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Evidence of Homology between the Pectate Lyase-Encoding *pelB* and *pelC* Genes in *Erwinia chrysanthemi*

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The genes for two of several pectate lyase isozymes produced by the phytopathogenic enterobacterium *Erwinia chrysanthemi* 1237 were subcloned and compared by DNA-DNA hybridization, and the encoded proteins were analyzed. The borders of the genes were located on a restriction map by incremental exonuclease III deletions. DNA-DNA hybridization studies revealed a low percentage of mismatch (7 to 17%) between *pelB* and *pelC*. No homology was detected between *pelC* and other regions of the *E. chrysanthemi* 1237 chromosome, in which three other isozyme genes apparently reside. The pectate lyase isozymes were readily purified by chromatofocusing or granulated-gel bed isoelectric focusing from the periplasmic shock fluids of *Escherichia coli* subclones. The molecular weights of PLb and PLc were 30,000 and 33,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Their isoelectric points were 7.6 and 8.1, respectively, as determined by equilibrium isoelectric focusing in ultrathin polyacrylamide gels. The K_m values for PLb and PLc were 0.20 and 0.32 mg/ml, respectively, with polygalacturonate as a substrate. Thin-layer chromatography of reaction products and viscometric assays revealed little difference between the two isozymes. All our data indicate that the genes are duplicates and that the proteins are isofunctional.

Pectic enzymes are produced by many plant pathogens and have been implicated as important disease factors, most notably in the soft-rot diseases caused by certain *Erwinia* species (5, 10, 11, 14, 30). The characteristic symptoms of soft rot are maceration of parenchymatous tissue and cell death. Isolated pectate lyase (PL) can also cause these symptoms (2, 3, 30, 43). Other evidence for the importance of PL as a disease factor includes the observation that recombinant *Escherichia coli* clones which produce PL show at least a limited ability to macerate potato tubers (12, 19) and that mutants of *Erwinia chrysanthemi* lacking the ability to produce extracellular PL are nonpathogenic (10).

E. chrysanthemi produces multiple isozymes of PL (6, 12, 14, 19, 21, 32, 44). Instances of isofunctional enzymes are not unknown in procaryotes (1, 9, 20, 39, 41), although they are rare compared with those in eucaryotes (24). The PL isozymes produced by *E. chrysanthemi*, which are distinguished by their isoelectric points, have many similar functional characteristics. They have alkaline pH optima, require divalent cations, cleave internal glycosidic linkages in polygalacturonic acid by β-elimination, and have varying abilities to macerate and kill plant tissue (5, 14).

Molecular and in vivo cloning has revealed that *E. chrysanthemi* 3937 and B374 have five *pel* genes arranged in two clusters (21, 33, 44). Two of the genes, *pelB* and *pelC*, are adjacent and encode two neutral (pI 7.7 to 8.3) isozymes, PLb and PLc. One acidic and two alkaline isozymes are produced from the *pelA*, *pelD*, and *pelE* genes, respectively, which form a second cluster. The *pelC* and *pelB* genes have also been cloned from *E. chrysanthemi* 1237, which appears to produce the same five isozymes (12).

The multiplicity of PL isozymes produced by *E. chrysanthemi* and the difficulty in separating those within a particular class have prevented characterization of individual isozymes. As a step toward understanding the origin and functional basis of PL isozyme multiplicity in *E. chrysanthemi*, we explored the relatedness of the *pelB* and *pelC* genes and the properties of the encoded proteins.

We confirmed by subcloning that the two neutral PL isozymes in *E. chrysanthemi* 1237 are encoded by two independently expressed structural genes. One border of each gene was defined by exonuclease III digestion. This enabled excision of the *pelC* gene for use in DNA-DNA hybridization experiments with the region containing *pelB*. We report a close genetic relationship between *pelC* and *pelB*. As the isozymes were produced independently by separate subclones, we were able to purify and characterize each isozyme.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. chrysanthemi* CUCPB 1237 (Rif^r Str^r) was the source of the cloned *pel* genes (12). The recipients for the cloned DNA were *E. coli* HB101 (*leu proA2 ara-14 xyl-5 galK2 mtl-1 lacY1 thi supE44 recA13 rpsL20 hsdS20*) (7) and *E. coli* JM107 [*endA1 gyrA96 thi hsdR17 supE44 relA* λ^- Δ(*lac-proAB*) F′ *traD36 proAB lacI*^{q}*A*Δ*M15*] (45). Recombinant plasmids were derived from pCSR6 (12) (Fig. 1). All recombinants were grown on LB medium containing ampicillin (50 μg/ml in broth, 100 μg/ml in solid medium). Growth of bacteria on pectate semisoft agar allowed detection of pectolytic phenotypes (42).

General DNA manipulations. Plasmid DNA was isolated and manipulated by standard techniques (25, 26). *E. chrysanthemi* chromosomal DNA was isolated by the method of Matthysse (28). Restriction sites were located by determining the order of overlapping fragments from single and double restriction endonuclease digestions.

Independent expression of PLb and PLc. The construction of plasmids containing *pelB* and *pelC* was accomplished by unidirectional, incremental digestion of recombinant plasmids pCS61951 and pCS6183, respectively, with exonuclease III by the method of Roberts and Lauer (36) (Fig. 1). Pectolytic activity of the subclones was characterized by a rapid isoelectric-focusing (IEF) technique. Day-old bacterial lawns were scraped from petri plates into microcentrifuge

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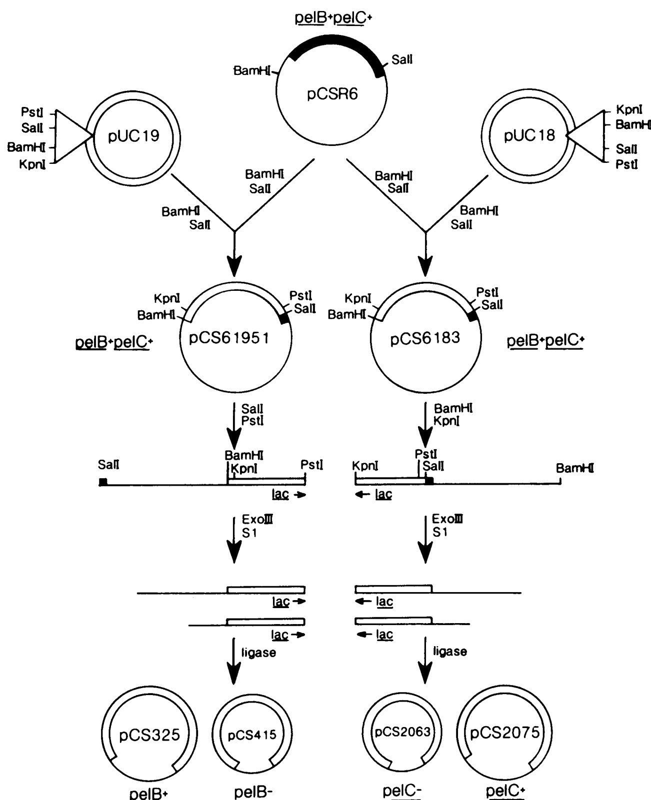

FIG. 1. Construction of subclones expressing *pelB* and *pelC*. Only restriction sites used in the subcloning are shown. Details of the subcloning are provided in the text. The thick solid and open bars represent vector DNA.

tubes containing 10 μl of distilled water. The tubes were vortexed vigorously and centrifuged for 5 min in an Eppendorf microcentrifuge. Supernatants were subjected to IEF as described previously (34). Induction experiments with pUC plasmid recombinants were performed by the method of Rodriguez and Tait (37). PL production was analyzed by spectrophotometric assays (12) and by activity-stained IEF of culture supernatants.

Hybridization conditions. The region of cloned DNA extending from 1.5 to 5.5 kilobases (kb) (Fig. 2) and containing both *pelB* and *pelC* was digested with various restriction endonucleases. The products of these digestions, after electrophoresis through a 1.2% agarose gel (Fig. 3A), were transferred to Gene-Screen nylon membranes by the Southern procedure (40) modified for Gene-Screen (New England Nuclear Corp., Boston, Mass.) Prehybridization and hybridization solutions contained 10% formamide for the low-stringency condition and 50% formamide for the high-stringency condition, 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), Denhardt reagent, 0.025 M sodium phosphate, and 100 μg of sonicated salmon sperm DNA per ml. Hybridization solutions contained 40 to 100 ng of denatured probe per ml (specific activity, 10^8 cpm/μg of DNA). The probe was the *Ava*I-*Eco*RI (*Eco*RI in the multiple cloning site of pUC18) fragment from pCS2063 which had been nick translated with [α-^{32}P]dATP for 1 h according to the Bethesda Research Laboratories nick translation system protocol. Hybridizations proceeded at effective temperatures of T_m − 38°C for 24 h and T_m − 10°C for 42 h. Blots were washed at the effective hybridization temperature and autoradiographed. The blot subjected to low-stringency hybridization (T_m − 38°C) was subsequently washed at increasing temperatures (T_m − 23°C, T_m − 10°C) and autoradiographed after each wash. A blot of *Eco*RI-*Ava*I-digested *E. chrysanthemi* 1237 DNA was allowed to hybridize with the probe at an effective temperature of T_m − 46°C. Washes were performed at T_m − 46°C and T_m − 36°C. The melting temperature (T_m) of *E. chrysanthemi* DNA was estimated to be 96°C in 0.33 M Na^+, based on the formula $T_m = 81.5 + 16.6(\log M) + 0.41(G+C\%) - 0.72(\%formamide)$ (29). M is the monovalent salt molarity and G+C% is the percentage of guanine plus cytosine residues in the DNA. The G+C% for *E. chrysanthemi* is 50 to 58% (23). An average value of 54% was used for melting temperature calculations.

Purification of the isozymes. PLb and PLc were obtained from the subclones producing each isozyme independently by the osmotic shock procedure of Heppel (16). The isozymes in the shock fluid were purified by either granulated-gel bed IEF (PLc) as previously described (13) or column chromatofocusing (PLb) (Pharmacia, Uppsala, Sweden), desalted on a BioGel P-6DG column (Bio-Rad Laboratories, Richmond, Calif.), and concentrated with a Millipore Immersible-CX Ultrafilter (Millipore Corp., Bedford, Mass.).

Physical properties of the isozymes. Isoelectric points were determined from an activity-stained pectate-agarose overlay after electrofocusing to equilibrium the purified proteins next to pI markers (FMC Corp., Philadelphia, Pa.) in ultrathin-layer polyacrylamide gels (12, 34). Equilibrium focusing was achieved in a 9-cm gel after 2.5 h at 6 W constant power and a 2,100 V limit. The gel was prefocused for 20 min at 2 W. Voltage after 20 min was 500 V. After prefocusing, the anodic wick was replaced with fresh 0.4% aspartic acid. Samples were applied in two portions on pieces of wick, one in the center of the anodic half of the gel and one in the center of the cathodic half of the gel. Molecular weights were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (22). The resolving and stacking gels contained 12.5% acrylamide–0.33% bisacrylamide and 5% acrylamide–0.13% bisacrylamide, respectively. The SDS

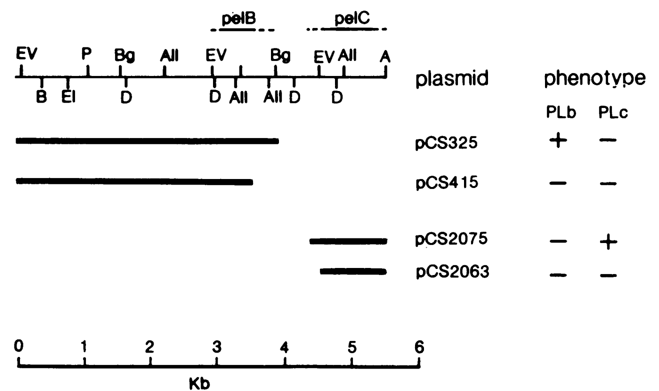

FIG. 2. Truncated products of exonuclease III digestion of plasmids containing *pelB* and *pelC* and their pectolytic phenotype. After exonuclease III digestion for various periods of time, the shortened plasmids were religated and introduced into *E. coli* by transformation. Expression of PL was detected on pectate semisoft agar (42). The plasmids for PL$^+$ and PL$^-$ colonies were extracted and digested with *Eco*RV and *Eco*RI (pUC18) or with *Eco*RV and *Hin*dIII (pUC19) to determine the extent of digestion. Solid bars represent DNA remaining after digestion. Restriction sites are: A, *Ava*I; A11, *Ava*II; B, *Bam*HI; Bg, *Bgl*I; D, *Dde*I; E1, *Eco*RI; EV, *Eco*RV; P, *Pvu*II.

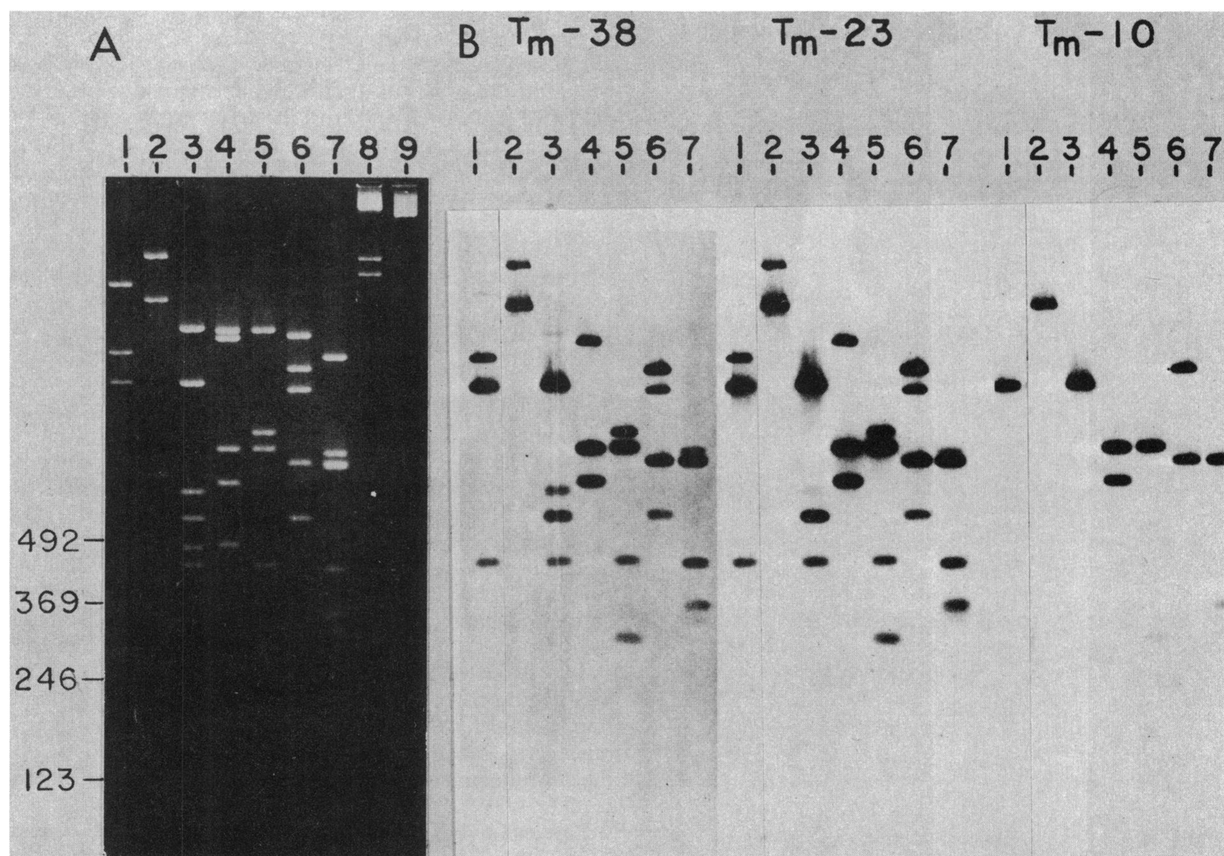

FIG. 3. Hybridization of *pelC* with cloned fragments containing *pelB*. (A) Ethidium bromide-stained 1.2% agarose gel of electrophoresed fragments resulting from various restriction endonuclease digestions of a 4.0-kb segment of *E. chrysanthemi* DNA containing *pelB* and *pelC*. The lanes are digestion products of the following enzymes: 1, *Eco*RV; 2, *Bgl*I; 3, *Eco*RV-*Bgl*I; 4, *Dde*I; 5, *Dde*I-*Eco*RV; 6, *Ava*II; 7, *Ava*II-*Eco*RV (band 7 is not visible in this photograph); 8, *Hin*dIII-cut λ markers; 9, 123-base-pair ladder; sizes of smaller fragments (in base pairs) are labeled to the left of lane 1. (B) Autoradiographs of a Southern blot of the gel shown in panel A. Hybridization was done at an effective temperature of T_m − 38°C with a ^{32}P-labeled (specific activity, 10^8 cpm/μg) pCS2063 fragment approximating *pelC*. Subsequent washes were done at T_m − 23°C and T_m − 10°C as indicated. Lanes are as in panel A.

(Bio-Rad) concentration was 0.1% in running buffer and gel. Samples consisted of 62.5 mM Tris hydrochloride (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.003% bromophenol blue, and approximately 0.1 μg of PL isozyme or 0.1 μg each of five marker proteins (Sigma Chemical Co., St. Louis, Mo.) (see Fig. 5). The samples were boiled for 90 s and then electrophoresed at 10 mA for 11 h through a 0.75-mm gel in a Hoeffer SE400 vertical slab unit (14 by 16 cm) (Hoeffer Scientific Instruments). The gel was immediately silver stained by the procedure of Heukeshoven and Dernick (17).

Enzymatic properties of the isozymes. Reaction products were identified by thin-layer chromatography (46) after polygalacturonic acid was incubated with PL at various concentrations (1,000 to 0.001 U/ml). One unit of PL activity is defined as that amount of activity necessary to produce 1 μmol of product per min under optimal conditions at 25°C (12).

The K_m of each isozyme was calculated from Lineweaver-Burk plots of rates of increase in A_{230} at various substrate concentrations. The reaction mixtures contained 1.40 to 0.014 mg of polygalacturonic acid per ml, 30 mM Tris hydrochloride (pH 8.8), 0.1 mM $CaCl_2$, and 0.3% (vol/vol) enzyme sample.

The rates at which PLb and PLc reduced the viscosity of a 12-mg/ml polygalacturonate solution were measured in an Ostwald-Fenske 200 viscometer (4). The reaction mixtures consisted of normalized samples of purified enzyme (1 U/μl), 0.12 M Tris hydrochloride (pH 8.5), 0.4 mM $CaCl_2$, and 12 mg of polygalacturonic acid per ml and were incubated at 30°C for various intervals.

RESULTS

Location and independent expression of *pelB* and *pelC*. The right border of *pelB* and the left border of *pelC* were located by incremental exonuclease digestion from opposite sides of the *Bam*HI-*Sal*I fragment of pCSR6 (Fig. 1). After ligation of this fragment into pUC18, the resulting plasmid, pCS6183, was cleaved with *Bam*HI and *Kpn*I between the pUC18 *lac* promoter and the left border of the insert. The *Bam*HI-*Sal*I fragment from pCSR6 was ligated also into pUC19 producing pCS61951, which was cleaved similarly but with *Sal*I and *Pst*I whose sites were to the right of the right insert border and to the left of the *lac* promoter. Exonuclease III digestion was allowed to continue for periods varying from 5 min to 2 h. The truncated products were blunt-end ligated and introduced into *E. coli* HB101 by transformation. A total of 24 pectolytic and 24 nonpectolytic transformants were selected for analysis. In each case, small-scale plasmid isolations

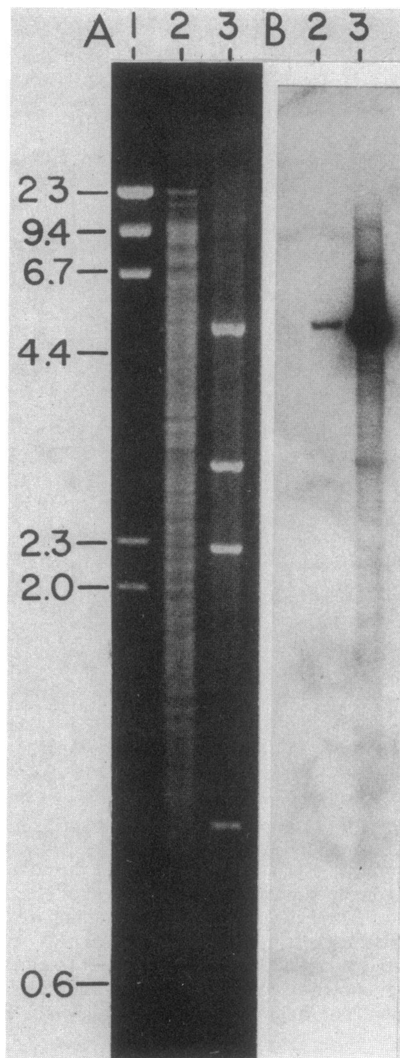

FIG. 4. Hybridization of *pelC* with *E. chrysanthemi* chromosomal DNA. (A) Ethidium bromide-stained 0.8% gel after electrophoresis of *Eco*RI-*Ava*I-cut *E. chrysanthemi* 1237 DNA (lane 2) and pCSR6 (lane 3). Lane 1 is *Hin*dIII-cut λ; sizes of fragments (in kilobases) are indicated on the left. (B) Autoradiographed Southern blot of the gel in the left panel that was allowed to hybridize with the labeled pCS2063 fragment approximating *pelC*. The dark band in the right lane is the DNA fragment from pCSR6 that contains both *pelB* and *pelC*. The left lane shows the corresponding band within the chromosomal digest.

were performed, and the plasmid DNA was digested with *Eco*RV to determine the extent of deletion. The plasmid pCS2063 is *pelC*$^-$ while plasmid pCS2075 expresses *pelC*, so the left boundary of *pelC* is between 4.4 and 4.5 kb on the map (Fig. 2). After ligation, the *lac* promoter is immediately upstream from the truncated end. Therefore, the *pel* gene will not be inactivated until the deletion extends into the structural gene itself, regardless of the orientation of the gene or its proximity to a native promoter. The protein has an apparent molecular weight of 33,000 (see Fig. 5) which requires ca. 0.9 kb of encoding DNA. Therefore, the position of *pelC* is ca. 4.4 to 5.3 kb on the restriction map (Fig. 2). The sizes of pCS325 and pCS415 demonstrate by similar logic the location of *pelB*. The apparent molecular weight of PLb is 30,000 which requires ca. 0.8 kb of encoding DNA (see Fig. 5); therefore, *pelB* was located to ca. 3.0 to 3.8 kb on the restriction map.

Addition of the *lac* inducer isopropyl-β-D-thiogalactopyranoside to *E. coli* JM107 clones containing pCS325 and pCS2075 had no effect on the level of expression of either isozyme, which indicates that expression is occurring from native promoters.

Homology of *pelB* and *pelC*. Restriction endonuclease fragments of the segment extending from 1.5 to 5.5 kb (Fig. 2) hybridized to a ^{32}P-labeled *pelC* fragment (Fig. 3). Hybridization with *pelB* fragments was abolished when the blot was washed at 86°C (T_m − 10°C) (Fig. 3). For example, see lane 1, band 4, which is the *Eco*RV fragment located at 2.9 to 3.3 kb, and the adjacent *Eco*RV-*Bgl*I fragment (lane 3, band 4). Assuming that each 1% base mismatch lowers the effective T_m by 1.4°C (18), the effective T_m for hybrids of *pelC* and all fragments making up *pelB* indicates a mismatch of 7 to 17% (8, 27). The *E. chrysanthemi* 1237 chromosome was probed for additional regions of homology to the *pelC* gene. Chromosomal DNA was digested with *Ava*I and *Eco*RI so that *pelB* and *pelC* would be on the same fragment. pCSR6 was digested with *Ava*I and *Eco*RI and electrophoresed in the lane next to the chromosomal DNA so that the *pelB-pelC*-containing band could be identified in the chromosomal lane (Fig. 4). At T_m − 46°C regions with less than 33% mismatch should be detectable. After washes at T_m − 46°C, specific hybridization was obscured by high nonspecific hybridization, so the blot was washed at T_m − 36°C (less than 25% mismatch). No hybridization was detectable on any region of the chromosome other than the band containing *pelB* and *pelC* (Fig. 4).

Physical and enzymatic properties of PLb and PLc. The PL

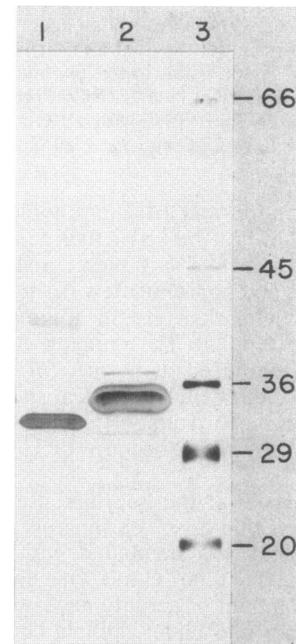

FIG. 5. SDS-polyacrylamide gel electrophoresis of PLb and PLc. Samples of chromatofocused PLb (lane 1) and PLc purified by granulated-gel bed IEF (lane 2) were electrophoresed through a 12.5% polyacrylamide-SDS gel and then silver stained. The molecular sizes of the marker proteins (lane 3) in kilodaltons (bovine serum albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsin inhibitor) are indicated to the right of the lane.

isozymes were substantially purified from the periplasmic shock fluid of *E. coli* subclones by a single step of either preparative IEF or chromatofocusing. The success of this protocol can be attributed to the high expression of the *pel* genes and the lack of other major *E. coli* periplasmic proteins with alkaline or slightly alkaline isoelectric points (data not shown). PLb and PLc had molecular weights of 30,000 and 33,000, respectively, as determined by SDS-polyacrylamide gel electrophoresis (Fig. 5). Isoelectric points were 7.6 and 8.1, respectively (Fig. 6). K_m values for PLb and PLc were 0.20 and 0.32 mg of polygalacturonic acid per ml, respectively. The enzymes were equally effective at depolymerizing the substrate, polygalacturonate, as observed from viscosity reduction. The viscosity index value for each enzyme was 568 (viscosity index = the reciprocal of the time in minutes required for 1 U of PL to cause 50% reduction in a 12-mg/ml polygalacturonate solution, multiplied by 10^3) (4). The same mixture of products (dimers, trimers, oligomers) was generated by both PLb and PLc as observed at given time points after initiation of the enzymatic reaction (Fig. 7).

DISCUSSION

We located on a more detailed restriction map the genes for the two neutral isozymes of PL produced by *E. chrysanthemi* 1237. The restriction sites in the multiple cloning site of pUC18, the vector in which the *pelC* gene was cloned, allowed us to excise a fragment which closely approximates this gene for use as a hybridization probe. To detect homology to various regions within *pelB*, a DNA segment spanning *pelC* and *pelB* was digested with various restriction endonucleases and blotted after electrophoresis. The hybridization results suggest a high degree of homology along the entire length of the two genes.

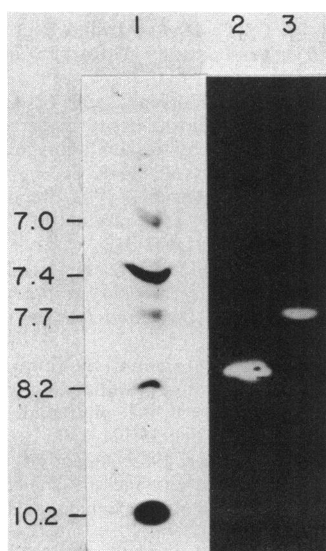

FIG. 6. Determination of the isoelectric points of PLb and PLc by ultrathin-layer polyacrylamide gel IEF. All samples were applied in two portions at opposite ends of their respective lanes. After focusing to equilibrium, the marker proteins (lane 1) were stained with Coomassie brilliant blue; the PL isozymes (PLc in lane 2 and PLb in lane 3) were actively stained with a substrate-agarose overlay. The pI markers were horse myoglobin, minor and major bands, whale myoglobin, minor and major bands, and cytochrome *c* (FMC Corp.).

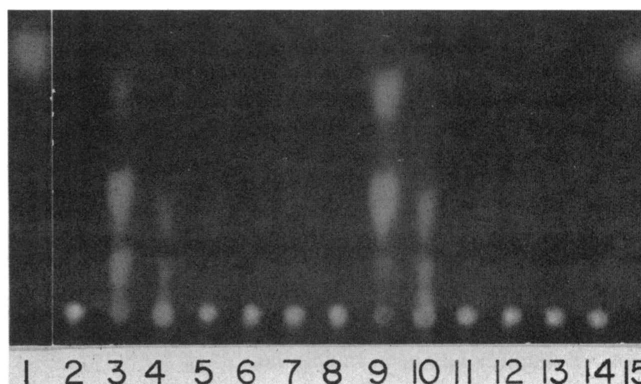

FIG. 7. Action pattern of PLb and PLc. Samples (50 μl) of substrate were incubated for 12 h with enzyme, after which 20 μl of each was spotted onto a Kodak Chromagram (no. 13255 cellulose) and developed for 8 h. The chromatogram was stained with bromophenol blue. Lanes: 1, monomer standard; galacturonate (20 μl of a 25% solution); 2, 20 μl of substrate without enzyme; 3, 1 U of PLb; 4, 0.1 U of PLb; 5, 0.03 U of PLb; 6, 0.01 U of PLb; 7, 0.003 U of PLb; 8, 0.001 U of PLb; 9, 1 U of PLc; 10, 0.1 U of PLc; 11, 0.03 U of PLc; 12, 0.01 U of PLc; 13, 0.003 U of PLc; 14, 0.001 U of PLc; 15, galacturonate (20 μl of a 25% solution).

The *E. chrysanthemi* PL isozymes fall into three distinct classes based on pI values (32). The more alkaline isozymes are larger (12, 19), better able to reduce the viscosity of pectate solutions, and more effective at macerating host tissue (14). We observed differences in the two neutral isozymes, PLb and PLc, but as would be predicted from the genetic similarity, they were relatively minor. A 3,000-dalton difference in molecular size was determined by SDS-polyacrylamide gel electrophoresis. Both isozymes were similarly efficient at reducing the viscosity of a pectate solution. The action patterns for PLb and PLc were also similar. These findings argue against distinct functions for each of these isozymes.

Evolutionary studies have shown isofunctional genes to be relatively rare in procaryotes. Those that are known appear to be the result of convergence of function or gene duplication (1, 9, 15, 20, 31, 35, 39, 41, 47). Hybridization studies reported here demonstrate extensive homology between *pelB* and *pelC* DNA sequences. The apparent homology between *pelB* and *pelC* suggests a gene duplication. Other strains of *E. chrysanthemi* express a pair of neutral isozymes (21, 44; N. T. Keen and A. K. Chatterjee, personal communications). This would indicate a relatively ancient duplication and strict conservation of the duplicates. Alternatively, duplications in other strains may have arisen independently. Interestingly, restriction maps for the regions containing the *pelB* and *pelC* genes of strains 1237, EC16, and 3937 bear little resemblance (Fig. 2) (19, 21).

No homology was detected between *pelC* and the other classes of isozymes (alkaline and acidic). It is therefore unlikely that a close genetic relationship exists between the neutral and the other classes of isozymes. This is not surprising in view of the differences between the respective proteins.

The unusual multiplicity of genes suggests that PL is important in the life cycle of *E. chrysanthemi*. Multiple genes may allow for a more complex regulatory strategy, allow degradation of pectate in diverse environments, or simply provide for higher levels of PL production through increased gene dosage. However, *pelB* and *pelC* mutants of

E. chrysanthemi 1237 have failed to reveal any difference in the ability of the mutants to utilize polygalacturonate as a carbon source or to cause soft rot in intact potato tubers under experimental conditions (38; D. L. Roeder and A. Collmer, *in* E. L. Civerolo, A. Collmer, R. E. Davis, and A. G. Gillaspie, ed., *Proceedings of the Sixth International Conference on Plant Pathogenic Bacteria*, in press). Moreover, the present data suggest that the genes are duplicates and that the proteins are isofunctional and redundant. It is interesting that representative strains from the six taxonomic subdivisions within *E. chrysanthemi* all produce a pair of neutral isozymes (J. L. Ried and A. Collmer, Appl. Environ. Microbiol., in press). The question remains why this apparent redundancy has been maintained.

ACKNOWLEDGMENTS

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