. These results indicate that the signal sequence and the three host-specific processing sites of penicillinase on the secretion vector were essential for effective expression of the enzyme in *B. subtilis*. Whenever the same vector plasmids were used, the presence of processing sites on the cartridge seemed to be the cause of an anomaly in the peptide cleavage, hence resulting in the reduced accumulation of penicillinase in the supernatant.

Analysis of extracellular penicillinase

Extracellular penicillinase produced by *B. subtilis* was analysed by PAGE (Fig. 7). The strain carrying pPTB50 produced two types of extracellular penicillinase, exo-large (exo-L; molecular mass 30.5 kDa) and exo-small (exo-S; molecular mass 29.5 kDa) enzymes (Imanaka *et al.*, 1983) at 18 h (Fig. 7, lanes 4, 11), although the production of exo-S enzyme was very slight at 12 h (Fig. 7, lanes 1, 7).

Fig. 6. Growth curves and penicillinase production of *B. subtilis* strains carrying *penP* plasmids. □, Optical density; ●, total penicillinase activity; ○, penicillinase activity in the supernatant.

Table 2. Penicillinase production by *Bacillus subtilis* carrying recombinant plasmids

Plasmid	Late exponential phase (h)	Penicillinase (units ml ⁻¹)		Supernatant/total (%)
		Total	Supernatant	
pTBS20X	12	1000	40	4
pTBS20L	12	1000	90	9
pTBS22S	12	1100	70	6
pTBS31X	12	4600	870	19
pTBS31L	10	1600	60	4
pTBS35X	12	4500	890	20
pTBS35L	12	3900	420	11
pTBS42X	12	5400	1300	24
pTBS42L	12	5900	850	14
pTBS45S	12	5200	700	13
pPTB50 (control)	12	5500	1500	27

Fig. 7. SDS-PAGE of extracellular penicillinase. Unless otherwise noted, each penicillinase was purified from a 12 h culture. Lanes 1 and 7, penicillinase from pPTB50 (control); lanes 4 and 11, from pPTB50 (18 h); lane 2, from pTBS31X; lane 3, from pTBS31L; lane 5, from pTBS35X; lane 6, from pTBS35L; lane 8, from pTBS42X; lane 9, from pTBS42L; lane 10, from pTBS45S. Molecular mass is shown in kDal.

B. subtilis strains carrying either pTBS31X or pTBS35X produced a specific penicillinase (designated as *exo-X*; molecular mass 28.5 kDal) which was smaller than *exo-L* and *exo-S* (Fig. 7, lanes 2, 5). This result might be due to the fact that pTBS31X lacked both the L and S cleavage sites, and also that pTBS35X lacked the S site, the L site possibly being influenced by the positive charge of arginine (see Fig. 5).

Cells carrying either pTBS31L or pTBS35L produced *exo-L* and *exo-S*, because the *penP* cartridge contained both processing sites corresponding to *exo-L* and *exo-S*, and also produced *exo-X*. The appearance of *exo-X* penicillinase, which is smaller than *exo-S*, suggests a new cleavage site of the enzyme for a host-specific protease.

Cells carrying pTBS42X produced *exo-X* and another penicillinase (molecular mass 30 kDal), although only three amino acids in the predicted sequence of penicillinase from pTBS42X were different from those of the wild-type at position S1 (see Fig. 5). Despite the presence of the L site on the vector, *exo-L* enzyme did not appear. This fact suggests that a proper amino acid sequence is needed in the vicinity of the processing site itself for the normal cleavage of penicillinase to proceed.

B. subtilis carrying either pTBS42L or pTBS45S produced another penicillinase (designated as *exo-LL*; molecular mass ~32 kDal) which was larger than *exo-L* (Fig. 7, lanes 9, 10). It was inferred that the enzyme *exo-LL* was obtained by the cleavage of prepenicillinase at a particular processing site on the secretion vector.

DISCUSSION

Extracellular enzymes are transported through the cytoplasmic membrane and are processed by host-specific proteases. To evaluate the contribution of the signal sequence and the cleavage sites to this process of transportation, we have constructed secretion vectors and *penP* cartridges from the *B. licheniformis* penicillinase gene, and have joined them together.

A series of experiments on the expression of fused genes in *B. subtilis* revealed that the signal sequence and the processing sites on the secretion vector need to be wild-type for effective gene expression. In contrast, the presence of processing sites on the cartridge adversely affected the amount of penicillinase accumulation in the culture medium.

In this context, Talmadge *et al.* (1980) also constructed fused genes from the signal sequence of *E. coli* penicillinase and rat insulin, and concluded that either the *E. coli* signal on the vector or the eukaryotic signal on the cartridge is sufficient for transporting rat insulin antigen into the periplasmic space in *E. coli*. This apparently inconsistent conclusion might be due to the difference in host strains, *B. subtilis* and *E. coli*.

The membrane penicillinase of *B. licheniformis* is a glyceride-cystein lipoprotein that could be, but would not necessarily be, an intermediate in the formation of *exo-enzyme* (Mézes *et al.*, 1983). To examine the role of the lipophilic modification, Mézes *et al.* (1983) prepared a deletion mutant, *penPAI*, whose product lacked residues of Ala₂₃-Leu-Ala-Gly-Cys₂₇. This sequence includes the cysteine residue that undergoes lipophilic modification and that is the site of cleavage by signal peptidase.

The amount of penicillinase synthesized by *penPAI* clones was nearly equal to that of the original *penP* clones in *B. subtilis* (Mézes *et al.*, 1983). In contrast, in the work described in this

paper the amount of penicillinase synthesized by pTBS20X, pTBS20L or pTBS22S, which at least lacked Cys₂₁-Val-Ala-Leu-Ala-Gly-Cys₂₇, was considerably smaller than that of pPTB50 in *B. subtilis*. The above-mentioned difference might be because charged amino acid residues, such as positively charged arginine, added to the hydrophobic segment of the signal sequence of pTBS plasmids result in either an anomaly of membrane transport of penicillinase, or jamming of the enzyme in the membrane. In addition, the low penicillinase production for pTBS20X, pTBS20L and pTBS22S might be attributed to reduced gene expression due to either instability or low translational efficiency of the mRNA.

Plasmids pTBS31X and pTBS31L could be constructed in *B. subtilis*. However, neither pPSC32S nor pTBS32S (putative recombinant plasmids) could be obtained in *E. coli* or *B. subtilis*, respectively. The important difference between the putative plasmid pTBS32S and plasmids pTBS31X and pTBS31L is in the amino acid residues arising from the inserted linker. For pTBS31X and pTBS31L, two positively charged amino acids, histidine and arginine, arose in connecting the vector and the cartridge. In contrast, negatively charged amino acids (two aspartate residues) could have arisen in the putative plasmid pTBS32S from the inserted linker. Thus, the negatively charged neighbouring signal sequence might have repelled the negative charge of the membrane phospholipid, resulting in the failure to construct pTBS32S.

Although pPSC plasmids for 20X, 20L and 22S, whose processing site MB was deleted, could not be constructed in *E. coli*, pTBS plasmids for each of them could be obtained in *B. subtilis*. The deletion of processing site MB might have been offset by other processing systems present in *B. subtilis*.

To establish a general secretion system, the cloning in this series of vector plasmids of foreign DNA from a higher vertebrate and the study of protein secretion are now in progress.

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