Cloning and Sequencing of pel Gene Responsible for CMCase Activity from *Erwinia chrysanthemi* PY35

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The phytopathogenic bacterium *Erwinia chrysanthemi* secretes multiple isozymes of plant cell wall disrupting enzymes such as pectate lyase and endoglucanase. We cloned genomic DNA from *Erwinia chrysanthemi* PY35. One of the *E. coli* XL1-Blue clones contained a 5.1-kb BamHI fragment and hydrolyzed carboxymethyl cellulose and polygalacturanic acid. By subsequent subcloning, we obtained a 2.9-kb fragment (pPY100) that contained the *pel* gene responsible for CMCase and pectate lyase activities. The *pel* gene had an open reading frame (ORF) of 1,278 bp encoding 425 amino acids with a signal peptide of 25 amino acids. Since the deduced amino acid sequence of this protein was very similar to that of PelL of *E. chrysanthemi* EC16, we concluded that it belonged to the pectate lyase family EC 4.2.2.2, and we designated it PelL1. Sequencing showed that the PelL1 protein contains 400 amino acids and has a calculated pI of 7.15 and a molecular mass of 42,925 Da. The molecular mass of PelL1 protein expressed in *E. coli* XL1-Blue, as analyzed by SDS-PAGE, appeared to be 43 kDa. The optimum pH for its enzymatic activity was 9, and the optimum temperature was about 40°C.

Key words: *Erwinia chrysanthemi*; pectate lyase gene; *pelL1*; CMCase

*Erwinia* is a phytopathogenic enterobacterium that causes soft rot on a variety of important crops. Its pathogenicity is due to its ability to secrete several extracellular enzymes that include pectinases, cellulases, and proteases. These extracellular enzymes attack cell walls and membranes of plants leading to plant tissue maceration. Among the pectinases, pectate lyase (PL) seems to be one of the most important determinants of the bacterium’s pathogenicity.¹,²)

Some *E. chrysanthemi* enzymes such as the pectinases, proteases, and cellulases exist in multiple enzymatic forms.³,⁴) As many as eight *E. chrysanthemi* 3937 endo-Pels have been characterized, PelA, PelB, PelC, PelD, PelE, PelL, and PelZ.³,⁵) The corresponding genes are organized in four clusters on the bacterial chromosome (*pel*A-*pelE-*pelD, *pel*B-*pelC-*pelZ, *pelL*, and *pelL1*). The pectate lyases are classified into five different families based on their amino acid sequences.⁶) This bacterial strategy to synthesize several iso-enzymes may help in the effective degradation of the complex polysaccharides in the cell walls of plants and thus be advantageous to the bacterium. The digestion of cell wall components by pectate lyase and cellulase in co-operation may directly increase the bacterial penetration into the plant tissue and, furthermore, make the degradation of cell wall materials easier for other cell wall degrading enzymes and/or aid in the release of cell wall components that could be used directly to nourish the bacterium.

*E. chrysanthemi* PY35, used in this study, was originally isolated from Chinese cabbage tissue showing soft rot symptoms. To understand the roles played by the pectic and cellulolytic isozymes of *E. chrysanthemi* PY35 in pathogenicity, it is necessary to study each one of the isoforms. In this report we describe the cloning of a pectate lyase (EC 4.2.2.2) gene responsible for CMCase activity in the *E. chrysanthemi* PY35. The properties of the pectate lyase, referred to as *pelL1*, and the complete nucleotide sequence of the corresponding structural gene are presented. The deduced primary structure of the protein is being compared to other pectate lyases.

Materials and Methods

**Bacterial strains and growth conditions.** The *E. chrysanthemi* PY35 was routinely cultured in tryptone-yeast extract medium. *E. coli* XL1-Blue cells and recombinant *E. coli* cells were cultured in LB containing appropriate antibiotics (ampicillin, 50 µg/ml; tetracycline, 10 µg/ml).

**Pectate lyase assay.** For the routine measurements of pectate lyase activity in *E. chrysanthemi* PY35 or *E. coli* XL1-Blue harboring pectate lyase DNA, a

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Abbreviations: CMCase, carboxymethylcellulase; *pel* gene, pectate lyase gene; *cel* gene, cellulase gene; CMCase gene, carboxymethylcellulase gene
plate assay was developed where sodium polypectate was incorporated into the growth medium. For the E. coli XL1-Blue polypectate lyase clones, 0.7% sodium polypectate was used, as previously described by Lim et al. and for Erwinia, 0.25% sodium polypectate was used in TY medium. To make the halos formed due to polypectate lyase activity visible, the plates were flooded with 10% of a saturated solution of copper acetate for 30 min. After excess stain was washed off, a halo against a blue background became visible. Observations were recorded within 30 min.

Purification of polypectate lyase. The polypectate lyase was purified as described by Fierobe et al. The specific activity of the polypectate lyase was measured spectrophotometrically as previously described. The breakdown of polygalacturonate yielding unsaturated uronides of various sizes was monitored by recording the increase in UV absorbance at 235 nm. The optimal pH and temperature for polypectate lyase activity were found using 2 mM of enzyme and 1% (w/v) polygalacturonate in Tris-Cl buffer. Cytoplasmic and periplasmic fractions were obtained as described by Sashihara et al. Protein was measured by the method of Bradford.

Assay of extracellular cellulase activity. To screen for cellulase activity in E. chrysanthemi PY35 or E. coli XL1-Blue harboring cloned cellulase genes, bacterial colonies were grown on LB plates with appropriate antibiotics and 1% carboxymethylcellulose (CMC, low viscosity, Sigma, St. Louis, MO, USA). After an incubation of 3 days, the plates were stained with 0.5% Congo red for 30 min, rinsed with water, and then washed twice with 1 M NaCl. Clones positive for extracellular cellulase activity were surrounded by a yellow halo against a red background.

Chemicals and reagents. Restriction enzymes and DNA modifying enzymes were purchased from Gibco-BRL (Gaithersburg, MD, USA) and Boehringer Mannheim (Indianapolis, IN, USA). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Recombinant DNA techniques. Standard procedures for restriction endonuclease digestion, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning-related techniques were used as described by Sambrook et al.

DNA sequencing. Nucleotide sequencing was done by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CN, USA). The samples were analyzed with an automated DNA sequencer (model 310; Applied Biosystems, Foster City, CA, USA). Assembly of the nucleotide sequences and the amino acid sequence analysis were done with the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada). The DNA sequence reported here has been deposited in the GenBank database under accession number AF171228 (pelL1).

Results and Discussion

Isolation and restriction map of the pelL1 gene
A shotgun method was used to clone the CMCase and polypectate lyase gene cluster. The genomic DNA of E. chrysanthemi PY35 was partially digested with Sau3AI. Three to five-kb fragments were then ligated into the BamHI site of pBluescript II SK+ and the construct introduced into the E. coli XL1-Blue host. The bacteria were then plated on CMCase and polypectate lyase activity indicator medium. From among three thousand transformants, one positive colony surrounded by the yellow and clear ring that indicates CMCase and polypectate lyase activity was isolated. The clone contained a 5.1-kb DNA insert. However, since this plasmid had no suitable enzyme site for subcloning, the 5.1-kb DNA insert was partially digested with Sau3AI and the digests were ligated into the BamHI site of pBluescript II SK+. Transformants were screened on the same indicator medium. One of them contained a 2.9-kb fragment (pPY100) responsible for both polypectate lyase and CMCase activities (Fig. 2A, lane 2; 2B, lane 5). The size of the inserted DNA and the orientation of restriction cleavage sites were identified. The inserted DNA of pPY100 contains single restriction sites for Dral, PstI, and BglI. A 1.6-kb fragment (pPY300) was defined and designated pelL1 (Fig. 1), since this fragment was sufficient for polypectate lyase activity as measured on polygalacturonic acid indicator medium (Fig. 2A, lane 5). Many individual isozymes of polypectate lyase and endoglucanase of Erwinia have been reported, but this is the first report of a clone harboring a pel gene responsible for CMCase activity in tandem. Other like peptate lyases, proteases, and Out proteins genes of Erwinia sp. have been described in tandem structures.

Nucleotide sequence of pelL1
The 1.6-kb inserted fragment in pPY300 was sequenced using the dideoxy chain-termination method. It contains a complete open reading frame (ORF). Figure 3 depicts the pelL1 structural gene with its flanking regions. The open reading frame contains 1,278 nucleotides and encodes a protein of 425 amino acid residues with a predicted molecular mass of 45,000 Da. The ATG initiation codon at nucleotide position 238 is preceded by a putative Shine-Dalgarno sequence, GAGG. The open reading frame ends with the ochre stop codon TAA at posi-
Fig. 1. Physical Map of the *Erwinia chrysanthemi* PY35 pelL1 Gene. The cleavage sites of the restriction enzymes *DraI*, *PstI*, *SacII*, and *BglII* are shown. pPY100 was constructed by subcloning a 2.9-kb fragment from a 5.1-kb insert DNA of *E. chrysanthemi* PY35 into the *BamHI* site of the plBluescript II SK+ vector. pPY300 (pelL1) was derived by subcloning a 1.6-kb fragment from pBluescript II SK+.

Fig. 2. Detection of Pectate Lyase and CMCase Positive Clones by the Agar Diffusion Method.

The cells were incubated for 3 days at 37°C. A, pectate lyase test: 1, negative control, pBluescript II SK+; 2, pPY100; 3, pPY200; 4, pPY400; 5, pPY300; 6, positive control, *E. chrysanthemi* PY35. B, CMCase test: 1, negative control, pBluescript II SK+; 2, *E. coli* harboring cellA of *Erwinia carotovora* subsp. *carotovora* LY34 for the positive control; 3, *E. coli* harboring cellB of *Erwinia carotovora* subsp. *carotovora* LY34 for the positive control; 4, pPY300; 5, pPY100; 6, positive control, *E. chrysanthemi* PY35.

Amino acid sequence similarities between pelL1 and other pel proteins

The comparison of the predicted amino acid sequence of PelL1 to the sequences deposited in the data bases showed that PelL1 shares significant sequence similarity with other Pel proteins.8,22,23 The results of the amino acid residues 135 to 342 of PelL1 of *E. chrysanthemi* EC1623 showed 39.5% similarity and 31.7% identity, respectively (Fig. 4). Pectate lyases are generally classified into five different families according to their amino acid sequences.8,22 PelL1 belongs to family 4, which contains PelL22 and PelX of *E. chrysanthemi* based on their amino acid sequences.

Fig. 3. Nucleotide and Deduced Amino Acid Sequence of the *pelL1* Gene of *E. chrysanthemi* PY35 with Its Flanking Regions. The putative Shine-Dalgarno (SD) box is indicated. −10 region and −35 region was underlined.
Enzymatic characterization of PelL1

A clone harboring pPY300 formed clear zones around its colonies on the assay plates containing sodium polypectate. This indicated that this clone supported not only the expression of the pectate lyase gene but also the secretion of the enzyme into the medium. To confirm secretion of the cloned pectate lyase by the E. coli XL1-Blue host cells, a clone with pPY300 was cultured aerobically in L-broth containing 0.1% (w/v) polygalacturonic acid. Pectate lyase activity was then assayed for the extracellular, periplasmic, and cytoplasmic fraction. About 12% of the total pectate lyase activity happened in the cytoplasmic space, while 65% occurred in the broth. No pectate lyase activity was detected in the corresponding fractions prepared from cells harboring pBluescript II SK+ without the insert. The results, therefore, suggest that the cloned PelL1 protein expressed in E. coli XL1-Blue is an extracellular pectate lyase.

For the purification of the enzyme, E. coli XL1-Blue harboring pPY300 was grown at 37°C. After centrifugation of the culture at 10,000 × g for 10 min at 4°C, the culture supernatant was precipitated with 60% ammonium sulfate. After dialysis against 10 mM Tris-HCl (pH 7.5), the extract was fractionated on Q-Sepharose in the presence of 2 M urea. The samples showing the best specific activity were dialyzed against 10 mM Tris-HCl (pH 7.5) and put on a phenyl-Sepharose column. The most interesting fraction was analyzed by SDS-PAGE. The result showed a single band and a protein of about 43 kDa (Fig. 5). This size was in agreement with that deduced from the nucleotide sequence of the pelL1 gene in pPY300. The effect of pH on the activity of the purified PelL1 expressed in E. coli XL1-Blue was tested at 40°C using polygalacturonic acid and various buffers ranging from pH 2 to pH 10. The enzyme was active at all pHs tested, but maximal activity was observed at pH 9. The temperature dependence of the PelL1 activity on polygalacturonate was tested at pH 9 by measuring the enzyme's activity at various temperatures. The activity was observed at temperatures between 20°C and 60°C with maximal activity at 40°C. As shown in Table 1, the PelL1 activity differed for different pectic substrates, and it was affected by the presence of CaCl2 or EDTA. PelL1 had an absolute requirement for calcium and was inactive on highly methoxylated pectin. This is characteristic also of other pectate lyases.

A clone harboring pPY100 had both activities of the pectate lyase and the CMCase. Deletion of the
Table 1. The Relative Activity of Pectate Lyase by Purified PelL1 from E. coli harboring pPY100

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme Activity (μmoles/min/mg-protein)</th>
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<tbody>
<tr>
<td>Polygalacturonate</td>
<td>270</td>
</tr>
<tr>
<td>Polygalacturonate + 1.5 mM CaCl₂</td>
<td>690</td>
</tr>
<tr>
<td>Polygalacturonate + 10 mM EDTA</td>
<td>0</td>
</tr>
<tr>
<td>Pectin (30% methoxylated) + 1.5 mM CaCl₂</td>
<td>840</td>
</tr>
<tr>
<td>Pectin (70% methoxylated) + 1.5 mM CaCl₂</td>
<td>270</td>
</tr>
<tr>
<td>Pectin (90% methoxylated) + 1.5 mM CaCl₂</td>
<td>30</td>
</tr>
</tbody>
</table>

* The enzyme reaction mixture was incubated at 40°C. Activity is defined as micromoles of unsaturated product released per minute per mg of purified enzyme. The reaction mixture consisted of 0.5%, (wt/vol) substrate and 10 mM Tris-Cl buffer (pH 9).

pPY100 downstream of pel/L1 resulting in loss of the CMCase activity (PY200, PY300, and PY500) implies that there may be a cel gene of E. chrysanthemi (Fig. 1). The functional significance for this tandem arrangement of the pel and cel genes has remained elusive. The variety of cell-wall degrading enzymes may result from separate functional adaptations in the enzymatic activities of the bacterium to plant conditions that the bacterium may encounter during the first step of infection or that may develop during tissue maceration. The current concept is that pectic and cellulolytic enzymes released by phytopathogens are involved in the enzymatic hydrolysis of plant cell walls. It is generally believed that these enzymes serve as cell wall-modifying enzymes, since their actions may also render other polysaccharide components in the cell walls more susceptible to hydrolysis. A study of the exact roles of these enzymes is thus essential to the understanding of the mechanism of host-parasite interaction in the disease cycle. We are now addressing the characterization of the putative CMCase gene downstream of the pel/L1 gene by introducing reporter transposon insertions into these two genes to explore their relative levels of expression and roles during pathogenesis.

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References


