Molecular Cloning and Sequencing of the Extracellular Pectate Lyase II Gene from *Erwinia carotovora* Er

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*Erwinia carotovora* Er produces three extra-cellular pectate lyases (PL I, II, and III). The gene for pectate lyase II (pelII) of *E. carotovora* Er was cloned and expressed both in *Escherichia coli* and *E. carotovora* Er. Localization experiments in *E. coli* showed that PL II was exclusively in the cytoplasmic space, while PL II was excreted into the culture medium. The complete nucleotides of the *pelII* gene were sequenced and found to include one open reading frame of 1122 bp coding for a protein of 374 amino acid residues. From comparison of the N-terminal amino acid sequence between the purified PL II and the deduced protein from the nucleotide sequence we reached the conclusion that the mature protein is composed of 352 amino acids with a calculated molecular weight of 38,169 and is preceded by a typical signal sequence of 22 amino acid residues. PL II had 90.1% and 82.9% homologies with PL I and PL III in amino acid sequence, respectively.

*Erwinia carotovora* is a Gram-negative phytopathogenic bacterium, a causative organism of soft-rot disease on potato, carrot, and other vegetables.1) This bacterium produces various extracellular enzymes, for example, pectinases, proteases, and cellulases.2) Plant tissue maceration from the degradation of the middle lamella and primary cell walls by these enzymes is the major symptom of soft-rot disease. Especially, pectate lyase (PL), which is one of the pectinases, seems to be a key enzyme in the pathogenicity to degrade pectic substances of plant cell walls.3) PL randomly cleaves the α-1,4-glycosidic bonds of pectate by a β-elimination reaction to yield a series of oligomers that are 4,5-unsaturated at the nonreducing end.

Ried et al. showed that several strains of *E. carotovora* have three extracellular PL isozymes,4) and we also identified three extracellular PLs of *E. carotovora* Er.5) The production of these PLs is inducible by the metabolites of pectate6) and is subject to catabolite repression.7) To investigate the role of each PL in pathogenicity and the molecular mechanism of each PL production, the pectate lyase gene (*pel*) from *E. carotovora* has been studied extensively by several researchers.8–13) We also reported previously the cloning of *pelI*14) and *pelIII,15)* and the nucleotide sequences of *pelI*15) and *pelIII.5) However, the structures of all three PLs from one strain of *E. carotovora* have not been reported.

In this report, we describes the cloning and nucleotide sequencing of *pelII*, and the comparison of the deduced amino acid sequences of all three PLs of *E. carotovora* Er. The expression of *pelII* in *E. carotovora* Er and the localization of the gene products of *pelII* in *E. coli* are also described.

Materials and Methods

*Bacterial strains and growth conditions. *E. carotovora* Er16) was grown at 30°C in either LB medium17) or M9 medium13) supplemented with 0.2% pectate and 0.2% casamino acids. *E. coli* HB10117) and DH5α13) were used as hosts for plasmids, and *E. coli* MV118418) was used for single-stranded template DNA preparation. *E. coli* was grown at 37°C in LB or 2×YT medium17) supplemented with 50 μg/ml ampicillin and 70 μg/ml kanamycin.

DNA manipulation. Standard methods for analysis of DNA, including restriction digestion, ligation, *E. coli* transformation, and isolation of plasmid DNA, were done essentially as described by Maniatis et al.17) Cells of *E. carotovora* Er were made competent and transformed with plasmid DNA by the procedure of Reverchon.19)

Cloning of *pelII*. Chromosomal DNA was isolated from *E. carotovora* Er by the method of Rodriguez et al.20) and digested completely with the restriction enzyme *Dral*. The DNA fragments from 1.7 to 2.0 kb in size were electroeluted after agarose gel electrophoresis and ligated to Smal-digested pUC11918) to create a partial genomic library. After transformation into *E. coli* DH5α, the resulting partial library was screened by the plate assay for pectate lyase activity as described previously.21

Analysis of nucleotide sequence. The *Dral* insert in plasmid pNN2 containing the *E. carotovora* Er *pelII* gene (identified as described in Results) was cleaved with various restriction enzymes and subcloned into pUC11818) and pUC119 in *E. coli* MV1184. After single stranded template DNA was prepared by the method of Vieira et al.,18) nucleotides were sequenced on both strands by using an automatic DNA sequencer, model 373A Applied Biosystems Inc., U.S.A. The *HpaI–Dral* region containing *pelII* in plasmid pNN10122) was also sequenced. Nucleotide and amino acid sequences were analyzed with the programs of GENETYX (Software Development Co., Ltd., Tokyo, Japan).

Cell fractionation and enzyme assays. Cell fractionation was done as described previously.5) The procedure for the measurement of PL activity has been described previously.21) The reaction mixture contained 0.25% pectate, 25 mm Tris–HCl (pH 9.0), and 0.2 mm CaCl2, β-lactamase and β-galactosidase activities were measured by the methods of Sawai et al.23) and Miller,24 respectively.

Results

Isolation and identification of *pelII* gene from *E. carotovora* Er

Three *Dral* segments of DNA, which were 1.7, 1.9, and 2.2 kb in size were detected by genomic Southern analysis with the probe II5) (data not shown). Since previous results

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suggested that the 1.7-kb and 2.2-kb segments contained pelI and pelIII, respectively.\textsuperscript{115} it appeared that the 1.9-kb segment might retain pelII. DNA fragments from 1.8 to 2.0 kb were isolated to construct a Dral plasmid library in pUC119, as described in Materials and Methods. Three \textit{E. coli} clones among about 1500 recombinants had peclu lyase activity in the assay. Plasmids

Fig. 1. Restriction Map of the pelII Region and Nucleotide Sequencing Strategy.

Fig. 2. Separation of Three Pectate Lyases by Hydroxylapatite Column Chromatography.

Culture fluids of \textit{E. carotovora} Er (---) and \textit{E. carotovora} Er harboring pNN2 (---) were analyzed.

Fig. 3. Nucleotide Sequence of the pelII Gene and Flanking Regions from \textit{E. carotovora} Er.

The deduced amino acid sequence defined by the open reading frame of 374 residues is indicated by single-letter code under the nucleotide sequence. The arrowhead designates the proposed cleavage site of the leader peptide. A putative ribosomal binding site upstream the open reading frame is underlined, and a downstream transcriptional termination signal is indicated by the horizontal arrows.

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1 AAAAGAAAAATGTTTTCTATTATAAATTCACTAAGTATTCCATCACTACATAGATTAAAAAATGTTTCTACCCCTTTAAATTCAATACCTTTACCTTTATCTCCTAATCTTTCCATTTTTGGGTTTTGAAATTGGATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
from these three clones were isolated and shown to be identical from their digestion patterns by various restriction enzymes. This plasmid was designated pNN2, and the restriction map of the 1.9-kb DraI insert is shown in Fig. 1.

The 1.9-kb PstI–EcoRI fragment from pNN2 was cloned into pUC118 to carry the fragment in the opposite direction, and this plasmid was designated pNN28. The pel gene in both pNN2 and pNN28 expressed its own gene in E. coli, indicating that the pel gene in the plasmid had its own promoter recognized in E. coli.

We had shown that three extracellular PLs of *E. carotovora* Er could be separated by hydroxylapatite column chromatography.15,25 To identify the cloned fragment as the pelII gene, elution patterns of extracellular PLs from the columns of *E. carotovora* Er with and without pNN2 were compared (Fig. 2). Cells of *E. carotovora* Er were grown in LB medium to the early stationary phase, and the culture fluid was put on the column after dialysis with 10 mM potassium phosphate buffer (pH 7.2). The PL II activity was eluted at a NaCl concentration of about 170 mM, and only this activity was significantly increased in *E. carotovora* Er harboring pNN2. This result clearly shows that the cloned pel is pelII.

**Analysis of pel II nucleotide sequence**

The nucleotides of the cloned 1853-bp DraI fragment were sequenced on both strands with the sequencing strategy shown in Fig. 1. As shown in Fig. 3, the nucleotide sequence had an open reading frame, which is preceded by a tentative ribosomal binding site. On the basis of the deduced amino acid sequence and the N-terminal 12 amino acid sequence of PL II, it was definitively proved that the 1122-bp tract contained the structural gene of the PL II. The gene of PL II encodes a polypeptide composed of 374 amino acid residues beginning with a 22 amino acid leader peptide that was directly demonstrated from the N-terminal amino acid sequence of purified PL II. A leader peptide region from MetI to Ala22 is further supported by established properties of bacterial leader peptide: a basic residue near the amino terminus (Lys2), an adjacent hydrophobic stretch (Leu4 through Ala17), and an Ala-X at the cleavage site (Ala22-Ala23).24 The mature PL II is shown to be 352 amino acid residues beginning with Ala23, and the calculated molecular weight of 38,169 agreed well with that of 39,000 measured by SDS–PAGE.25 A large inverted repeat following the pelII stop codon could form a hairpin-like structure with a T cluster and may be the RNA-independent transcriptional termination signal. The free energy of this structure is —16.5 kcal/mol according to the base-pairing rules of Tinoco et al.26

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**Fig. 4.** Nucleotide Sequence of the pel I Gene and Flanking Regions from *E. carotovora* Er.

The arrowhead indicates the proposed cleavage site of the leader peptide. The deduced amino acid sequence is shown by single-letter code below the open reading frame. The potential ribosomal binding site is underlined, and an inverted repeat is indicated by the horizontal arrows.
Table I. Distribution of Enzyme Activities in Subcellular Fractions of E. coli HB101

<table>
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<th>Enzymes</th>
<th>Plasmid</th>
<th>Extracellular activity (U)</th>
<th>Periplasmic activity (U)</th>
<th>Cytoplasmic activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectate lyase II</td>
<td>pNN2B</td>
<td>0.0 (0.0)</td>
<td>0.0 (0.0)</td>
<td>419.0 (100.0)</td>
</tr>
<tr>
<td></td>
<td>pBR329</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>β-Lactamase</td>
<td>pNN2B</td>
<td>290.2 (15.7)</td>
<td>1402.0 (75.6)</td>
<td>162.1 (8.7)</td>
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<tr>
<td></td>
<td>pBR329</td>
<td>291.7 (14.6)</td>
<td>1621.2 (81.1)</td>
<td>85.2 (4.3)</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>pNN2B</td>
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<td>0.00 (0.0)</td>
<td>1.48 (100.0)</td>
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<tr>
<td></td>
<td>pBR329</td>
<td>0.00 (0.0)</td>
<td>0.00 (0.0)</td>
<td>1.42 (100.0)</td>
</tr>
</tbody>
</table>

* Values in parentheses indicate percent distribution of the enzyme is each strain.

Alteration of nucleotide sequence of pelI

Nucleotide sequence of the 1.3-kb HpaI–Ddel region containing pelI had been reported by Ito et al. previously. But the comparison of the nucleotide sequence of pelI with that of pelII or pelIII indicated that some frame shifts existed in the nucleotide sequence of pelI. Then, the 1.6-kb HpaI–Ddel region of pelI was sequenced once more and is shown in Fig. 4. The sequence contains a single open reading frame of 1122 bp, beginning with a start codon at positions 125 to 126 and ending with a stop codon at positions 1248 to 1250. The N-terminal 10 amino acid sequence of PL I indicated the presence of a leader peptide of 22 amino acid residues. The presumed cleavage site of the leader peptide was between Ala22 and Ala23, and this was different from the previous result. The molecular weight of mature PL I protein was calculated to be 37,926. Search for secondary structures found an inverted repeat downstream of the open reading frame that would allow the formation of a hairpin-like mRNA structure followed by several T bases with the free energy of -13.0 kcal/mol.

Localization of pelII product in E. coli

PL II is secreted into the culture fluid from E. carotovora Er and the presence of a leader peptide in PL II suggests an extracytoplasmic location for it. To investigate whether the product of pelII is transferred to the extracytoplasmic space in E. coli, a localization experiment was done (Table I). Since the high copy number of the vector pUC119 disturbed the normal distribution of enzymes, the 1.8 kb HindIII–EcoRI fragment from pNN2 was cloned into the same sites of pBR329 and designated pNN2B. Cells of E. coli HB101 were transformed with pNN2B or pBR329 and grown to the early stationary phase. β-Galactosidase activity was observed in the cytoplasm of the cells and most of the β-lactamase activity was detected in the periplasm, while all of the PL II activity was found in the cytoplasm of E. coli cells harboring pNN2B, unlike E. carotovora Er.

Discussion

We have described the cloning and characterization of pelII gene of E. carotovora Er. Isolation of all three pel genes from E. carotovora Er has been done by the cloning of pelII described in this report. Therefore, the comparison of the primary structures of all three PLs became possible. The deduced amino acid sequences of PL I, PL II, and PL III are given in Fig. 5 in single letter code. All three PLs consisted of 374 amino acid residues and the few differences in amino acid residues among the three PLs were scattered throughout their sequences: PL I has homologies of 90.1% and 88.0% with PL II and PL III, respectively, and PL II showed 82.9% homology with PL III. Although each PL had a leader peptide of 22 amino acid residues, the sequence of PL II was slightly different from those of the others. But this difference didn't greatly affect the secretion of PLs by E. carotovora Er.

The nucleotide sequences of the three pel structural genes also showed high homology of more than 80% with each other. This high level of nucleotide identity appeared to be an evidence for a gene duplication sometime in the past, with slight subsequent divergence. Previous studies suggested that pelI is about 5.6 kb upstream of pelIII and the incomplete pel gene designated pelX is between them. And a genomic Southern analysis using a portion of pelIII as a probe showed that no additional region hybridized except pelI, pelIII, and pelX. It was incomprehensible that the pelII region could not be detected by a genomic Southern analysis. Then chromosomal DNA of E. carotovora Er was newly prepared and various genomic Southern analyses were done. The results suggested that pelI, pelII, and pelIII...
are close together and pelX doesn’t exist originally. As shown in this report, pelII was cloned from newly prepared chromosomal DNA and sequenced. The nucleotide sequence at positions from 96 to 839 of pelII shown in Fig. 2 was more than 99% identical to that of pelX reported by Ito et al.21 and the nucleotide sequence at positions from 1248 to 1853 was identical to that of the region upstream from pelIII (data not shown). These results suggest that a mutation happened to occur in the pelII structural gene at positions from 839 to 1248, which was cloned from the chromosomal DNA previously prepared, although the reason for this mutation is not clear. Since it was reported that the PL production of this strain sometimes decreased when the strain was preserved in rich medium for more than two months,28 such mutations may occur spontaneously. Wandersman et al. also reported that the production of extracellular proteases in Erwinia chrysanthemi B374 was very unstable and spontaneous mutation of protease production was observed.29 This decrease of PL activity could be avoided by selecting the colonies on the plate of M9 medium supplemented with 0.2% peptone and 0.2% casamino acids.

The nucleotide sequence analysis suggests that pelI, pelII, and pelIII are in order and form a tandem cluster, and all these genes constitute independent transcriptional units in the same direction. As such, our results are similar to those reported by Lei et al.8 with E. carotovora EC, although minor differences are observed between them.

PL II is secreted from E. carotovora ER and has a typical leader peptide of prokaryotes. The product of pelII, however, was not excreted from E. coli. This phenomenon was nearly the same as the cases of pelI and pelIII.21 These results suggest that a kind of leader peptide of E. carotovora might not function well in E. coli and the pel products could not be translocated and excreted across the inner membranes.

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References