Comparative Studies on Extracellular Penicillinases of the Same Structural Gene, *penP*, Expressed in *Bacillus licheniformis* and *Bacillus subtilis*

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Extracellular penicillinases produced by *Bacillus licheniformis* ATCC 9945A and *Bacillus subtilis* from the same structural gene, *penP*, were compared. The two strains secreted the same exo-large penicillinase (mol. wt, 30 500; isoelectric point, pI = 5.00–5.04; NH₂-terminal amino acid, Ser). In contrast, the exo-small enzyme from *Bacillus subtilis* (mol. wt, 29 500; pI = 5.00–5.04; NH₂-terminal amino acid, Glu or Asn) was slightly different from that of *Bacillus licheniformis* (mol. wt, 29 500; pI = 5.13; NH₂-terminal amino acid, Lys). The difference in the NH₂-terminal residue is most probably due to differences in degradation by host-specific proteolytic enzymes.

INTRODUCTION

By using vector plasmids, we have cloned the structural gene for penicillinase, *penP*, from *Bacillus licheniformis* ATCC 9945A in *Bacillus subtilis* and *B. licheniformis* (Imanaka *et al.*, 1981). Although both strains carrying the *penP* gene produced nearly the same total amount of penicillinase, the fraction of secreted penicillinase for *B. subtilis* and *B. licheniformis* amounted to around 30 and 60% of the total activities, respectively (Imanaka *et al.*, 1981).

It has been reported that *B. licheniformis* 749/C, containing only a single structural gene for penicillinase (Sherratt & Collins, 1973), produces both an extracellular penicillinase and a membrane-bound form which can be converted to the extracellular enzyme by proteolytic cleavage (Yamamoto & Lampen, 1976). That prepenicillinase made *in vitro* was larger than the membrane-bound form (Sarvas *et al.*, 1978), and that two related extracellular penicillinases, exo-large and exo-small, were present in a culture supernatant (Izui *et al.*, 1980), is evidence of host-specific processing. The processing occurs by modification and cleavage of amino acid residues at the NH₂-terminal sequence of the penicillinase (Simons *et al.*, 1978; Smith *et al.*, 1981).

The purpose of this paper is to demonstrate that peptide products from the same structural gene are processed differently by host-specific peptidases.

METHODS

Materials. Tetracycline, chloramphenicol and standard dansyl amino acids (arginine, glutamic acid, glycine and serine) were purchased from Sigma. Kanamycin and benzylpenicillin were from Meiji Seika Co., Tokyo, Japan. Restriction endonucleases (*Eco*RI, *Pst*I, *Bgl*II, *Hinf*I, *Hae*III), T4 DNA ligase and T4 polynucleotide kinase were from Takara Shuzo Co., Kyoto, Japan. Yeast extract, Bacto-tryptone and Casamino acids were from Difco. Ampholine (pH 4–6; 3–10) was from LKB, Sweden. Protein molecular weight standards were from Boehringer Mannheim. Low melting point agarose was from Bethesda Research Laboratories, Maryland, U.S.A. Cellulose phosphate P1 was from Whatman. Carboxymethylcellulose was from Serva Feinbiochemica, Heidelberg, Germany. Sephadex G-100 was from Pharmacia. Polyamide layer sheet was from Seikagaku Kogyo Co., Tokyo, Japan. All other reagents were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Table 1. *Bacterial strains and plasmids*

Bacteria	Properties*
<i>Bacillus licheniformis</i> ATCC 9945A M015-1	<i>pepA1 penI10 penP81 penP83</i> Sm ^r Ile ⁻ Rec ⁻
<i>Bacillus subtilis</i> M112	<i>leuA8 arg-15 thr-5 recE4</i> r _M ⁻ m _M ⁻
<i>Escherichia coli</i> C600-1	<i>leu-6 thr-1 thi-1 supE44 lacY1 tonA21 hsdR hsdM</i> Trp ⁻
Plasmids	
pTTB32	Km ^r <i>penP</i> ⁺ <i>penI10</i>
pTTE11	Tc ^r <i>penP</i> ⁺ <i>penI10</i>

* See Imanaka *et al.* (1981) for details. Sm, Streptomycin; Km, Kanamycin; Tc, Tetracycline.

Bacterial strains and plasmids. Strains and plasmids used in this work are listed in Table 1. Both *B. licheniformis* ATCC 9945A M015-1 and *B. subtilis* M112 are recombination deficient and do not produce any detectable penicillinase. Penicillinase genes from a constitutive strain of *B. licheniformis* ATCC 9945A have been cloned in *Escherichia coli* with pMB9 (Tc^r) as vector plasmid, and the cloned genes were transferred into *Bacillus* strains with pTB53 (Km^r Tc^r). These recombinant plasmids were designated as pTTE11 and pTTB32, respectively (Imanaka *et al.*, 1981). *penP* and *penI* are, respectively, the structural gene and the repressor gene for penicillinase, and are coded on the same *Eco*R1 fragment (2.8 MDal, Imanaka *et al.*, 1981).

Media. L broth contained 10 g Bacto-tryptone, 5 g yeast extract, and 5 g NaCl in 1 litre of deionized water; it was adjusted to pH 7.0 with NaOH and solidified with 15 g agar per litre. Kanamycin (5 µg ml⁻¹) and tetracycline (20 µg ml⁻¹) were added to L broth to ensure plasmid stability for *Bacillus* strains carrying pTTB32 and *E. coli* carrying pTTE11, respectively. Modified M9 medium contained 6 g Na₂HPO₄, 3 g KH₂PO₄, 5 g NaCl, 1 g NH₄Cl, 20 g Casamino acids, 0.2 g glucose, 0.1 g MgSO₄·7H₂O, 15 mg CaCl₂·2H₂O and 10 mg thiamin in 1 litre of deionized water (Imanaka *et al.*, 1980). CH/S medium (Pollock, 1965) contained Casamino acids (10 g l⁻¹), KH₂PO₄ (2.8 g l⁻¹) and salt solution.

Penicillinase assay. Penicillinase was assayed by the iodometric method as described previously (Imanaka *et al.*, 1981). One unit of penicillinase was defined as the quantity required to hydrolyse 1 µmol benzylpenicillin per h at 30 °C.

Protein assay. Protein concentration was measured by the Lowry method using bovine serum albumin as standard.

Preparation of extracellular penicillinase. The procedure described by Yamamoto & Lampen (1976) was used to purify extracellular penicillinase, except that the culture broth was directly adjusted to pH 5.0 without alkaline (pH 9.0) treatment. Host strains (*B. licheniformis* and *B. subtilis*) carrying pTTB32 were cultivated to the end of the exponential growth phase in CH/S medium (5 l) in a 10-l fermenter, and the cells were removed by centrifugation (10000 g, 10 min). The supernatant liquid was adjusted to pH 5.0 with acetic acid.

The following purification procedures were carried out at about 4 °C. Phosphocellulose (Whatman P1, H⁺ form) was added to the supernatant (final concentration, 10 g l⁻¹) and the suspension was gently stirred overnight. The phosphocellulose was collected and washed twice with 0.01 M-KH₂PO₄/NaOH (pH 5.0), and the adsorbed enzyme was eluted with 0.2 M-KH₂PO₄/NaOH (pH 7.6). The enzyme solution was adjusted to pH 4.8 with acetic acid, and was dialysed overnight against 0.01 M-ammonium acetate buffer (pH 4.8). The enzyme solution was applied to a carboxymethylcellulose column (2.5 × 20 cm) equilibrated against 0.01 M-ammonium acetate buffer (pH 4.8) and the column was washed with three volumes of the same buffer. The column was eluted with a 1-litre linear gradient of 0.01 M, pH 4.8 to 0.3 M, pH 8.5, ammonium acetate buffer. Enzyme fractions were pooled in a dialysis tube and dehydrated in polyethylene glycol solution. The concentrated solution was applied to a Sephadex G-100 column (2.5 × 100 cm) which had been equilibrated with 0.01 M-ammonium acetate buffer, pH 8.0, and eluted with the same buffer.

SDS-PAGE. To determine the molecular weight of the enzyme, the continuous SDS system of Weber & Osborn (1969) was used. The gel was 7.5% (w/v) polyacrylamide. The discontinuous SDS system of Laemmli (1970) was also used, for which the gel was 12.5% polyacrylamide. Gels were fixed in 25% (w/v) TCA, stained in 0.5% Coomassie Brilliant Blue R for 40 min, and destained. The protein standards included *E. coli* RNA polymerase β'-subunit (mol. wt 165000) and β-subunit (155000), bovine serum albumin (68000), RNA polymerase α-subunit (39000), trypsin inhibitor (21500) and cytochrome *c* (12500).

Isoelectric focusing. Sucrose density gradient (0–50%) electrofocusing of penicillinase was performed by using a 110 ml vertical column. Ampholine (pH 4–6/pH 3–10; 4:1) was added [final concentration 1% (w/v)]. The anode strip was saturated with 1 M-H₃PO₄, and the cathode strip was saturated with 1 M-NaOH. The protein was focused at a constant voltage, 600 V for 48 h at 0 °C.

Determination of the NH₂-terminal amino acid residue. The NH₂-terminal amino acid residue of penicillinase was determined by using dansyl chloride (1-dimethylaminonaphthalene-5-sulphonyl chloride) (Gray, 1972). Dansyl derivatives of the NH₂-terminal amino acid were spotted on one side of a polyamide sheet. A mixture of the sample and standard dansyl amino acids (dansyl-Arg, -Glu, -Gly, -Ser) was spotted on the reverse side, followed by two-dimensional TLC. The first solvent was 1.5% (v/v) formic acid, and the second was benzene/acetic acid (9:1, v/v). The third separation was done with ethyl acetate/methanol/acetic acid (20:1:1, by vol.).

DNA sequencing. DNA sequencing was performed as described by Maxam & Gilbert (1977). The DNA fragment sequenced was isolated from the 2.8 MDal *Eco*RI fragment of recombinant plasmid pTTE11.

Other procedures. Preparation of plasmid pTTE11 from *E. coli*, digestion of plasmid DNA with restriction endonucleases, and agarose gel electrophoresis were carried out as described previously (Imanaka *et al.*, 1980, 1981, 1982).

RESULTS

Purification of extracellular penicillinase

A typical enzyme purification from the culture supernatant of *B. licheniformis* ATCC 9945A M015-1 carrying plasmid pTTB32 is summarized in Table 2. More than 90% of the total penicillinase activity in the culture supernatant was adsorbed by the phosphocellulose. Only a single peak of penicillinase activity was detected during the carboxymethylcellulose chromatography and after gel filtration on Sephadex G-100 (data not shown). The final recovery of purified penicillinase was 58%. The specific activity of the purified enzyme, 350 units ($\mu\text{g protein}^{-1}$), is similar to that previously reported for *B. licheniformis* 749/C [330 units ($\mu\text{g protein}^{-1}$), Pollock (1965); 355 units ($\mu\text{g protein}^{-1}$), Yamamoto & Lampen (1976); 330–350 units ($\mu\text{g protein}^{-1}$), Simons *et al.* (1978)].

The purification of the extracellular penicillinase produced by *B. subtilis* MI112(pTTB32) is also summarized in Table 2. The increase in specific activity during the purification was similar to that for the enzyme from *B. licheniformis* and the final specific activity of the purified penicillinase was virtually identical at 346 units ($\mu\text{g protein}^{-1}$).

Determination of molecular weight

Purified extracellular penicillinases produced by both *B. licheniformis* and *B. subtilis* were subjected to SDS-polyacrylamide gel electrophoresis for the determination of molecular weight. The enzyme from each strain exhibited two protein bands at the same positions (Fig. 1, lanes B and C). The molecular weights for the two bands corresponded to 30 500 and 29 500, respectively. Accordingly, the two proteins were considered to be the related extracellular penicillinases, *exo*-large and *exo*-small (Izui *et al.*, 1980).

Table 2. Summary of purification of penicillinase

Source	Fraction	$10^{-6} \times$ Total enzyme activity (units)	Total protein (mg)	Specific activity [units ($\mu\text{g protein}^{-1}$)]	Yield (%)
<i>B. licheniformis</i> ATCC 9945A M015-1(pTTB32)	Supernatant	14.4			100
	Phosphocellulose treatment	13.5	171	79	94
	Carboxymethylcellulose chromatography	12.6	43	290	88
	Sephadex G-100 gel filtration	8.4	24	350	58
<i>B. subtilis</i> MI112(pTTB32)	Supernatant	6.31			100
	Phosphocellulose treatment	6.15	70	88	97
	Carboxymethylcellulose chromatography	4.33	15	297	69
	Sephadex G-100 gel filtration	3.41	10	346	54

Fig. 1. SDS-PAGE of purified extracellular penicillinases. A, protein standards; B, penicillinase from *B. licheniformis* M015-1(pTTB32); C, penicillinase from *B. subtilis* MI112(pTTB32). Standards: a, β' -subunit and β -subunit of RNA polymerase from *E. coli* (mol. wts 165000 and 155000, respectively); b, bovine serum albumin (68000); c, RNA polymerase α -subunit (39000); d, trypsin inhibitor (21500); e, cytochrome c (12500).

Fig. 2. Isoelectric focusing of purified penicillinase. (a) *B. licheniformis* M015-1(pTTB32), 2 ml per fraction; (b) *B. subtilis* MI112(pTTB32), 1 ml per fraction. O, penicillinase activity.

Isoelectric focusing of extracellular penicillinases

To separate the exo-large and exo-small penicillinases and determine their isoelectric points, isoelectric focusing of penicillinase was performed. Extracellular penicillinase from *B. licheniformis* showed two peaks of enzyme activity (Fig. 2a). It was confirmed by SDS-PAGE that the major peak (fraction 18, pH 5.00) corresponded to an exo-large penicillinase, while the minor peak (fraction 20, pH 5.13) to an exo-small type (Fig. 2a).

Figure 2(b) shows isoelectric focusing of extracellular penicillinase from *B. subtilis*. A single peak of enzyme activity is apparent. It was also confirmed from SDS-PAGE that the peak (pH 5.00–5.04) was composed of both exo-large and exo-small penicillinases (Fig. 2b).

These results suggest that the penicillinases secreted by *B. licheniformis* and *B. subtilis* differed subtly and the difference was beyond the scope of detection by the SDS-PAGE (Fig. 1). Hence, a partial nucleotide sequence was determined to obtain information on the amino acid sequence at the NH_2 -terminus of the *penP* gene product.

Nucleotide sequence of NH_2 -terminal region of *penP*

Plasmid pTTE11 was extracted from *E. coli* and digested with restriction endonuclease *EcoRI*. The 2.8 MDal (4300 bp) *EcoRI* fragment was separated by electrophoresis on a low melting point agarose gel (1%). The region of the gel containing the 2.8 MDal fragment was isolated, and the gel was melted at 65 °C and extracted three times with phenol saturated with 0.1 M-Tris/HCl (pH 8.0). To remove phenol, the water layer was treated with ether, and DNA was precipitated with ethanol. Since this fragment carrying *penP* from *B. licheniformis*

Fig. 3. Restriction map of the penicillinase gene region for *B. licheniformis* ATCC 9945A, a strategy for DNA sequencing, and amino acid sequence of the NH₂-terminal portion of penicillinase. The structural gene of penicillinase is indicated as a box, the open section corresponding to the signal peptide region, and the filled section to the extracellular penicillinase region. The stretches of DNA sequenced are indicated by full arrows, and dotted lines show non-sequenced regions. Vertical arrows at L, S3, S2 and S1 indicate proteolytic cleavage sites.

ATCC 9945A was quite similar to that from *B. licheniformis* 749/C with respect to the molecular size and restriction map, the nucleotide sequence of *penP* from strain 749/C (Neugebauer *et al.*, 1981) was considered relevant. The DNA sequencing strategy for the NH₂-terminal region of penicillinase from *B. licheniformis* ATCC 9945A is summarized in Fig. 3. The *HinfI*-digested fragment (580 bp) containing the desired region was isolated by PAGE and further analysed.

The nucleotide sequence of this 580 bp fragment was completely identical with that from strain 749/C (Neugebauer *et al.*, 1981). It has also been reported that penicillinase produced by *B. licheniformis* 9945A was enzymically and immunologically indistinguishable from that produced by strain 749 (Sherratt & Collins, 1973; Imanaka *et al.*, 1981). Accordingly, it might be inferred that the NH₂-terminal amino acid sequence and their processing were the same for extracellular penicillinases from strains ATCC 9945A and 749/C of *B. licheniformis*. The NH₂-terminal amino acid sequence derived from the nucleotide sequence for *B. licheniformis* ATCC 9945A is shown in Fig. 3; it is identical to the sequence already published by Neugebauer *et al.* (1981) for *B. licheniformis* 749/C.

Determination of the NH₂-terminal amino acid

Extracellular penicillinases from *B. licheniformis* and *B. subtilis* were analysed for their NH₂-terminal amino acids. Figure 4(a) shows a representation of the chromatogram obtained for penicillinase from *B. licheniformis*. By reference to the chromatograms presented by Gray (1972) the unknown spot on the left-hand chromatogram was judged to be that for lysine. Hence the NH₂-terminal amino acids for the penicillinase from *B. licheniformis* were serine and lysine.

The NH₂-terminal amino acids for the penicillinase from *B. subtilis* are represented in Fig. 4(b). The unknown spot on the left-hand chromatogram was identified as that for aspartic acid. Thus the three spots on the right-hand side correspond to serine, glutamic acid and aspartic acid. However, since in this procedure NH₂-terminal glutamine or asparagine are converted to dansyl-glutamic acid and dansyl-aspartic acid, respectively, the amides cannot be distinguished from the unsubstituted acids in these experiments. However, from inspection of the previous

Fig. 4. TLC of dansyl amino acids on polyamide sheets. Penicillinases from *B. licheniformis* M015-1 (pTTB32) (a) and *B. subtilis* MI112(pTTB32) (b) were analysed. Left-hand side for each: the chromatogram for a mixture of the standards and a sample of penicillinase. Right-hand side for each: chromatogram of the penicillinase sample only. The crosses on the sketches represent the origins. The filled areas correspond to standard dansyl amino acids; the open areas are the derivatives of dansyl chloride such as dansylamide (DNS-NH₂) and dansyl acid (DNS-OH) (Gray, 1972). Numbers 1, 2 and 3 with arrows indicate the directions of first, second and third separations, respectively.

data on the molecular weight of the penicillinase (30 500 for exo-large and 29 500 for exo-small), the isoelectric point (5.00–5.04) and the amino acid sequence (Fig. 3), the most probable NH₂-terminal amino acids for exo-small penicillinase from *B. subtilis* were taken as glutamic acid and asparagine (see Discussion).

DISCUSSION

Some properties of extracellular penicillinases produced by strains 749/C and ATCC 9945A of *B. licheniformis* and by *B. subtilis* are summarized in Table 3. Based on the similarity of

Table 3. *Properties of extracellular penicillinase*

Strain	Molecular weight	Isoelectric point	NH ₂ -terminal residue
<i>B. licheniformis</i> 749/C	30 500*	4.95–5.05†	Ser‡
	29 500*		Lys‡
<i>B. licheniformis</i> ATCC 9945A	30 500	~5.00	Ser
M015-1(pTTB32)	29 500	~5.13	Lys
<i>B. subtilis</i> MI112(pTTB32)	30 500	5.00–5.04	Ser
	29 500		Glu, Asn

* Izui *et al.* (1980). † Crane & Lampen (1974). ‡ Nielsen & Lampen (1982).

proteins from *B. licheniformis* 749/C and ATCC 9945A, the cleavage sites of exo-large and exo-small penicillinases for strain ATCC 9945A were designated as L and S1 in Fig. 3. It is evident that the exo-large penicillinase contains a more negatively-charged sequence than the exo-small enzyme. Consequently, the experimental fact that the isoelectric point for the exo-large enzyme (around 5.00) was lower than that (5.10–5.13) for the exo-small enzyme (Table 3) could be explained by the slight difference in the negative charge between the exo-large and exo-small enzymes.

On the other hand, the cleavage sites of exo-large and exo-small enzymes from *B. subtilis* were most likely to be L, and S2 or S3, respectively (Fig. 3). On this basis, the experimental findings of nearly the same isoelectric points for these enzymes and their difference of molecular weight were understandable, because one positively-charged and one negatively-charged amino acid were simultaneously deleted from the exo-large enzyme, resulting in no charge variation between the exo-large and exo-small penicillinases.

Aiyappa *et al.* (1977) reported that membrane penicillinase of *B. licheniformis* 749/C was converted to extracellular enzyme by a penicillinase-releasing protease. Furthermore, Izui *et al.* (1980) have shown that exo-large penicillinase of the same strain was converted to exo-small form by proteolytic cleavage. The different cleavage of extracellular penicillinase produced by *B. subtilis* and *B. licheniformis* ATCC 9945A might have been due to the difference in host-specific proteases. Indeed, when the culture broth of *B. licheniformis* ATCC 9945A was kept at pH 9.0 for 30 min prior to the enzyme preparation, a new exo-small enzyme (mol. wt 28000) appeared. In contrast, such a new enzyme did not appear for *B. subtilis* after the same alkaline treatment (data not shown). These results support the above-mentioned proposal.

Our results emphasize that it is important that any peptide product should be carefully examined when it is produced by different host strains, even if the product is expressed from the same structural gene.

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