Molecular Cloning and Sequencing of a Pectate Lyase Gene from Yersinia pseudotuberculosis

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A pectate lyase gene (pelY) from Yersinia pseudotuberculosis was cloned in Escherichia coli DH-5a. The gene was expressed in either orientation in pUC plasmids, indicating that the insert DNA carried a Y. pseudotuberculosis promoter which functioned in E. coli. However, when cloned in the orientation which placed the coding region downstream of the vector lac promoter, expression of pelY was nine times higher than it was in the opposite orientation and the growth of E. coli cells was inhibited. Nucleotide sequence analysis of the pelY gene disclosed an open reading frame of 1,623 base pairs (PLY). The peptide sequence at the amino-terminal end of the protein contains a typical signal peptide sequence, consistent with the observation that the mature PLY protein accumulated largely in the periplasmic space of E. coli. The pl of PLY produced in E. coli cells was 4.5, and its activity was inhibited 90% or more by EDTA. The enzyme macerated cucumber tissue about 1,000 times less efficiently than did PLE from Erwinia chrysanthemi EC16. The pelY gene has no sequence similarity to the pel genes thus far sequenced from Erwinia spp.

Pectate lyase (PL) is one of the most important enzymes involved in the maceration of plant tissues by soft-rotting Erwinia strains (5, 6, 11). A consistent feature of these bacteria is the occurrence of multigene families encoding functionally similar PLs. Several of these pel genes have been cloned and sequenced from strains of E. chrysanthemi (2, 9, 12; S. Tamaki, S. Gold, M. Robeson, S. Manulis, and N. T. Keen, submitted for publication) and E. carotovora (14, 15), and marker exchange mutagenesis experiments have shown that several individual pel genes are required for high pathogenicity (11). Surprisingly, PLs are also produced by other nonphytopathogenic enterobacteria, such as members of the genera Klebsiella and Yersinia (1, 4, 22). Since it would be of interest to compare the structure, organization, and regulation of pel genes in these organisms with those of Erwinia spp., we undertook the cloning and characterization of a pel gene(s) from Yersinia pseudotuberculosis (4). We report here that there is no detectable homology between the single detected Yersinia pelY gene and any of the families of clustered pel genes thus far sequenced from Erwinia spp.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and the plasmids used and constructed in this work are given in Table 1.

Media and culture conditions. Bacterial strains were grown and maintained on Luria medium containing ampicillin at a concentration of 50 μg/ml (broth) or 100 μg/ml (solid medium). Y. pseudotuberculosis was grown at 30°C, and Escherichia coli strains were grown at 37°C. Cultures of E. coli for enzyme assays were grown at 28 to 30°C.

PL assays. PL activity in culture fluids or in periplasmic fractions was determined by monitoring the A232 as described previously (9). The specific activity of PLY on pectin was assayed at 235 nm with 1% citrus pectin (P9135; Sigma Chemical Co.) instead of polygalacturonic acid (P3889; Sigma). Purified PLE (10) from E. chrysanthemi EC16 was used as a control. The effect of Ca²⁺ on PL activity was determined with reaction mixtures lacking CaCl₂ or with 3 or 0.5 mM EDTA added instead of CaCl₂.

The screening of E. coli clones for PL production was done on YC agar plates (9) containing 100 μg of ampicillin per ml. The cells were incubated at 30°C for 24 h and were then lysed by exposing the plates to chloroform vapor for 20 min. The plates were then transferred to 30°C for 2 h. Positive PL clones were detected by the overlay method of Ried and Collmer (18) or by flooding the plates with 1 M CaCl₂ (9).

Standard DNA techniques. Restriction enzyme digestions, ligation conditions, preparation of competent cells, transformation procedures, and gel electrophoresis are described by Maniatis et al. (16) or Keen et al. (9). Large-scale isolation of plasmid DNA was done by the alkaline lysis procedure (16). Plasmid constructs were checked by miniboil plasmid extractions and restriction with the appropriate enzymes (7, 9). For subcloning and plasmid constructions, the desired DNA fragments were recovered from low-melting-point agarose gels by the method of Crouse et al. (7) or by electroelution from ultrapure agarose (Bio-Rad Laboratories) with 0.3 mM sodium acetate (pH 8.0) at 220 V.

Total-DNA isolation and construction of genomic libraries. Y. pseudotuberculosis chromosomal DNA was isolated as described previously (9). The chromosomal DNA was digested to completion with either EcoRI or BamHI and was ligated with pUC19 cut with EcoRI or BamHI, respectively. The ligated DNA was transformed into E. coli DH-5a, and transformants were selected on LB-ampicillin plates. From each library, 3,500 colonies were screened for PL production on polygalacturonic agar plates (9).

Subcloning. Plasmid DNA from a PL-positive clone (pPELY11) was further subcloned. The DNA was partially digested with Sau3A, and 5-kilobase (kb) fragments were purified from an agarose gel by electroelution. These fragments were ligated into the BamHI site of plasmid pUC119, which had been dephosphorylated with calf intestine alkaline phosphatase.

DNA sequence analysis. Exonuclease III deletions were generated from either end of the insert fragment of pPELY14.
and pPELY15 by the method of Henikoff (8). Deletions from the 5' and 3' ends of the gene were done in plasmids pPELY14 and pPELY15, respectively. After religation, the deletion plasmids were transformed into E. coli DH-5α. Appropriate deletions were assayed on YC plates for PL activity and transformed into E. coli MV1193. These bacteria were transfected with AM13K07, and templates of single-stranded plasmid DNA were isolated (20).

The DNA sequences of overlapping deletions were determined by the dideoxy chain termination method (19). All data were confirmed by sequencing both strands and were analyzed by the computer program of Pustell and Kafatos (17).

**Characterization of the PL produced by pPELY15 clone.**

The isoelectric point (pl) of the PLY protein was determined by preparative isoelectric focusing (9). *E. coli* DH-5α cells containing pPELY15 were grown in 30 ml of LB broth with 50 µg of ampicillin per ml for 16 h at 28°C. The periplasmic fraction was prepared as described previously (9). This fraction was dialyzed against 5 mM Tris hydrochloride (pH 8.0) and purified on an LKB 8100 preparative isoelectric focusing column with Bio-Rad 3-10 or 3-5 Ampholines. The runs were done at 650 V for 48 h at 4°C. Fractions (2.5 ml) were collected from the column and assayed for pH, PL activity, and protein concentration. Protein was determined by the method of Bradford (3) with bovine serum albumin as a standard.

Molecular weight was determined on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels by the method of Laemmli (13). The electrophoresis was done on whole-cell proteins of *E. coli* DH-5α containing desired deleted plasmids. Cultures were grown for 16 h at 28°C on 5 ml of LB broth with 50 µg of ampicillin per ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM was added at the time of culture initiation. Portions (1.5 ml) of these cultures were centrifuged, and the cells (ca. 12 mg) were washed once with 1.5 ml of 0.01 M Tris hydrochloride (pH 7.5). The pellets were then suspended in 100 µl of water, an equal volume of 2X sample buffer (13) was added, and the samples were boiled for 5 min. The gels were run at 160 V for 5 h, with phosphorylase b (97,400 daltons [Da]), bovine serum albumin (66,200 Da), ovalbumin (42,700 Da), carbonic anhydrase (31,000 Da), and soybean trypsin inhibitor (21,500 Da) as molecular mass standards (Bio-Rad). The gels were stained with Coomassie brilliant blue R250.

**Plant tissue maceration.**

Maceration was determined by incubating five thin cucumber mesocarp slices (ca. 0.5 by 1 cm, 0.1 to 1 mm thick) with various dilutions of periplasmic fractions or purified PL. The assays were done in a total volume of 1 ml of 0.02 M Tris hydrochloride (pH 8.5) at 30°C for 1 to 5 h. Loss of tissue cohesiveness was tested with a spatula. The maceration index was scored on a scale of 0 to 5, where 0 indicated no maceration and 5 denoted complete tissue softening and disintegration. Controls were enzymes boiled for 5 min or buffer alone.

**RESULTS**

**Cloning of the pELY gene.**

Two libraries of *Y. pseudotuberculosis* were constructed in pUC19 and screened in *E. coli* DH-5α. Two PL-positive clones were recovered from the EcoRI library, but none from the BamHI library. Only one of the two positive clones gave consistent responses on pectate agar plates, and it was therefore selected for further study. When plasmid DNA was isolated and transformed into *E. coli* DH-5α, all of the resultant transformants were pectolytic. Restriction of this plasmid (pPELY11) with EcoRI showed the presence of plasmid pUC19 and a single insert fragment of about 20 kb (Fig. 1). A 4.5-kb *XbaI*-SacI fragment subcloned in pUC19 (pPELY12) produced about the same level of PL activity in DH-5α as did pPELY11. No activity was detected when the *SacI*-EcoRI or EcoRI-*XbaI* fragment was similarly subcloned (Fig. 1).

**Subcloning.**

pPELY11 was partially digested with *Sau3A*, and ca. 5-kb fragments were subcloned into the BamHI site of pUC119. Of 350 transformants screened, 11 were PL positive. The purified plasmids isolated from these clones were restricted with BamHI and HindIII. All of the PL-positive plasmids had a common 500-base-pair BamHI-HindIII fragment (Fig. 1). This data, as well as results from the construction of pPELY12, indicated that the 20-kb

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**TABLE 1. Bacterial strains, bacteriophage, and plasmids used**

<table>
<thead>
<tr>
<th>Strain, plasmid, or phage</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH-5α</td>
<td></td>
<td>Bethesda Research Laboratories, Inc. 20</td>
</tr>
<tr>
<td>MV1193</td>
<td><em>Δ</em>(lac-proAB) thi supE44 <em>Δ</em>(sr1-recA)06::Tn10 (Tet') (F' traD36 proAB lacZΔM15)</td>
<td>20</td>
</tr>
<tr>
<td><strong>Y. pseudotuberculosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICPB 3821</td>
<td></td>
<td>A. Chatterjee (4)</td>
</tr>
<tr>
<td>Phage M13K07</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>pUC119 and pUC118</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>pPELY11</td>
<td>20-kb <em>EcoR</em> fragment from <em>Y. pseudotuberculosis</em> DNA cloned in pUC19; PL positive</td>
<td>This report</td>
</tr>
<tr>
<td>pPELY12</td>
<td>4.5-kb <em>XbaI</em>-SacI fragment from pPELY11 cloned in pUC19; PL positive</td>
<td>This report</td>
</tr>
<tr>
<td>pPELY14</td>
<td>3.6-kb fragment from partial <em>Sau3A</em> digest of pPELY11 cloned in pUC19 in the orientation opposite to that of the vector lac promoter; moderately PL positive</td>
<td>This report</td>
</tr>
<tr>
<td>pPELY15</td>
<td>3.6-kb fragment from pPELY14 cloned in pUC118 (downstream of the lac promoter); strongly PL positive</td>
<td>This report</td>
</tr>
</tbody>
</table>
EcoRI insert of pPELY11 contained only one pel gene. The PL-positive plasmid with the smallest Sau3A insert was named pPELY14 and used for further study.

To determine the orientation of the pel gene, pPELY14 was cut with EcoRI and PstI and the insert was subcloned into pUC118 restricted with the same enzymes. The resultant construct was designated pPELY15. All transformants carrying pPELY15 produced higher levels of PL than did those with pPELY14 (Table 2). In addition, IPTG increased expression of the gene only in pPELY15 (Table 2). These results suggested that the 3.6-kb insert of pPELY15 carries a Y. pseudotuberculosis promoter sequence which is functional in E. coli and that the pelY coding region in pPELY15 is oriented downstream from the vector lac promoter.

E. coli cells carrying pPELY15 formed smaller colonies than did cells with pPELY14, suggesting that overproduction of the Yersinia PL exerted a toxic effect on E. coli cells. This could also explain the relatively low number of PL-positive clones recovered from the EcoRI library (2 of 3,500) since clones in which the pelY gene was oriented downstream from the vector lac promoter probably did not grow well and were missed.

Characterization of the PL produced by the clones. The results presented in Table 2 demonstrate that more than 90% of the PL activity produced by E. coli cells carrying pPELY15 was secreted into the periplasm. Electrofocusing of periplasmic fractions from E. coli cells carrying pPELY11 or pPELY15 (Fig. 2) disclosed only one detectable PL, with a pI of 4.5. This is consistent with the production of the protein by Y. pseudotuberculosis (4). SDS-gel electrophoresis of whole-cell proteins from E. coli cells carrying pPELY15 (Fig. 3) showed an intense band at ca. 55,000 Da. This band was also predominant in the periplasmic fraction but was absent in cells carrying only pUC118. Since EDTA completely inhibits the activity of PLs produced by Erwinia spp. (6), we examined its effect on PLY activity.

TABLE 2. Production of PL by E. coli DH-5α cells containing pPELY14 or pPELY15

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>IPTG</th>
<th>PL activity (U/g of cells) in fraction</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Periplasmic</td>
</tr>
<tr>
<td>pPELY14</td>
<td>-</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>23</td>
</tr>
<tr>
<td>pPELY15</td>
<td>-</td>
<td>249</td>
</tr>
</tbody>
</table>

* Cultures were grown for 16 h in 15 ml of L broth with 50 μg of ampicillin per ml. IPTG at 1 mM was added at the time of initiation of growth. The cells were centrifuged, and the supernatant was dialyzed. The periplasmic fractions were prepared as described in the text.

* The pelleted spheroplasts were lysed with 5 ml of 0.01 M Tris hydrochloride (pH 8.0), centrifuged, and assayed for PL activity.

* ND, No activity was detected.

omission of Ca²⁺ from the reaction mixture caused a 43% inhibition of the PL activity. The addition of 0.5 or 3 mM EDTA further reduced the activity by 90 and 92%, respectively. PLY was 1.9 times more active on pectate than on...
pectin, whereas PLe was 3.5 times more active. Thus, PLY differs considerably from the Erwinia PLs in enzymatic properties.

**Plant tissue maceration.** To determine the ability of PLY to macerate, 10 U of the enzyme was incubated with cucumber slices. PLe (0.1 U) from *E. chrysanthemi* EC16 was used as a positive control. After 1 h, the maceration indices for PLY and PLe were 0 and 3, respectively, and after 5 h they were 3 and 5. The results of several experiments with various concentrations of PLY and PLe indicated that PLY is less than 0.001 as efficient as the PLe protein in plant maceration.

**DNA sequence of the pelY gene.** To map the endpoints of the pelY gene more precisely and to generate templates for DNA sequence determination, exonuclease III deletions were generated from either end of the DNA fragment in pPELY14 and pPELY15. The positions of some of the relevant deletions are shown in Fig. 4. Deletion 5.2 was presumed to occur in the 3′ noncoding end of the gene since it had no effect on PL activity. Deletions 5.3 and 5.4 totally destroyed activity and thus appeared to occur in or near coding regions. These two deletions were then recloned in pUC118 (downstream of the lac promoter), and cells were grown with IPTG. Deletion 5.4 was completely inactive, whereas deletion 5.3 exhibited low PL activity in the peptate plate assay and the spectrophotometric assay (4.5 U/g of cells, compared with ca. 400 U/g for deletion 5.2).

Deletions which defined the 3′ end of the pelY gene included 3.2 and 3.3 (Fig. 4), which totally destroyed activity. Deletion 3.1 and all other deletions which mapped to the right of it had no effect on activity.

The DNA sequence presented in Fig. 4 revealed a single long open reading frame between nucleotides 369 and 1991. This is consistent with the deletion analysis, and the open reading frame is therefore believed to encode the PLY prepolyprotein. The ATG designated as the presumptive start of the open reading frame is preceded by a purine-rich sequence which should function as a ribosome-binding site. This is the only in-frame ATG in the region defined by deletions 5.3 and 5.4 that is preceded by a purine-rich sequence. Furthermore, the peptide sequence at the amino-terminal end of the putative prepolyprotein strongly resembles a signal peptide sequence (21) and explains the fact that the PLY protein is efficiently secreted to the periplasm of *E. coli* cells. The deduced cleavage point of the putative signal peptide is between two alanines (amino acids 23 and 24), but this has not been confirmed by N-terminal amino acid sequencing of the mature PLY protein. The translated prepolyprotein has 541 amino acids, and the calculated molecular mass is 60,716 Da. By subtracting the mass of the putative signal peptide sequence, the mass of the mature enzyme was calculated to be 58,228 Da. This agrees closely with the value of 55,000 Da estimated by SDS-gel electrophoresis (Fig. 3). Deletion 3.6 at the 3′ end of the gene (Fig. 4) did not lead to an active PL, but *E. coli* cells carrying this DNA yielded an intense band at 43,000 Da, which corresponded well with the calculated molecular mass (44,550 Da) of the truncated protein (data not shown). Possible transcriptional initiation signals were found between deletions 5.2 (PL positive) and 5.3 (PL negative). These sequences are positioned at bases 238 to 243 and 257 to 261 (Fig. 4). No sequence which could function as a catabolic activator protein-binding site was found. This is consistent with the study of Chatterjee et al. (4), in which repression of PLY production was not observed in *Y. pseudotuberculosis*. In the 3′ end downstream of the translational stop of pelY (positions 2028 and 2044) there are sequences which may form stem-loop structures and thus may function as terminators of transcription.

**DISCUSSION**

The soft-rot erwinias produce four or more PL isozymes which are encoded by a corresponding number of clustered pel genes (6, 11). Although we cannot rule out the possibility of other pel genes that were not cloned, we were able to select clones from *Y. pseudotuberculosis* which contained only the pelY gene. Deletion analyses of the insert DNA in pPELY11 suggested that only one pel gene was present in this clone, unlike results with *E. chrysanthemi* (11). One or possibly two PLs have also been reported for *Yersinia enterocolitica* and *Klebsiella oxytoca* (1). It appears, therefore, that the pel gene organization is less complex in these organisms than in *Erwinia* spp.

Most PL activity occurred in the periplasmic fraction when the cloned pelY gene was expressed in *E. coli* (Table 2). This is consistent with the occurrence of a putative signal peptide sequence in the gene product (Fig. 4) and agrees with the previous results of Chatterjee et al. (4).

The *Yersinia* PL exhibited several differences from the *Erwinia* PLs. The molecular mass of the mature PLY was 58,228 Da, which is considerably higher than the ca. 39-kDa mass observed for the mature PLs of *Erwinia* spp. (6, 11). Our data for the *Yersinia* PL are in agreement with the value of 55 kDa previously reported for the PL of *Y. enterocolitica* (1). Another difference from the *Erwinia* PLs is that the *Yersinia* enzyme is 1.9 times less active on pectin than on peptate, whereas the *E. chrysanthemi* PL was 3.5 times less active. Similar to the *Erwinia* PLs, the *Yersinia* enzyme is calcium dependent. However, whereas EDTA completely abolishes activity of the *Erwinia* enzymes, a maximum inhibition of ca. 90% was observed with the *Yersinia* enzyme. Our results also conflict with those of Bagley et al. (1), who reported that PLs from *K. oxytoca* and *Y. enterocolitica* do not require divalent cations for activity.

The acidic pI value for PLY is similar to those reported for the PLs of *K. oxytoca* and *Y. enterocolitica* (1). All of these enzymes are inefficient macerators of plant tissue, similar to PLa from *E. chrysanthemi*, which also has a low isoelectric point (2). Although it is possible that the low pI values of these PLs are solely responsible for their low maceration efficiency, other factors may also be involved, since efficient macerating enzymes with low pI values have been described for other organisms (6).

Computer searching disclosed no homology between the amino acid sequence of PLY and those of the protein products of sequenced *Erwinia pel* genes. Thus, these genes appear to have evolved from different lineages. Since three distinct families of pel genes have thus far been recovered from *Erwinia* spp. (11; Tamaki et al., submitted; D. and N. T. Keen, Trollinger, unpublished data), at least three families of pel genes exist in the family *Enterobacteriaceae*. It is not clear why such catalytically similar proteins evolved independently. Perhaps this reflects different functions in nature. For instance, it has been speculated (1, 4) that the PLs of *Yersinia* and *Klebsiella* spp. might be advantageous to their survival as saprophytes. Chatterjee et al. (4) also suggested that the PLs of *Yersinia* and *Klebsiella* spp. might have an extremely catabolic function related to bacterial nutrition, whereas the *Erwinia* enzymes might also have cytoytic and other specific functions in plant hosts. This possibility is supported by our findings that pelY is structurally distinct from the *Erwinia pel* genes, does not occur as a multigene
FIG. 4. Nucleotide sequence determined for the pelY gene. The predicted amino acid sequence of the preprotein is shown, and selected restriction sites are noted. The positions of selected exomuclease III deletions referred to in the text are indicated with arrows and numbers. For deletions at the 5' end of the sequence, DNA to the left of the arrow was deleted. For deletions at the 3' end, DNA to the right of the arrow was deleted. The presumed leader peptide is underlined, and the possible cleavage site is indicated by an arrow. The putative ribosome-binding site and other possible signal sequences discussed in the text are underlined.
family, and encodes a PL which inefficiently macerates plant tissue.

ACKNOWLEDGMENTS

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LITERATURE CITED


