Cloning and Characterization of the Pectate Lyase III Gene of *Erwinia carotovora* Er

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A pectate lyase gene III (*pel* III) of *Erwinia carotovora* Er was cloned. The gene was expressed independently of a vector promoter in both *E. carotovora* Er and *Escherichia coli*. The *pel* III product was largely excreted in the culture medium of *E. carotovora* Er, while the product was only exported to the periplasmic space and was not excreted in the medium of *E. coli*. Nucleotide sequence analysis of *pel* III disclosed an open reading frame of 1,122 bp encoding a protein of 374 amino acids. The deduced amino acid sequence contained the N-terminal 30 amino acid sequence from the purified pectate lyase III (PL III) indicating the presence of a 22-amino-acid signal peptide. A putative ribosome-binding site was found to be 9 bp upstream of the start codon. The location of *pel* III was about 5.6 kb downstream of *pel* I. The PL III showed 80% homology in the amino acid sequence with the PL I of *E. carotovora* Er.⁹⁾

Erwinia carotovora and *Erwinia chrysanthemi* are phytopathogenic bacteria that cause soft-rot diseases in many plant species.²⁾ Their pathogenicity is due to their ability to produce several extracellular enzymes including pectinases, cellulases and proteases.³⁾ These extracellular enzymes attack plant cell walls and membrane components and lead to plant tissue maceration. Among pectinases, pectate lyase (PL) has been extensively studied and seems to be one of the most important determinants of the pathogenicity.⁴⁾

E. carotovora produces two to four isozymes of PL,⁵⁾ while *E. chrysanthemi* produces at least five PLs.⁶⁾ To study the regulation of PL production and the role of PL isozymes in pathogenicity, several laboratories have isolated genes of PL (*pels*).^{8,18,24,29-34)} In soft-rot *Erwinia*, the production of PLs is induced by polygalacturonate and its metabolites, and repressed by glucose.

Previous work in our laboratory indicated

that *E. carotovora* Er secreted at least three different PLs (PL I, PL II, and PL III). PL I and PL II were purified and characterized by Sugiura *et al.*,⁷⁾ and *pel* I was cloned and sequenced.^{8,9)} To identify the whole structures of the *pel* genes, we tried to clone other *pel* genes using a part of *pel* I as a probe. In this report, we describe the cloning and the characterization of *pel* III.

Materials and Methods

Materials. Restriction endonucleases, sequencing reagents, and other DNA modifying enzymes were purchased from Takara Shuzo Co., Ltd., Kyoto and Toyobo Co., Ltd., Osaka. All ³²P-labeled nucleotides were obtained from Amersham Co., Ltd., Buckinghamshire, U.K.

Bacterial strains, plasmids, and phage. The bacterial strains, plasmids, and phage used in this study are listed in Table I. *E. coli* HB101 was used for the construction of the genomic library. *E. coli* JM109 was used as the host of recombinant plasmids. *E. coli* MV1184 was used for the preparation of single-stranded template DNA for sequen-

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Table I. BACTERIAL STRAINS, PLASMIDS, AND PHAGE

Strain and plasmid	Genotype or description	Source or reference
<i>Escherichia coli</i>		
HB101	F ⁻ , <i>hsdS20, recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44, leuB6, thi-1</i>	14
JM109	<i>recA1, Δ (lac-proAB), endA1, gyr A96, thi-1, hsdR17, relA1, supE44, [F' traD36, proAB, lacI^q ΔM15]</i>	10
MV1184	<i>ara⁻, thi⁻, strA, Δ (srl-recA) 306::Tn10, (φ80, lacZΔM15), [F' traD36, proAB, lacI^q ZΔM15]</i>	11
<i>Erwinia carotovora</i>		
Er (IAM1068)	wild type	1
Plasmids		
pUC118 and pUC119	Cloning plasmid	11
pBR329	Cloning plasmid	12
pNN3	4.1 kb <i>Hind</i> III- <i>Pst</i> I fragment containing <i>pel</i> III, cloned in pUC119	This study
pNN3B	4.1 kb <i>Hind</i> III- <i>Bam</i> HI fragment of pNN3 cloned into the same site of pBR329	This study
pNN1	8.7 kb <i>Eco</i> RI fragment containing <i>Pel</i> I and <i>pel</i> X, cloned in pBR329	8
pNN101	5.1 kb <i>Eco</i> RI- <i>Hind</i> III fragment of pNN1 cloned into the same site of pBR329	8
pNN11	1.9 kb <i>Hind</i> III- <i>Pst</i> I fragment of pNN1 cloned into the same site of pUC119	This study
Phage		
M13 K07	M13 helper phage	11

cing.

Media and growth conditions. *E. carotovora* Er was usually grown aerobically in LB medium¹⁴⁾ at 30°C. *E. coli* strains were grown aerobically in LB medium or 2 × YT medium¹³⁾ at 37°C. If required, 50 µg/ml of ampicillin and 70 µg/ml of kanamycin were added to the medium.

Southern and colony hybridization. Chromosomal DNA from *E. carotovora* Er was prepared by the method of Rodriguez *et al.*¹⁵⁾ After digestion of DNA with several restriction endonucleases, the fragments were resolved in an agarose gel and transferred to a nylon membrane for Southern blot analysis.

E. coli colonies were grown on nylon membranes, and filters were treated by the instruction of the maker (Amersham Co., Ltd.).

As hybridization probes, two kinds of DNA fragments were used. One was the 0.4-kb *Pst* I-*Hpa* II fragment of pNN1 (probe I) and the other was the 0.3-kb *Pst* I-*Eco* RI fragment of pNN1 (probe II) (Fig. 1A). These probes were labeled with [γ -³²P]ATP using T4 polynucleotide kinase after dephosphorylation. Hybridization was done at 65°C with washing according to the manufacturer's standard conditions (Amersham Co., Ltd.).

DNA sequencing. DNA was sequenced by the dideoxy chain termination method of Sanger *et al.*¹⁶⁾ DNA

fragments from pNN3 were subcloned in pUC118 or pUC119, and the single-stranded template DNA was prepared by infection of *E. coli* MV1184 with the M13 K07 helper phage. Sequencing reactions were done according to the protocols supplied with the M13 Sequencing Kit or 7-DEAZA Sequencing Kit (Takara Shuzo Co., Ltd.).

Construction of pNN3. The 1.9-kb *Hind* III-*Pst* I fragment obtained by digestion of pNN1 was cloned into the *Hind* III and *Pst* I sites of pUC119, and the resulting plasmid was designated pNN11. On the other hand, *E. carotovora* Er chromosomal DNA was completely digested with *Pst* I and the DNA was size-fractionated by agarose gel electrophoresis. DNA fragments of 2.0–2.5 kb were ligated to the *Pst* I site of pNN11, and the resulting plasmids were used to transform *E. coli* HB101. Ampicillin-resistant colonies were screened by both colony hybridization with probe II and plate assay for PL production.

Measurements of enzyme activities. PL activity in fluid was measured by monitoring the increase of optical density at 235 nm as described previously.¹⁷⁾

A plate assay to screen the *E. coli* colonies for PL production was done on YC agar plates containing 0.7% sodium polygalacturonate and 50 µg/ml ampicillin.^{18,19)} The clones were grown on the plate and lysed by exposing the colonies to chloroform vapor. After leaving the plates

Fig. 1. (A) Restriction Map of the Cloned *pel* I-*pel* III Region.

Thin lines represent the cloned fragments in different pBR329 or pUC119 derivatives. Thick lines indicate the fragments of probe I and probe II, respectively.

(B) Sequencing Strategy for *pel* III.

Arrows mark the extent and the direction of nucleotide sequence runs. Two BAL31 nuclease-deleted clones are marked with asterisks.

at 30°C for more than 2 hr, positive clones were detected by flooding the plates with 1 M CaCl_2 .

Activities of β -lactamase and β -galactosidase were assayed by the methods of Sawai *et al.*²⁰⁾ and Miller,²¹⁾ respectively.

Fractionation of extracellular, periplasmic, and cytoplasmic PLs in *E. coli* cells. Fractionation of PL was done by the method of Cornelis *et al.*²²⁾ *E. coli* cells were grown in LB medium, harvested at the early stationary phase, and washed twice with 10 mM Tris-HCl buffer (pH 7.5) containing 25% sucrose. The washed cells were suspended in the same buffer containing 25% sucrose and 1 mM EDTA, and the suspension was gently shaken for 10 min at room temperature. After centrifugation at $3,000 \times g$ for 10 min, the cells were quickly and vigorously suspended in ice-cold water. The suspension was further shaken for 10 min at 4°C and centrifuged at $8,000 \times g$ for 10 min. The supernatant contained the periplasmic enzymes of the cells. The precipitated cells suspended in 10 mM Tris-HCl buffer (pH 7.5) and disrupted with a French pressure cell ($500\text{--}600 \text{ kg/cm}^2$). The cytoplasmic fraction was obtained from the disrupted cell fraction by centrifugation at $22,000 \times g$ for 10 min.

Results

Cloning of *pel* III

Previous work from our laboratory indicated that *E. carotovora* Er excreted a large amount of PL I and PL II in the culture medium, while only a small amount of PL III was produced. PL I and PL II were purified by Sugiura *et al.*,⁷⁾ but PL III has not been purified because of its small quantity. The N-terminal 10 amino acids of PL I and PL II were identical.⁷⁾ To search for *pel* II and *pel* III, Southern blot analysis of pNN1 using probe I, which encoded the N-terminal region of PL I was done. As shown in Fig. 2A, two fragments were detected in *Eco*RI and *Hind*III digests of pNN1 (lane 2). It was expected that the 2.2-kb *Hind*III-*Eco*RI fragment might contain *pel* II or *pel* III, because the 5.1-kb *Eco*RI-*Hind*III fragment included *pel* I (Fig. 1A). Furthermore, the 0.3-kb *Pst*I-*Eco*RI fragment in the 2.2-kb

Fig. 2. Autoradiograms of Southern Blot Analysis.

(A) Detection of the DNA region homologous to *pelI* with probe I. Lanes: 1, *Ban*II plus *Pst*I digests of the 2.2-kb *Hind*III-*Eco*RI fragment in pNN1; 2, *Eco*RI plus *Hind*III digests of pNN1; 3, *Eco*RI, *Hind*III plus *Pst*I digests of pNN101; 4, *Eco*RI digests of pNN1. Approximate sizes in kb are indicated on the right. (B) Detection of the DNA fragment containing the 0.3-kb *Pst*I-*Eco*RI region with probe II. Chromosomal DNA was digested with following restriction endonucleases. Lanes: 1, *Eco*RI; 2, *Pst*I; 3, *Hind*III; 4, *Eco*RI plus *Hind*III. Approximate sizes in kb are indicated on the right.

*Hind*III-*Eco*RI region strongly hybridized with the probe (lane 1). An analysis of the nucleotide sequence of the 0.3-kb *Pst*I-*Eco*RI fragment found an open reading frame of 303 bp encoding 101 amino acid residues. Its amino acid sequence had a high homology with that of PL I. And the N-terminal 10 amino acids were also detected in the 0.3-kb *Pst*I-*Eco*RI fragment. Since this region was a part of the gene due to *Eco*RI cutting (Fig. 1A), Southern blot analysis for the chromosomal DNA was done using probe II to obtain recombinants covering the whole gene. Probe II hybridized to both a 6.7-kb *Pst*I-*Pst*I fragment and a 2.2-kb *Pst*I-*Pst*I fragment (Fig. 2B). From the restriction map of 8.7-kb *Eco*RI-*Eco*RI fragment shown in Fig. 1A, the 2.2-kb *Pst*I-*Pst*I fragment was concluded to contain the 0.3-kb *Pst*I-*Eco*RI region.

To construct the *pel* gene, *E. carotovora* Er chromosomal DNA was digested with *Pst*I completely and DNA fragments of 2.0–2.5 kb were ligated to the *Pst*I site of pNN11 as described in Materials and Methods.

Among 1,200 clones tested, 38 clones were found to hybridize with probe II, and 17 clones among the 38 positive clones showed PL activities by the plate assay. Some of these 17 clones had an identical recombinant plasmid containing a 4.1-kb segment of chromosomal DNA and it was designated pNN3. A restriction map of pNN3 is shown in Fig. 1A.

Identification of PL isozyme cloned in pNN3

E. carotovora Er was transformed with pNN3 by the method of Reverchon.²³⁾ Extracellular PLs produced by *E. carotovora* Er were separated and characterized by hydroxyapatite column chromatography as described previously.⁹⁾ Elution profiles of PL activities in culture fluids of *E. carotovora* Er strains harboring pNN3 and without pNN3 in hydroxyapatite column chromatography are shown in Fig. 3. *E. carotovora* Er harboring pNN3 raised the level of PL III activity about 50-fold in comparison with the host strain, but it didn't affect levels of PL I and PL II activities. From these results, the *pel* gene encoded in

pNN3 was *pel* III alone. When the cell extract of *E. coli* HB101 harboring pNN3 was analyzed by the same method, the elution point of the PL activity was approximately the same as PL III in the concentration of potassium phosphate buffer (data not shown).

Expression of *pel* III and cellular localization of PL III in *E. coli*

A 2.2-kb *Dra*I–*Dra*I fragment containing the 203 bp sequence upstream of the Shine-Dalgarno sequence and the *pel* III structural gene was cloned into the *Sma*I site of pUC119.

Fig. 3. Elution Profiles of Pectate Lyases on Hydroxyapatite Column Chromatography.

The activities of *E. carotovora* Er (○) and *E. carotovora* Er harboring pNN3 (●) were analyzed.

Two recombinant plasmids carrying the insert in the same or the opposite direction to the *lac* promoter were obtained. Both of the *E. coli* HB101 transformants carrying the these two kinds of plasmids had PL III activity. These results indicate that the 203-bp sequence upstream of the Shine-Dalgarno sequence contains a promoter which is recognized by *E. coli* (data not shown).

In our preliminary experiments, the PL activity was detected in the culture fluid at the stationary phase of *E. coli* HB101 harboring pNN3, and at the same time, a large amount of β -lactamase activity was also found. These phenomena seemed to come from the result of overproduction of these enzymes according to the high copy number of the vector pUC119. Then a 4.1-kb *Hind*III–*Bam*HI fragment of pNN3 was ligated to *Hind*III and *Bam*HI sites of pBR329, and this plasmid was designated pNN3B. Unlike *E. coli* HB101 harboring pNN3, little β -lactamase activity was found in the culture fluid of *E. coli* HB101 harboring pNN3B throughout its growth phase (data not shown).

E. coli HB101 harboring pNN101, pNN3B, or pBR329 was grown until the early stationary phase. The cells were harvested by centrifugation and washed, and the cellular fractions were prepared as described in Materials and Methods. β -Lactamase (a periplasmic marker

Table II. LOCALIZATION OF PECTATE LYASE AND OTHER ENZYMES IN *E. coli* HB101 CARRYING pNN101, pNN3B OR pBR329

Enzymes	Plasmid	Extracellular activity (U)	Periplasmic activity (U)	Cytoplasmic activity (U)
Pectate lyase	pNN101	0.0 (0.0) ^a	0.0 (0.0)	185.3 (100.0)
	pNN3B	0.0 (0.0)	730.0 (31.5)	1584.4 (68.5)
	pBR329	0.0	0.0	0.0
β -Lactamase	pNN101	87.0 (8.1)	911.5 (84.9)	75.4 (7.0)
	pNN3B	52.3 (8.0)	551.1 (83.8)	54.2 (8.2)
	pBR329	191.1 (7.9)	2120.4 (87.6)	109.0 (4.5)
β -Galactosidase	pNN 101	0.00 (0.0)	0.072 (3.8)	1.81 (96.2)
	pNN3B	0.00 (0.0)	0.067 (3.5)	1.86 (96.5)
	pBR329	0.00 (0.0)	0.064 (4.5)	1.37 (95.5)

^a Values in parentheses indicate percent distribution of the enzyme in each strain.

enzyme) and β -galactosidase (a cytoplasmic marker enzyme) activities in different cellular compartments were measured (Table II). About 30% of PL III activity was found in the periplasm, while no PL III activity was detected in the culture fluid. On the other hand, most of β -lactamase activity and β -galactosidase activity were found in the periplasm and the cytoplasm, respectively. It appeared, therefore, that the PL III was exported to the periplasm by a specific transport mechanism.

Nucleotide sequence of *pel III*

Restriction endonuclease-digested subclones and BAL31 nuclease-deleted subclones of pNN3 were used for DNA sequencing. The overall sequencing strategy is shown in Fig. 1B. The sequence obtained covered a 1,415-bp region, and an open reading frame was found as shown in Fig. 4. The open reading frame encodes 374 amino acid residues starting from the initiation codon at bases 218 to 220. The sequence AGGAG, 9 bases upstream of the

Fig. 4. Nucleotide Sequence of the *pel III* Region and the Predicted Amino Acid Sequence of the Encoded PL III.

A possible ribosome-binding site is underlined. The signal peptide processing site is indicated by an arrow.

initiation codon, is likely to be a ribosome-binding site. The amino acid sequence from Ala23 to Asp52 matched exactly with the N-terminal 30 amino acid sequence of PL III purified from the culture medium of *E. carotovora* Er harboring pNN3.³⁶⁾ Therefore, it appeared that PL III had a 22-amino-acid signal peptide, which is typical for prokaryotic proteins both in size and the distribution of hydrophobic and hydrophilic amino acid residues. The amino acid sequence from Try289 to Thr296 was also identical to that of a tryptic polypeptide of the purified PL III.³⁶⁾

The calculated molecular weight of the precursor form and the processed form of PL III were 40,480 and 38,253, respectively, and the calculated molecular weight of the processed form corresponded generally to the molecular weight of 39,500 estimated by SDS-PAGE of the purified PL III.³⁶⁾

Discussion

Although more than two PLs of *E. carotovora* are secreted into the culture medium, the regulatory mechanism of each PL production and the roles of individual PLs in pathogenicity are not clear. Recently, to characterize an individual PL, several *pel* genes were cloned.^{8,24,29,34)}

We previously reported the cloning of *pel* I of *E. carotovora* Er and its nucleotide sequence.^{8,9)} Southern blot analysis of pNN1, which contained *pel* I, suggested that another *pel* gene might be about 5.6 kb downstream of *pel* I. As expected, a *pel* gene was found on a 4.1-kb *Hind*III–*Pst*I fragment. This *pel* gene was identified as *pel* III from the analysis of the culture supernatant of *E. carotovora* Er containing the newly cloned *pel* gene by hydroxyapatite column chromatography. The *pel* III was about 5.6 kb downstream of *pel* I, and both *pel* I and *pel* III had individual transcription units in the same direction. We previously found a *pel* X gene 0.6 kb downstream of *pel* I. It was highly homologous to *pel* I, but its function is not clear. Since there is about 2.2 kb between *pel* X and *pel* III,

another *pel* gene might be found in this region. Actually, a 0.9-kb *Hind*III–*Ban*II region was hybridized with probe II in Southern blot analysis (Fig. 2A).

Lei *et al.* cloned three *pels* which are a tandem cluster in the same transcriptional direction in *E. carotovora* EC,²⁴⁾ and sequenced the nucleotides of *pel* A and *pel* B.^{25,26)} The comparison of nucleotide sequences of *pel* III with those of *pel* A or *pel* B suggested that *pel* III might correspond to *pel* C. Recently, Hinton *et al.* also cloned and sequenced *pel* C of *E. carotovora* SCRI193,³⁴⁾ and there was 98.7% homology between PL C and PL III in the amino acid sequence. Moreover, the comparison in the nucleotide sequence between 1812 bp of *pel* C and 1415 bp of *pel* III showed 93% homology, and the homology extended not only to structural genes but also to 5'- and 3'-noncoding regions. We have purified PL III, and we showed that the actual signal peptide cleavage site of PL III is different from that of PL C deduced from the nucleotide sequence. Purification and characterization of PL III are described in the accompanying paper.³⁶⁾

The nucleotide sequence of *pel* III was compared with that of *pel* I, and considerable homology between them was detected. Both PL I and PL III seemed to start from a methionine residue in the same position, but PL III had 8 extra amino acids compared with PL I in the C-terminal portion. The processed PL I and PL III contained 345 and 352 amino acids, respectively. The homology between them was 80%.

pel III was highly expressed in *E. coli* independent of the *lac* promoter, while *pel* I was only poorly expressed (Table II). But cloned *pel* I was expressed in *E. carotovora* Er as well as cloned *pel* III (data not shown). Since several sequences which were very similar to the *E. coli* promoter were found in 5'-noncoding 217 bp region of *pel* III, these sequences might be recognized in *E. coli* (Fig. 4). And it is possible that the true promoter sequence of *E. carotovora* is a little different from that of *E. coli*.

The comparison of the N-terminal amino

acid sequence of purified PL III with the deduced amino acid sequence from the nucleotide sequence of *pel* III suggested that PL III had a 22-amino-acid signal peptide.³⁶⁾ This signal peptide was the same length as that of PL I.⁹⁾ It was reported that the *pel* I product was accumulated in the cytoplasm of *E. coli*.⁹⁾ The *pel* III product was not secreted into the culture fluid, either, but excreted into the periplasmic space. The *pel* I product might be accumulated in the cytoplasmic space according to the low expression of *pel* I in *E. coli*. Andro *et al.* reported that one or more steps of secretion process might exist in *E. chrysanthemi* using the Out⁻ mutant, which was defective in the secretion of pectinases and cellulases.³⁵⁾ *E. carotovora* also seems to have a complex secretion mechanism which is not explained clearly by the signal hypothesis and requires some other factors.

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