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# Cloning and Characterization of a Pectate Lyase Gene from *Erwinia carotovora* EC153

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**A *pel* gene cloned from strain EC153 of *Erwinia carotovora* encoded a pectate lyase that macerated plant tissue with moderate efficiency. This gene, called *pel*153, was sequenced and found to possess considerable homology with a pectate lyase gene from *Yersinia pseudotuberculosis*. The *Yersinia* protein, however, was truncated at the carboxyl terminal end relative to the *Erwinia* gene product and had a lower isoelectric point. The *Erwinia pel*153 gene was overexpressed in cells of *Escherichia coli*, and a 56-kDa protein was observed on sodium dodecyl sulfate-polyacrylamide gels. This compares with a molecular weight of 61 kDa for the mature, secreted protein as determined from sequencing data. Southern blot analysis disclosed the presence of the *pel*153 gene in three different strains of *E. carotovora*, but mutation of the gene in strain EC153 did not affect its ability to soft-rot potato tubers.**

*Additional keywords*: DNA sequencing, gene cloning, gene overexpression, maceration, marker exchange mutagenesis, soft rotting, Southern blots.

*Pel* genes encoding pectate lyase (PL) proteins have been cloned from several members of the Enterobacteriaceae (Collmer and Keen 1986; Kotoujansky 1987). Two distinct families of *pel* genes have been isolated from *Erwinia chrysanthemi* that encode proteins with very limited amino acid homology (Tamaki et al. 1988), despite the fact that their catalytic properties are similar *in vitro* (Barras *et al.* 1987). Several *pel* genes cloned from isolates of *E. carotovora* have considerable homology to the *pel*B/C gene family of *E. chrysanthemi* (Ito *et al.* 1988; Lei *et al.* 1987, 1988; Tamaki *et al.* 1988). On the other hand, we recently sequenced the *pel*Y gene from *Yersinia pseudotuberculosis* (Manulis *et al.* 1988) that had no significant homology with the *pel* genes of *E. chrysanthemi* and encoded a larger protein product.

In this paper we report the cloning of a *pel* gene (called *pel*153) from strain EC153 of *E. carotovora* and show that it has surprisingly high homology with the *Yersinia pel*Y gene. In addition, we characterized the *pel*153 gene product and mutated the *pel*153 gene in strain EC153 by a gene replacement method.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, culture media, and PL assays.** Strains and plasmids used and constructed in this work are shown in Table 1. Bacterial strains were grown and maintained on Luria media (Maniatis *et al.* 1982). Strain EC153 of *E. carotovora* was grown at 28° C, and strains of *E. coli* were grown at 37° C except cells grown for enzyme production, which were grown at 28° C in shaken liquid media. Ampicillin was used at 75 μg/ml, tetracycline at 25 μg/ml, chloramphenicol at 35 μg/ml, rifampicin at 75 μg/ml, and kanamycin at 50 μg/ml as required. Cells of *E. coli* were grown at 28° C on 15 ml of L broth in 50-ml Delong flasks shaken at 150 cycles per min. EC153 cells of *E. carotovora* were grown on the minimal salts medium of Chatterjee *et al.* (1979) containing 0.5% polygalacturonic acid.

PL activity in culture fluids or in various cell fractions was determined by monitoring the change in absorbance of sodium polygalacturonate at 232 nm as previously described (Keen *et al.* 1984). One PL unit denotes the activity that liberates 1 μmole of reducing equivalents from sodium polygalacturonate (P3889; Sigma Chemical Co., St. Louis) per min at 22° C and pH 8.5. Activity on pectin was determined by using the same assay but substituting citrus pectin (P9135; Sigma) for polygalacturonate.

The PL encoded by *pel*153 as well as PLc of *E. chrysanthemi* EC16 (Tamaki *et al.* 1988) were also assayed viscosimetrically by using size 100 Ostwald Viscosimeters. Both enzyme preparations were the dialyzed periplasmic fraction of D1210λ cells of *E. coli*. Sodium polypectate (2%) in 20 mM Tris-HCl, pH 8.3, containing 3 mM $CaCl_2$ (5 ml), was mixed with 0.5 ml of enzyme (0.5–3.0 units). This reaction mixture was added to the viscosimeter, and efflux times were taken at intervals over a 2-hr period at room temperature.

PL production by bacteria was also determined on YC agar plates containing sodium polypectate (Keen *et al.* 1984). After colonies had grown, it was necessary in some cases to invert the plates over chloroform for 30 min and continue incubation at 37° C for an additional 2 hr before developing by flooding with 1 M $CaCl_2$ (Keen *et al.* 1984). When plates developed with calcium chloride were dried at room temperature for several hours with the covers removed, marked depressions surrounding PL-positive colonies became visible.This "pitting" was more clearly visualized by incubating plates at 42° C following calcium chloride treatment and drying.

**Conjugations and marker exchange mutagenesis.** Plasmid pBR325 carrying various insert fragments was introduced into *E. carotovora* EC153 *rif* by triparental

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Nucleotide and/or amino acid sequence data is to be submitted to GenBank as accession number J03673.

matings. One of the plasmids was pPEL153-8 (Table 1), containing the *pel*153 gene mutated by insertion of a DNA fragment of about 1.7 kb from Tn*903* carrying a neomycin phosphotransferase (*npt*) gene (Oka *et al.* 1981) into an internal *Stu*I site. Approximately $10^9$ cells of EC153 *rif*, *E. coli* DH$\alpha$ containing pPEL153-1 or pPEL153-8 (see Table 1), and *E. coli* HB101 (pRK2013) (Ditta *et al.* 1980) were mixed on an area of about 3 cm square on the surface of an L agar plate without antibiotics. The mixture was incubated for about 16 hr at 31° C, and about $10^9$ cells of the mating mixture were then plated onto L agar containing kanamycin and rifampicin and the cells grown at 31° C. Resultant single colonies were restreaked on L kanamycin medium and single colony isolates tested for PL production on YC-sodium polypectate plates. More than 95% of the colonies were PL positive and resistant to ampicillin, tetracycline, kanamycin, and rifampicin, but sensitive to chloramphenicol, indicating that they were strain EC153 containing pPEL153-8. pPEL153-8 was stable in strain EC153 through several single colony transfers on L agar, even in the absence of antibiotic selection. When the plasmid was recovered from strain EC153 and transformed into *E. coli* DH5$\alpha$, it consistently gave the expected restriction patterns with various enzymes.

Roeder and Collmer (1985) observed that minimal culture medium with limiting phosphate concentrations rendered

**Table 1.** Bacterial strains, phage, and plasmids utilized and constructed

| Bacteria | Description | Reference |
|---|---|---|
| *Erwinia carotovora* EC153 | | Chatterjee *et al.* 1979 |
| *E. carotovora* EC153 *rif* | Spontaneous rifampicin-resistant mutant | This paper |
| *E. carotovora* EC153 *pel*153::npt | Marker exchange mutant deficient in PL153 | This paper |
| *E. carotovora* 0285-11 | | D. Cooksey |
| *E. carotovora* 73-22 | | L. Moore |
| *E. chrysanthemi* EC16 | | Keen *et al.* 1984 |
| *E. chrysanthemi* 3937 | | Reverchon *et al.* 1986 |
| *Escherichia coli* | | |
| HB101 | | Maniatis *et al.* 1982 |
| DH5$\alpha$ | | Bethesda Research Labs |
| MV1193 | | Vieira and Messing 1987 |
| D1210$\lambda$ | | Hasan and Szybalski 1987 |
| Phage | | |
| M13K09 | | Vieira and Messing 1987 |
| Plasmids | | |
| pUC118 and pUC119 | Cloning and sequencing vectors | Viera and Messing 1987 |
| pUC128 and pUC129 | Cloning and sequencing vectors | Keen *et al.* 1988 |
| pDSK509 | Broad host range plasmid | Keen *et al.* 1988 |
| pBR325 | 5.4-kb cloning vector | Bolivar 1978 |
| pNH18a | Invertible promoter expression vector | Hasan and Szybalski 1987 |
| pRK2013 | Helper plasmid for conjugations | Ditta *et al.* 1980 |
| pPEL410 | High-expression plasmid carrying the *pel*C gene of *Erwinia chrysanthemi* | Tamaki *et al.* 1988 |
| pPELY14 | 3.6-kb insert of *Y. pseudotuberculosis* DNA encoding *pel*Y; cloned in pUC119 in the orientation opposite to that of the vector *lac* promoter; low PL activity in *E. coli* | Manulis *et al.* 1988 |
| pPELY15 | 3.6-kb insert from pPELY14 cloned in pUC118 such that the gene is oriented downstream of the vector *lac* promoter; high PL activity in *E. coli* | Manulis *et al.* 1988 |
| pPELY16 | 3.6-kb *Sph*I/*Sma*I insert from pPELY14 subcloned into the *Sph*I/*Sma*I sites of pNH18a; the *pel*Y gene was thus oriented downstream from the vector *tac*/*lac* promoters following promoter inversion | This paper |
| pPEL153-1 | 5.2-kb *Eco*RI fragment of EC153 DNA cloned into pBR325; weakly PL positive | This paper |
| pPEL153-2 | 4.1-kb *Eco*RV fragment from pPEL153-1 cloned into the *Sma*I site of pUC119; PL positive | This paper |
| pPEL153-3 | 2.2-kb *Bam*HI fragment from pPEL153-2 cloned into the *Bam*HI site of pUC129 in the opposite orientation to the vector *lac* promoter; PL positive | This paper |
| pPEL153-4 | Same as pPEL153-3, except the 2.2-kb *Bam*HI fragment was cloned into pUC128 in the opposite orientation to the vector *lac* promoter; PL positive | This paper |
| pPEL153-5 | 2.2-kb *Bam*HI fragment from pPEL153-3 cloned into the *Bam*HI site of pNH18a such that the open-reading frame was oriented downstream of the vector *tac*/*lac* promoters after promoter inversion; strongly PL positive following induction. | This paper |
| pPEL153-6 | Approximately 1.8-kb *Mlu*I/*Pst*I fragment from pPELY15 ligated with pPEL153-3 cut with the same enzymes such that the resulting recombinant gene contained the 5′ end of *pel*153 and the 3′ end of *pel*Y; PL positive | This paper |
| pPEL153-7 | Approximately 3.3-kb DNA insert of pPEL153-6 removed with *Bam*HI and *Sal*I and cloned into the same sites of pNH18a such that the open-reading frame was oriented downstream of the vector promoters following promoter inversion; strongly PL positive | This paper |
| pPEL153-8 | pPEL153-1 restricted at the unique intragenic *Stu*I site (see Fig. 1) and ligated with a 1.7-kb *Pvu*II fragment (Oka *et al.* 1981) from pDSK509 encoding kanamycin resistance; PL negative | This paper |

pBR322 unstable in E. chrysanthemi such that a significant number of progeny cells had lost the plasmid. Because pBR322 and pBR325 share the same replicon, we tested whether pPEL153-8 (based on the latter plasmid) could be cured from strain EC153 of *E. carotovora* during phosphate starvation and the mutant *pel*153 gene in pPEL153-8 (see Table 1) would marker exchange for the wild-type gene. Cells of EC153 carrying pPEL153-8 were grown on the medium of Roeder and Collmer (1985) containing 250 $\mu$M potassium phosphate but without antibiotics at 28° C and with shaking for about 40 hr. Cells were then plated on L agar plates containing kanamycin, and single colonies were screened for the loss of tetracycline and ampicillin resistance by plating on the appropriate media. It was observed that greater than 95% of the recovered kanamycin-resistant colonies had lost resistance to the other two antibiotics, suggesting that plasmid pBR325 had been cured and that the mutant *pel* gene in pPEL153-8 had exchanged with the wild-type *pel*153 gene.

**DNA techniques, library construction, and subcloning.** Restriction enzyme digestions used salts recommended by the suppliers or 1× KGB salts (McClelland *et al.* 1988). Agarose gel electrophoresis, ligation conditions, preparation of competent cells of *E. coli*, and transformation techniques were as described by Maniatis *et al.* (1982) or Keen *et al.* (1984). In latter stages, the TSB method of Chung and Miller (1988) was employed for preparation of competent cells of *E. coli*. This procedure was simple and gave high transformation efficiencies either when cells were grown out in TSB medium or when using the heat shock/LB procedure previously employed (Keen *et al.* 1984). Total genomic DNA of *E. carotovora* EC153 and other *Erwinia* spp. was prepared as previously described (Keen *et al.* 1984).

For library construction, DNA of EC153 was restricted to completion with *Eco*RI and ligated to pBR325 DNA that had been restricted with the same enzyme. Following ligation and transformation of HB101 of *E. coli*, tetracycline-resistant but chloramphenicol-sensitive colonies were plated on YC plates containing sodium polypectate as above. After growth for 24 hr at 37° C, colonies were lysed by inverting plates over chloroform as described above. PL-positive clones were screened for the formation of halos around the bacterial colonies. During subcloning, plasmid constructs were checked by miniboil plasmid extractions (Keen and Tamaki 1986) and restriction with the appropriate enzymes before agarose gel electrophoresis. For subcloning and plasmid constructions, the desired DNA fragments were recovered from low-melting point agarose gels by the method of Crouse *et al.* (1983) or, in some cases, by electroelution from agarose gels into 0.3 M sodium acetate, pH 8.0, at 220 V followed by ethanol precipitation.

Southern blots were performed essentially as described by Maniatis *et al.* (1982). Chromosomal DNA of various strains of *E. carotovora* or *E. chrysanthemi* (about 5 $\mu$g) was restricted with *Bam*HI and, following electrophoresis on a 1% agarose gel, was blotted onto a Zeta-bind membrane (AMF Cuno, Meriden, CT) according to the manufacturer's directions. Blots were probed with the nick translated, $^{32}$P labeled 2.2-kb *Bam*HI insert fragment of pPEL153-3, containing the *pel*153 gene, by overnight incubation at 42° C in a standard hybridization solution containing 50% formamide. Blots were washed twice for 1 hr at 42° C with 0.1× saline sodium citrate and 0.1% sodium dodecyl sulfate (SDS; Maniatis *et al.* 1982) before a 16-hr exposure of X-ray film at −70° C with a DuPont Cronex intensifying screen.

**DNA sequencing.** Exonuclease III deletions (Henikoff 1984) were generated from the primer end of the insert fragments of desired plasmids, and resultant single stranded DNA was sequenced by the dideoxy method as described previously (Tamaki *et al.* 1988). All data were confirmed by comparison of overlapping sequence data for both strands. Data were analyzed by the computer program of Pustell and Kafatos (1984), by the Bio-net programs (Intelligenetics, Mountain View, CA), and by data base searching of the National Biomedical Research Foundation protein library (release January 1988).

**Electrophoresis and electrofocusing.** Whole cells of *E. coli* carrying various plasmids were suspended in 2.5× Laemmli electrophoresis sample solution, boiled for 5 min, and aliquots applied to 10% SDS-polyacrylamide gels (Laemmli 1970). These were electrophoresed and stained with Coomassie blue R250 as previously described (Tamaki *et al.* 1988).

Periplasmic fractions of *E. coli* or culture fluids of strains of *E. carotovora* were dialyzed against 5 mM Tris-HCl, pH 8.0, and concentrated to various degrees with Centricon devices. The concentrated preparations were applied to thin-layer electrofocusing gels on filter paper wicks and the gels developed at 4 watts with a maximum of 2000 V for about 40 min at 1° C. Gels were run on a Bio-Rad Biophoresis unit (Bio-Rad Laboratories, Richmond, CA) and prepared according to the manufacturer's instructions. Bio-Rad pI standards were also applied and the positions of selected marker bands noted. Polypectate overlays were then prepared according to Roeder and Collmer (1985) and exposed for various periods of time at 32° C before developing with 0.2% aqueous ruthenium red.

**Maceration and pathogenicity assays.** Dialyzed periplasmic fractions of *E. coli* and culture fluids of *E. carotovora* were assayed for maceration activity on cucumber mesocarp tissue as previously described (Keen and Tamaki 1986). Maceration activity was quantitated as the minimum PL activity of various enzymes required to produce detectable maceration of cucumber slices following incubation for 1 hr at 37° C in 0.01 M Tris-HCl, pH 8.0.

Pathogenicity tests for soft-rotting ability were performed on whole potato tubers by using the method of Roeder and Collmer (1985) in which 50 $\mu$l of bacterial suspensions were inoculated by means of disposable pipette tips. Inoculated tubers were incubated aerobically and at high humidity at 31° C in the dark and the results read after 48 hr. Tubers were sliced through the inoculation wounds and the amount of soft-rotted tissue removed and weighed from 12 replicate tubers (Roeder and Collmer 1985).

## RESULTS

**Cloning the *pel*153 gene.** One PL-positive clone was detected by screening 750 HB101 colonies of *E. coli* containing the *Eco*RI plasmid library of EC153 DNA. The positive plasmid clone, called pPEL153-1, contained a 5.2-kb *Eco*RI fragment that was restriction enzyme mapped (Fig. 1). Further subcloning led to the isolation of pPEL153-2, which contained a 4.1-kb *Eco*RV fragment. A 2.2-kb *Bam*HI fragment from pPEL153-2 also directed PL

production in *E. coli* cells, but only one insert orientation could be obtained in either pUC128 (called pPEL153-4) or pUC129 (called pPEL153-3). These results suggested that overexpression of the *pel*153 gene was toxic to cells of *E. coli* and implied that the putative *pel* gene in pPEL153-3 and pPEL153-4 might be oriented opposite to the vector *lac* promoters.

No additional *pel* genes were detected on the 5.2-kb insert fragment in pPEL153-1 by deletion analysis. Any deletion that removed part of the 5′ coding region of pPEL153 (see Figs. 1 and 2) resulted in the loss of detectable PL activity in *E. coli*.

**Sequencing of the *pel*153 gene.** A single long open-reading frame (ORF) was located on the insert DNA of pPEL153-3 and pPEL153-4 (Fig. 2), which was oriented opposite to the vector *lac* promoters as predicted by the observations above. The initiation codon was located 34 bp downstream from the *Bam*HI site of pPEL153-3, and DNA further 5′ to this site was sequenced from deletions prepared with pPEL153-2. The ORF of the *pel*153 gene was preceded by a Shine-Dalgarno sequence that was somewhat unusual in that it contained a T residue in an otherwise typical purine-rich region. The entire ORF encoded a protein product of 568 amino acids that gave a calculated molecular weight of 63,528 Da. Based on the sequence data, the first 19 amino acids are predicted to constitute a signal peptide leader sequence. If this assumption is correct, the mature, secreted protein of 549 amino acids has a calculated weight of 61,596 Da and a computer-calculated isoelectric point of 8.1. We have not, however, confirmed the cleavage site by N-terminal sequencing of the mature, secreted *pel*153 protein product.

**Homology of *pel*153 with the *pel*Y gene of *Y. pseudotuberculosis*.** Computer searching of the NBRF database failed to show significant homology between the protein product of the *pel*153 gene and previously sequenced proteins. However, the *pel*153 gene product showed considerable homology to that of the *pel*Y gene, recently sequenced in our laboratory (Manulis *et al.* 1988). Indeed, the protein products have 85% identical amino acid residues in the common regions (Fig. 3). The putative signal peptide sequences of the two proteins differed considerably and that

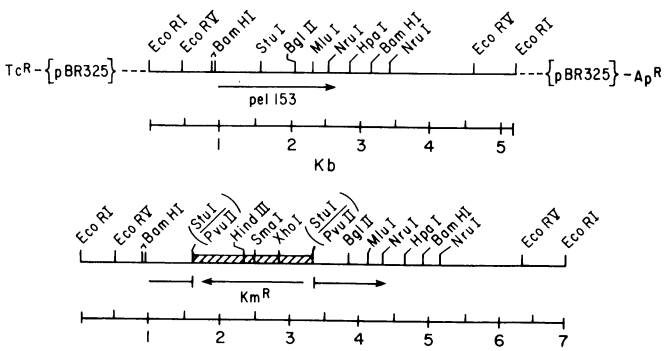


**Fig. 1. Upper:** Restriction map of the 5.2-kb *Eco*RI fragment from strain EC153 as originally cloned in pBR325 (pPEL153-1). The orientation of the fragment is denoted relative to the vector antibiotic genes. Map distances are shown in kilobases, and the arrow identifies the open-reading frame of *pel*153 as deduced by sequencing and expression studies. **Lower:** Map of pPEL153-8, showing a 1.7-kb DNA fragment encoding kanamycin resistance inserted into the *Stu*I site of *pel*153.

of the *pel*153 gene carried a four amino acid deletion relative to the *pel*Y gene product. In the coding regions of the predicted mature proteins, however, the two genes read co-linearly and contained large blocks of highly homologous amino acids (Fig. 3). The major difference was that the *pel*Y gene product was truncated by 31 amino acids at the carboxy terminus relative to that of *pel*153. To ensure that a sequencing error had not occurred at the 3′ end of the *pel*Y gene, we subcloned a 270 bp *Mlu*I/*Bst*XI fragment from pPELY15 containing the 3′ end of the *pel*Y gene (see Manulis *et al.* 1988) and sequenced both strands. This data confirmed that the previously reported sequence of the *pel*Y gene was correct. Comparison of the sequences of the 3′ ends of the coding regions of the *pel*Y gene and the *pel*153 gene (Fig. 2) suggests that the *pel*Y gene probably evolved from the *pel*153 gene or a common ancestor by deletion of a single G residue at position 1,983 of the *pel*153 sequence. Addition of a G following base 1,983 of the *pel*Y gene (Manulis *et al.* 1988) restores the reading frame so that the 3′ end of the *pel*Y gene now reads co-linearly with *pel*153; 26 of the 34 C terminal amino acid residues thus formed are identical to those in the *pel*153 product, and indeed the *pel*Y gene then terminates at precisely the same position as the *pel*153 gene.

To further confirm that the truncated *pel*Y gene was functional, the *pel*Y sequence 3′ to the unique and conserved *Mlu*I site (Fig. 2; Manulis *et al.* 1988) was used to replace the 3′ end of the *pel*153 gene, generating plasmid pPEL153-6. The resulting recombinant gene encoded a PL-active protein in cells of *E. coli* (Table 2). The chimeric protein was also overexpressed by cloning into the invertible promoter vector, pNH18a, to generate pPEL153-7 (Table 1).

**Overexpression of the *pel*153 and *pel*Y genes.** Because the 2.2-kb *Bam*HI fragment in pPEL153-3 and pPEL153-4 could not be cloned in the orientations that were downstream from the vector *lac* promoters and PL activity from cells carrying these plasmids was relatively low (Table 2), the 2.2-kb *Bam*HI insert of pPEL153-3 was cloned into pNH18a to yield pPEL153-5 (Table 1). Following inversion of the promoter cassette by exposure of D1210λ cells of *E. coli* to 42° C for 15 min, the *pel*153 gene was then oriented downstream from the tandem *tac*/*lac* promoters of pNH18a (Hasan and Szybalski 1987). Following induction, relatively high yields of PL activity were indeed observed with pPEL153-5, but much lower PL activity was observed in cells that were not heat induced (Table 2). Highest PL yields were obtained when the temperature induction was performed relatively late in the growth phase at 28° C, namely when cell densities were between 0.8 and 1.0 A at 500 nm. Induction at lower (about 0.5 A or less at 600 nm) or higher (1.2 A or higher at 500 nm) cell densities resulted in significantly less PL activity (data not shown).

The yields of PL activity from pPEL153-5 were nevertheless much lower than from pPEL410, carrying the EC16 *pel*C gene of *E. chrysanthemi* (Table 2). In part, this is due to a large difference in specific activities of the two proteins (unpublished observations). As expected, cells of *E. coli* carrying pPEL153-1 and pPEL153-3 produced relatively low PL activities (Table 2). Induced cultures carrying the *pel*Y gene (pPELY16) or the recombinant *pel*153/*pel*Y gene (pPEL153-7) produced considerable activity, but less than directed by pPEL153-5. The majority of the PL activity (90% or more) from all constructs was observed in the periplasmic fractions (data not shown), as

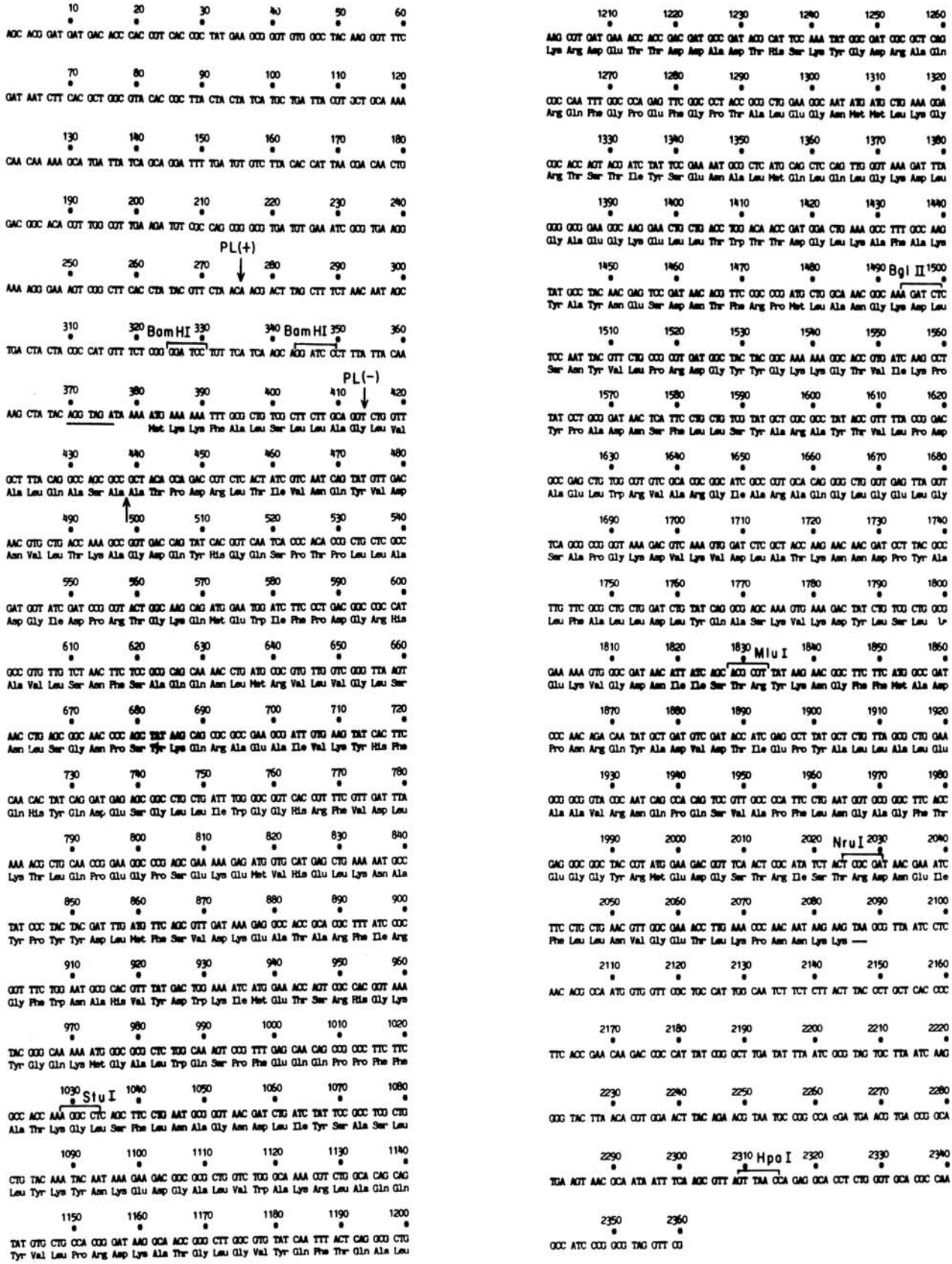


**Fig. 2.** DNA sequence of the *pel*153 gene and flanking DNA, showing selected restriction sites noted in Figure 1 and the text. The putative Shine-Dalgarno sequence is underscored. The noted *exo*III deletions at the 5′ end of the coding region did not affect or entirely destroy production of PL activity in *E. coli* as designated. An arrow denotes the assumed signal peptide cleavage site of the preprotein.

observed previously with the *pel*Y protein of *Y. pseudotuberculosis*, and consistent with the observed signal peptide sequence on the putative *pel*153 preprotein (Fig. 2).

**Characterization of the *pel*153 gene product from cells of *E. coli* carrying overexpression plasmids.** Cells of *E. coli* expressing the *pel*Y gene from *Y. pseudotuberculosis* or the *pel*153 gene from *E. carotovora* were electrophoresed on SDS-polyacrylamide gels and the protein products located by Coomassie blue staining (Fig. 4). The *pel*Y protein ran at about 54 kDa as previously observed (Manulis *et al.* 1988), whereas the *pel*153 protein gave an estimated weight of 56 kDa (Fig. 4). The same protein bands were observed specifically in periplasmic fractions of cells of *E. coli* carrying plasmids with the *pel*153 or *pel*Y genes, respectively (data not shown). Thus, the secreted *pel*153 protein runs on SDS gels at about 5 kDa less than the weight of the mature protein as determined from sequencing data. However, the *Yersinia* PL also runs at about 4 kDa less than the weight of 58 kDa predicted from sequencing data. Behavior of the protein products on SDS-polyacrylamide gels was therefore consistent with the occurrence of the 3′ deletion in the coding region of the *Yersinia pel*Y gene (Fig. 3).

Periplasmic fractions from cells of *E. coli* expressing *pel*153 yielded a single band of PL activity when polypectate overlays were performed on electrofocusing gels (Fig. 5). The pI of this protein was determined to be about 8.8 based on the standard proteins employed. This is somewhat higher

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PEL EC153   1 MKKFALSLLAGLVAL----QASAATPDRLTIVNQYVDNVLTKAGDQY
                ::: :: :    :         ::  :::: : :::::::: :: : :
PEL Y       1   MKKRALLLSMSVLAMLYIPAGQAAEIDRLTVVKQYVDNVLNKASDTY

44  HGQSPTPLLADGIDPRTGKQMEWIFPDGRHAVLSNFSAQQNLMRVLVGLSNLSGNP
    ::   : :::::: ::::: :::::::::: ::::::::::::::::  ::: ::: :
48  HGDKPSPLLADGVDPRTGQQMEWIFPDGRRAVLSNFSAQQNLMRVMSGLSELSGDP

100 SYKQRAEAIVKYHFQHYQDESGLLIWGGHRFVDLKTLQPEGPSEKEMVHELKNAYP
     :  ::: :: :: : ::: :::: :::::::::::::::::::::::: :::::::::
104 QYQKRAEDIVRYHGQNYQDNSGLLYWGGHRFVDLKTLQPEGPSEKEKVHELKNAYP

156 YYDLMFSVDKEATARFIRGFWNAHVYDWKIMETSRHGKYGQKMGALWQSPFEQQPP
    :::::::::  :: :::::::::::::: : :::::: ::  ::::: : ::::::
160 YYDLMFSVDSDATTRFIRGFWNAHVYDWRILETSRHGEYGKPMGALWESTFEQQPP

212 FFATKGLSFLNAGNDLIYSASLLYKYNKEDGALVWAKRLAQQYVLPRDKATGLGVY
    :::::::::::::::::::::::::::    :::::::::: :::::::  ::::::
216 FFATKGLSFLNAGNDLIYSASLLYKYQQDQGALVWAKRLADQYVLPRDAKTGLGVY

268 QFTQALKRDETTDDADTHSKYGDRAQRQFGPEFGPTALEGNMMLKGRTSTIYSENA
    :::::::: : ::::::::: :::::::::::::::::::::::::::::: :::::
272 QFTQALKREEPTDDADTHSKFGDRAQRQFGPEFGPTALEGNMMLKGRTSTLYSENA

324 LMQLQLGKDLGAEGKELLTWTTDGLKAFAKYAYNESDNTFRPMLANGKDLSNYVLP
    ::::::::::::  :  :: :: :::::::::: ::: ::::::: ::: ::::: ::
328 LMQLQLGKDLGGQGDDLLKWTVDGLKAFAKYGYNEQDNTFRPMIANGQDLSNYTLP

380 RDGYYGKKGTVIKPYPADNSFLLSYARAYTVLPDAELWRVARGIARAQGLGELGSA
    :::::::::: : ::: : : :: :::::: :  :  :: ::::::  ::::  :::
384 RDGYYGKKGSVLKPYKAGNEFLISYARAYAVDNDPLLWKVARGIASDQGLGDIGSA

436 PGKDVKVDLATKNNDPYALFALLDLYQASKVKDYLSLAEKVGDNIISTRYKNGFFM
    :::  :: : : : : :::::::::::::: :: :  : :::::: :::: :::  ::::
440 PGKEMKVKLDTTNSDPYALFALLDLYNASQVAEYRSLAEKVADNIIKTRYIDGFFM

492 ADPNRQYADVDTIEPYALLALEAAVRNQPQSVAPFLNGAGFTEGGYRMEDGSTRIS
    : : ::::::: :::::::::::  :: :: :::::::::::::
496 ASPDRQYADVDAIEPYALLALEASLRNKPQAVAPFLNGAGFTEVLT 541 PEL Y

548 TRDNEIFLLNVGETLKPNNKK 568 PEL EC153
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**Fig. 3.** Homology between the protein products of the *pel*Y and *pel*153 genes. Identical amino acids are denoted by (:) between them. The *pel*Y gene product is 27 amino acids shorter than that from *pel*153.

**Table 2.** Pectate lyase present in the periplasmic fractions of cells of *Escherichia coli* carrying plasmids with the *pel*153 gene from *Erwinia carotovora* EC153

| Plasmid | *Pel* gene | Induction[a] | Pectate lyase (units per gram of cells)[b] |
|---|---|---|---|
| pPEL153-1 | *pel*153 | − | 9.7 |
| pPEL153-3 | *pel*153 | − | 45.5 |
| | | + | 47.8 |
| pPEL153-5 | *pel*153 | − | 13.4 |
| | | + | 602.0 |
| pPEL153-6 | *pel*Y/*pel*153 | − | 29.0 |
| pPEL153-7 | *pel*Y/*pel*153 | − | 4.7 |
| | | + | 255.0 |
| pPEL153-8 | *pel*153::npt | − | <0.1 |
| pPELY16 | *pel*Y | − | 8.1 |
| | | + | 202.0 |
| pPEL410 | *pel*C | + | 12,400.0 |

[a] All cultures (15 ml in 50-ml DeLong flasks) were grown at 28° C for a total period of 20–26 hr, 10–16 hr of which were in stationary phase; pPEL153-1, 153-3, and 153-8 were grown in strain DH5α; all other plasmids were grown in strain D1210λ. Induction was with addition of IPTG to 1 mM at culture initiation in the case of pPEL153-3 and pPEL410; IPTG was added to all cultures carrying pPEL153-5, 153-7, and Y16, and induction in these cases was by a 10- to 15-min exposure of cultures to 42° C in a water bath (after 2–5 hr growth at 28° C to attain a density of about 0.9 A at 500 nm) before returning them to growth overnight at 28° C.

[b] Data reported are for periplasmic fractions, which were observed to contain 90% or more of the total PL activity. Cell weights are on a wet basis.

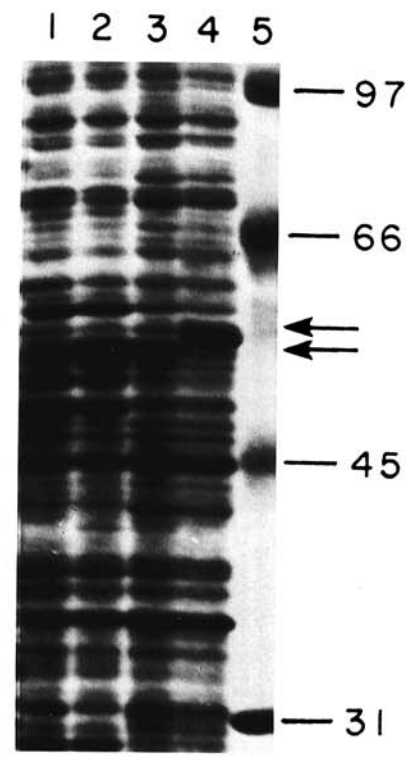


**Fig. 4.** Sodium dodecyl sulfate-polyacrylamide gel of proteins from D1210λ cells of *E. coli* carrying expression plasmids with various *pel* genes. Lane 1, cells of *E. coli* carrying pPELY14, encoding low levels of PLy; lane 2, cells carrying pPELY15, encoding PLy; lane 3, induced cells carrying pPELY16, encoding PLy, after temperature induction; lane 4, induced cells carrying pPEL153-5, encoding PL153, after temperature induction; lane 5, molecular weight markers with sizes in kDa; arrows denote positions of the PL153 (upper) and PLy (lower) proteins.

than the computer-generated value of 8.1 deduced above from sequence data, but we have observed similar discrepancies with PLs from *E. chrysanthemi* (Keen and Tamaki 1986; Tamaki *et al.* 1988).

The *pel*153 gene product preferred polypectate to pectin as substrate, but like the *pel*Y gene product (Manulis *et al.* 1988) was significantly more active on pectin than the *pel*E gene product of *E. chrysanthemi* (data not shown). In comparisons that used a viscosimetric assay, PL153 gave a three- to fourfold greater rate of viscosity reduction on sodium polypectate than did PLc, based on equivalent PL activities as determined with the spectrophotometric assay (data not shown). Because PLc is considered to be an endo-type enzyme, catalyzing random cleavage of the polypectate chain (Barras *et al.* 1987), the results indicate that PL153 also cleaves randomly.

**Mutagenesis of the *pel*153 gene in strain EC153.** Plasmid pPEL153-1 was restricted at the unique *Stu*I site occurring in the *pel*153 and a DNA fragment encoding a *npt* gene conferring kanamycin resistance was inserted to generate pPEL153-8 (see Table 1). Cells of *E. coli* carrying pPEL153-8 did not produce detectable PL activity (Table 2). This result confirms that the ORF identified in Figure 2 encodes PL activity; it also confirms earlier indications that only one functional *pel* gene occurs in clone pPEL153-1.

Plasmid pPEL153-8 was introduced into strain EC153 by conjugation, and kanamycin-resistant, tetracycline- and ampicillin-sensitive colonies were selected following growth on low phosphate medium without antibiotics. To confirm that mutation of the *pel*153 gene had occurred, DNA was purified from one of the mutant colonies as well as the wild-type strain EC153, two other isolates of *E. carotovora*, and two isolates of *E. chrysanthemi*. Following restriction with *Bam*HI, the DNA was separated on a 0.8% agarose gel and blotted onto a Zeta-bind membrane before probing with the *Bam*HI DNA fragment containing *pel*153 (Fig. 1). As shown in Figure 6, wild-type EC153 and strain 0285-11 of *E. carotovora* yielded a *Bam*HI band of about 2.2 kb that hybridized strongly to the *pel*153 probe. In addition, strain 73-22 of *E. carotovora* also yielded a strongly hybridizing 2.2-kb *Bam*HI band (data not shown). The mutant strain EC153 *pel*153::npt, however, contained only a strongly hybridizing band at about 4.0 kb, proving that mutation of the *pel*153 gene had occurred by insertion of the *npt* gene (Fig. 6). Neither of the two DNAs of *E. chrysanthemi* hybridized to the *pel*153 probe, indicating that these bacteria do not contain the *pel*153 gene.

Culture fluids of strain EC153, strain EC153 carrying plasmid pPEL153-8, and the mutant strain EC153 *pel*153::npt were electrofocused on thin polyacrylamide layers and PLs detected with polypectate overlays (Fig. 5). The major activity in all three strains was due to three or more highly basic PLs that focused together above pI 9.7. These are presumed analogous to the highly basic products of *pel* genes sequenced from other strains of *E. carotovora*. In no case was the product of the *pel*153 gene detected from culture fluids, and no differences were observed between the wild-type strain and the *pel*153 mutant strain. In Figure 5, the relatively light band seen for the *pel*153 protein from *E. coli* (lane 3) represents about 10 times more activity units applied to the electrofocusing gel than for lane 5 containing PLc of *E. chrysanthemi* EC16. Thus, failure to detect the *pel*153 gene product was due at least partly to the fact that the polypectate overlay technique is much less sensitive for detection of the *pel*153 and *Yersinia pel*Y gene products than of the lower molecular weight PLs.

**Maceration by PL153 and pathogenicity of EC153 *pel*153:npt.** Preparations of PL153 recovered from the periplasmic fraction of cells of *E. coli* overexpressing *pel*153 gave moderate maceration activity against cucumber mesocarp slices. When compared to preparations of PLe and PLc of *E. chrysanthemi* EC16, PL153 was about 40 times less active than PLe and about four times less active than PLc when all preparations were normalized for PL activity. PL153 was considerably more active, however, than PLa of *E. chrysanthemi* (Tamaki *et al.* 1988) or PLy from *Y. pseudotuberculosis* (Manulis *et al.* 1988).

Inoculation of potato tubers with either the wild-type EC153 or the EC153 *pel*153::npt mutant strain gave severe soft-rotting symptoms after 48 hr at 31° C (data not shown). No significant differences, however, were observed in the degree of soft-rotting by the two strains. Thus, mutation of the *pel*153 gene had no detectable effect on soft-rot pathogenesis.

## DISCUSSION

Strains of *E. chrysanthemi* produce PLs of about 40 kDa in size, but isolates of *E. carotovora* appear to produce more

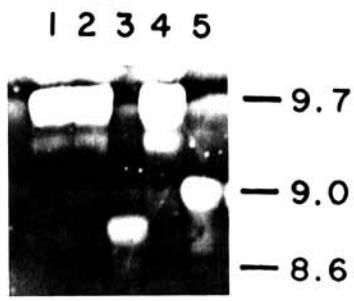


**Fig. 5.** Polygalacturonate overlay of electrofocusing gel with various pectate lyases. Lane 1, concentrated culture fluids of *E. carotovora* strain EC153 *pel*153::npt; lane 2, wild-type strain EC153 carrying pPEL153-8; lane 3, periplasmic fraction from *E. coli* cells D1210λ carrying pPEL153-5 and producing PL153; lane 4, wild-type EC153; lane 5, periplasmic fraction of D1210λ cells of *E. coli* carrying pPEL410, encoding PLc of *E. chrysanthemi* EC16. Isoelectric points of reference proteins are shown on the right.

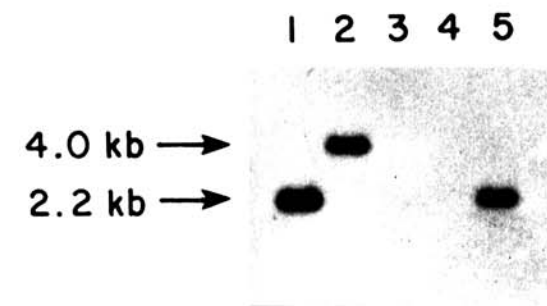


**Fig. 6.** Southern blot of total genomic DNA from several *Erwinia* strains restricted with *Bam*HI and separated on a 1.0% agarose gel. The blot was probed with a 2.2-kb *Bam*HI fragment carrying the *pel*153 gene (see Fig. 1.). Lane 1, wild-type strain EC153; lane 2, mutant strain EC153 *pel*153::npt; lane 3, strain EC16 of *E. chrysanthemi*; lane 4, strain 3937 of *E. chrysanthemi*; lane 5, strain 0285-11 of *E. carotovora*.

diverse PLs as well as enzymes with considerable homology to the *pel*B/C family of *E. chrysanthemi* (Tamaki *et al.* 1988). For example, isolates of *E. carotovora* have been observed to produce PLs of 78 kDa (Hu *et al.* 1987) and 61 kDa in the present case. Strain EC14 of *E. carotovora* has also been reported to produce a PL of about 31 kDa with a pI of 9.5 (Roberts *et al.* 1986). In addition, isolates of *E. carotovora* produce macerating polygalacturonases (Lei *et al.* 1985). Thus, isolates of *E. carotovora* appear to exhibit greater diversity in the pectic enzymes produced than *E. chrysanthemi*.

Another difference between the two groups is that the *pel*B/C genes of *E. chrysanthemi* encode neutral or slightly alkaline enzymes, but the homologous genes in *E. carotovora* encode more basic PLs with pIs above 9.0. The *pel*153 gene of *E. carotovora* that we have characterized therefore appears to be analogous to the *pel*B/C genes of *E. chrysanthemi* with respect to isoelectric point and maceration efficiency of the gene product. It is perhaps noteworthy in this regard that *E. chrysanthemi* does not contain DNA that hybridizes with the *pel*153 gene (Fig. 6).

Hybridization data showed that the strain EC153 *pel*153 gene occurs in two other strains of *E. carotovora* (Fig. 6); this gene also appears similar to a *pel* gene occurring in clone H2 of strain SCRI193 by Plastow *et al.* (1986). Indeed, recent comparisons of sequence data have confirmed that this gene is highly homologous to our *pel*153 gene (J. Hinton, personal communication). Ried and Collmer (1986) also noted the production of a slightly alkaline PL by three strains of *E. carotovora* and by three strains of *E. carotovora* subsp. *atroseptica*. However, we were not able to detect the *pel*153 gene product on thin-layer electrofocusing gels of EC153 culture fluids, presumably due to the poor sensitivity of polypectate overlays for detection of PL153.

We were surprised to find that the EC153 *pel*153 gene possessed considerable homology with the *pel*Y gene recently sequenced from *Y. pseudotuberculosis* (Manulis *et al.* 1988). It is significant that genes with such high homology occur in enteric bacteria that are pathogens of plants and animals. Despite their considerable differences in isoelectric point and the somewhat smaller size of the *Yersinia* PL, the *pel*Y and *pel*153 genes have several similarities. For instance, neither gene is closely linked to other pel genes (Manulis *et al.* 1988; this study).

This is in marked contrast to other pel genes previously described from *Erwinia* spp., which occur in clusters (Kotoujansky 1987). In addition, the protein products of both the *pel*153 and *pel*Y genes are not efficiently secreted by the bacteria from which they were cloned (Chatterjee *et al.* 1979) or from cells of *E. coli*, despite the fact that both proteins possess signal peptide sequences and are readily secreted to the periplasm of *E. coli*. The two protein products also exhibited toxic effects on cells of *E. coli*. Thus, constructs in which the genes were oriented downstream of the *lac* promoter of pUC plasmids decreased the growth rate of *E. coli* (in the case of the *Yersinia pel*Y gene), and the EC153 *pel* gene could not be cloned at all downstream of the *lac* promoter on pUC plasmids.

Despite this toxicity, the proteins were efficiently overexpressed in cells of *E. coli* by using an invertible promoter vector, pNH18a, constructed by Hasan and Szybalski (1987). With this plasmid, DNA fragments carrying a gene of interest are initially cloned in the opposite orientation to a vector promoter cassette containing the *lac* and *tac* promoters. The constructs are grown in the lysogenic strain D1210λ of *E. coli*, which produces the cI857 temperature-sensitive phage lambda repressor. Cells are grown to high density at 28° C, followed by a brief exposure to 42° C that inactivates the repressor and induces promoter inversion; cells are then returned to 28° C for protein production. In the case of both the *Yersinia* and strain EC153 *pel* genes, significant amounts of enzyme could be isolated from the periplasmic fractions of cells of *E. coli* in this way (Table 2).

It was surprising that the *pel*153 gene encoded a protein product that was 31 amino acids longer at the C terminus than that from the *pel*Y gene (Manulis *et al.* 1988). Members of the *pel*B/C and *pel*A/D/E gene families thus far sequenced from *E. chrysanthemi* (Tamaki *et al.* 1988) and *E. carotovora* (Ito *et al.* 1988; Lei *et al.* 1987, 1988) have completely conserved carboxyl termini; furthermore, the EC16 *pel*B gene of *E. chrysanthemi* (Keen and Tamaki 1986) did not produce an active product when it was truncated at a unique intragenic *Bgl*I site to form a gene product with six less C-terminal amino acids (Trollinger, unpublished data).

Cells of *E. coli* producing either the *Yersinia* or EC153 PLs did not readily secrete them to the medium, although the proteins were efficiently secreted into the periplasm. Thus, chloroform lysis was required to get readable pectate plate assays when cells of *E. coli* carried moderate-producing plasmid constructs of either gene. Whether the decreased secretion is due to the relatively large size of the proteins or other factors is not known. It is noteworthy in this regard that strain EC153 is recognized as a relatively inefficient secretor of PLs (Chatterjee *et al.* 1979). *Erwinia pel* gene products are generally secreted to the culture medium through the function of protein products from "out" genes (Thurn and Chatterjee 1985). It has not been established whether these proteins function in strain EC153 and whether they work in concert with the *pel*153 gene product.

The marker exchange mutant lacking *pel*153 gave soft-rotting symptoms in two potato tuber assays that were indistinguishable from the wild-type strain EC153. This indicates that the other PLs produced by strain EC153 are sufficient to produce maceration in the assays employed. However, the fact that the *pel*153 gene appears to be conserved in four different strains of *E. carotovora* leads to the speculation that the *pel*153 gene product may have some as yet unknown role in pathogenicity or saprophytic survival of the bacteria.

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