JOURNAL OF BACTERIOLOGY, Sept. 1984, p. 825–831
0021-9193/84/090825-07$02.00/0

Vol. 159, No. 3

Molecular Cloning of Pectate Lyase Genes from *Erwinia chrysanthemi* and Their Expression in *Escherichia coli*

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Received 13 April 1984/ Accepted 5 June 1984

A genomic library of *Erwinia chrysanthemi* EC16 was constructed in plasmid pHC79, and seven putative pectate lyase (PL) clones in *Escherichia coli* were selected on pectate agar. Six of the recombinant cosmids contained a common *PstI* fragment of ca. 8.2 kilobases (kb). Subcloning of this fragment in either orientation into the *PstI* site of plasmid pBR329 resulted in *E. coli* transformants that produced a PL of pI 9.8 which was indistinguishable from one of two PLs produced by strain EC16. A 6.6-kilobase *PstI* fragment from the remaining cosmid clone caused production of an *Erwinia* PL of pI 8.8 when the fragment was subcloned in either orientation into plasmid pBR329 and transformed into *E. coli*. Selected pBR329 subclones for the 8.2- and 6.6-kilobase *PstI* fragments showed no similarity in their restriction maps and did not cross-hybridize. All of the *E. coli* cosmid clones that produced large amounts of PL also caused soft-rot of potato tubers and tuber slices, thus confirming the role of the enzymes in plant tissue maceration. The *E. coli* cosmid clones and plasmid pBR329 subclones produced the PLs constitutively, unlike *Erwinia chrysanthemi*, which made the enzymes inducibly. However, catabolite repression appeared to function in the *E. coli* clones, and almost all of the PL activity occurred in the periplasm and culture fluids. Thus, the *Erwinia* PL clones appear to contain signal peptide sequences, transcription and translation signals, and a recognition sequence for the catabolite activator protein, all of which function efficiently in *E. coli*.

The production of extracellular enzymes that degrade pectic polymers of higher plant cell walls is important in the pathogenesis of several microorganisms, including members of the genus *Erwinia* (1, 2, 8, 20). These bacteria elaborate several pectate lyases (PL; EC 4.2.2.2) which when purified have been shown to randomly cleave pectic substrates and to macerate plant tissue. In addition, the enzymes liberate oligosaccharides from purified pectic substances and higher plant cell walls that function as elicitors of active plant defense reactions (5, 7). PL produced by *Erwinia* spp. are largely secreted into the medium (1, 2), and their synthesis is regulated by induction and cyclic AMP-mediated catabolite repression (2, 19). Further investigation of the mechanisms underlying all of these processes would be facilitated by molecular cloning of the structural and regulatory DNA sequences accounting for PL production. We report here the molecular cloning of genes from *Erwinia chrysanthemi* EC16 that code for two different PL and their expression in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Erwinia chrysanthemi* EC16 was kindly supplied by A. Chatterjee. Strains of *E. coli* employed and the plasmids utilized and constructed are summarized in Table 1.

Chemicals and DNA electrophoresis. Restriction enzymes and T4 DNA ligase were obtained from Bethesda Research Laboratories or New England Bio-Labs. All other chemicals except where noted were purchased from Sigma Chemical Co. Gel electrophoresis of DNA was performed in various concentrations of Sigma low-EEO agarose or Bio-Rad low-melting agarose (Bio-Rad Laboratories) in Tris-acetate buffer (13), and DNA was visualized by ethidium bromide incorporated into the gels or by poststaining. Phage λ DNA fragments cut with *Hin*dIII were used as size standards. The occurrence of plasmids in bacteria was confirmed by using a modified rapid boiling method (4) and digestion with the appropriate enzymes before electrophoresis.

Cosmid cloning. Strain EC16 was grown on 200 ml of L broth for 24 h at 30°C, and the pelleted cells were either stored at −20°C and thawed before extraction or used fresh. DNA was extracted by modification of the method of Marmur (14). The cells were suspended in 15 ml of TES (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA, 150 mM NaCl), the suspension was incubated at 37°C for 5 min, and then 1.2 ml of 20% sodium dodecyl sulfate was added, and the mixture was incubated for 5 min at 37°C. Phenol-chloroform-isoamyl alcohol (24:24:1 [vol/vol]; 15 ml) was added and mixed in vigorously. The mixture was centrifuged for 10 min at 15,000 × *g* in a swinging bucket head. The upper layer was collected, and 2.4 ml of 3 M sodium acetate and 12.8 ml of isopropanol were added. The mixture was mixed well in a beaker, and the DNA was spooled out onto a Pasteur pipette. The DNA was dispersed into small pieces and redissolved in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA by heating at 65°C for 1 to 3 h with occasional shaking. The DNA was then purified by two successive rounds of CsCl-ethidium bromide centrifugation (16 h in a Beckman VTi50 rotor at 16°C and 50,000 rpm). No plasmids were detected in strain EC16 by CsCl gradients or by electrophoresis. When electrophoresed on agarose gels, preparations of DNA from frozen EC16 cells exhibited an abnormally high degree of shearing, which could not be avoided by alterations in the extraction procedure. Although the clones described in this paper were isolated from such DNA, later experiments showed that extraction from freshly pelleted *Erwinia* spp. cells led to more satisfactory DNA preparations upon agarose gel electrophoresis.

All plasmids were isolated from stationary-phase *E. coli*

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Source or reference
E. coli	
DH-1	(13)
HB-101	(13)
C-600	(13)
Plasmid	
pHC79	(9)
pBR329	(3)
pPL1 to pPL7	pHC79 cosmid clones from EC16
pPL34	*Pst*I subclone of pPL3 in pBR329
pPL74	*Pst*I subclone of pPL7 in pBR329
pPL742	*Eco*RI subclone of pPL74 in pBR329
Erwinia chrysanthemi	
EC16	Gift from A. Chatterjee (1)

cells grown in 1 liter of L broth containing the appropriate antibiotic. The rapid boiling method (6) was employed, and plasmids were then purified by two or three successive CsCl-ethidium bromide centrifugations as above except in a VTi65 rotor run at 50,000 or 65,000 rpm for 6 to 16 h at 15°C. Purified DNA from strain EC16 was partially digested with *Pst*I and fractionated on sucrose gradients. DNA between ca. 35 and 50 kilobases (kb), as determined by gel electrophoresis of samples from the gradients, was pooled. After isopropanol precipitation, the DNA was dissolved in ligation buffer and ligated to plasmid pHC79 cut with *Pst*I, usually at a 10:1 insert/vector ratio. The resulting concatamers were packaged into phage λ heads by the method of Hohn and Murray (10). Strain DH-1 cells grown for 12 h on L broth including 0.4% maltose were transfected and plated onto L agar containing 20 μg of tetracycline ml^{-1}. Tetracycline-resistant *E. coli* colonies which were ampicillin sensitive (45 to 85% in several experiments) were found to contain plasmids with large DNA inserts (ca. 40 kb) by electrophoresis.

Plate assay for PL activity. A plate assay was devised to rapidly screen the *E. coli* clones for PL production. Colonies from the EC16 library were transferred onto plates containing agar plus 20 μg of tetracycline ml^{-1} and onto 0.7% sodium polygalacturonate (85 or 98% pure; Sigma) in either L agar or YC agar (2 g of ammonium sulfate, 0.2 g of magnesium sulfate · 7HOH, 3 g of Casamino acids, 2 g of yeast extract, water to 1 liter; the medium was adjusted to pH 8.0 with NaOH, 15 g of agar was added, and the medium was autoclaved). The omission of antibiotics from the media did not influence results, and accordingly, the antibiotics were usually omitted. After 16 to 24 h at 37°C, the plates were flooded with 5 ml of 1 M $CaCl_2$ and allowed to stand at room temperature. After 5 to 30 min, distinct cloudy halos appeared around putative PL clones and control EC16 colonies grown on the same medium but without antibiotics and at 30°C. After cultures were left standing with the lids removed for 5 to 12 h, pronounced depressions were observed in the agar medium of positive clones as the plates dried. Although both media were satisfactory, the YC medium permitted greater sensitivity for detection of positive clones. The screening of 570 pHC79 clones with EC16 inserts yielded 7 putative PL clones which were investigated further.

Subcloning. In some experiments, selected PL cosmid DNA (2 μg) and plasmid pBR329 (0.5 μg) were digested with restriction endonuclease(s), and the pBR329 DNA was treated with calf alkaline phosphatase (Boehringer Mannheim Biochemicals) by the method of Crouse et al. (4). After incubation at 68°C for 15 min, the DNA samples were electrophoresed separately on low-melting agarose gels (0.7%; Bio-Rad) containing ethidium bromide at 0.5 μg ml^{-1} in a mini-sub cell (Bio-Rad). The appropriate bands were then cut from the gel and mixed. The agarose was melted at 68°C, and the DNA was directly ligated by the method of Crouse et al. (4) except that sterile gelatin was added to 100 μg ml^{-1}. Portions of the ligated DNA were then transformed into *E. coli* HB-101 or C-600 cells by the method of Morrison (18) with competent cells stored at −80°C. Transformants were screened on polygalacturonate agar plates, and PL-positive subclones were further characterized by electrophoresis of the intact and restricted plasmids.

Characterization of PL produced by the clones. Culture fluids from 8- to 16-h cultures grown on 50 or 200 ml of L broth or YC broth were directly assayed after centrifugation of the cells. The inclusion of antibiotics did not significantly influence enzyme production. The pelleted cells were then weighed, and sphaeroplasts were prepared by suspending each 200 mg of washed, freshly packed cells in 1 ml of 0.2 M Tris hydrochloride (pH 8.0) and proceeding exactly by the technique of Witholt et al. (24). The periplasmic fraction was collected after centrifugation of the mixture for 5 min at 10,000 × *g*, and the pelleted sphaeroplasts were lysed with an equivalent volume of 0.01 M Tris hydrochloride (pH 8.0). After centrifugation of the lysate for 10 min at 10,000 × *g*, the supernatant constituted the cellular fraction.

Erwinia chrysanthemi EC16 produced PL strictly extracellularly, and the enzyme was accordingly purified from culture fluids grown to late log phase at 30°C for ca. 24 h on the medium of Garibaldi and Bateman (8) containing 0.7% sodium polygalacturonate (85%; Sigma). The resulting culture fluids (usually batches of ca. 1 liter) were dialyzed for 24 to 48 h against several changes of 5 mM Tris hydrochloride (pH 8.0). Extracellular culture fluids and periplasmic fractions from the *E. coli* clones were dialyzed in the same manner.

Preparations of PL from EC16 or the *E. coli* clones were adjusted to pH 8.0 after dialysis and then pumped onto a column (2.75 by 10 cm) of phosphocellulose (Sigma) in 5 mM Tris hydrochloride (pH 8.0). After application of the enzyme in 50 to 1,500 ml of 5 mM Tris (pH 8.0), the column was washed with the same buffer until little or no additional material absorbing at 280 nm eluted, and the enzyme was then displaced with the same buffer containing 0.25 M NaCl. Fractions of 12 ml were checked for their absorbance at 280 nm and their PL activity; peak enzyme fractions were pooled and dialyzed against water or 5 mM Tris hydrochloride (pH 8.0).

Preparations of PL from the phosphocellulose column were further purified on an LKB 8100 preparative electrofocus column with LKB 3-10 or 9-11 Ampholines. The runs were made in linear sucrose gradients (1 to 10%) at 700 V for 48 h at 1°C. The column was eluted, and fractions (ca. 2.5 ml) were assayed for pH, absorbance at 280 nm, and PL activity. Peak enzyme fractions were pooled, dialyzed extensively against water, and lyophilized.

Preparations of PL purified by electrofocusing were run on 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gels (1.5 mm thick) by methods previously published (11). Proteins were detected by silver staining (16).

PL assay. Activity was determined by monitoring the absorbance of reaction mixtures containing sodium polygalacturonate at 232 nm with a Beckman Kintrac VII kinetics

spectrophotometer at 22°C. Reaction mixtures contained 0.5 ml of appropriately diluted enzyme, 0.5 ml of 0.2 M Tris hydrochloride (pH 8.5), 1 ml of 3 mM $CaCl_2$, and, to start the reaction, 1 ml of 1% (wt/vol) sodium polygalacturonate. Polygalacturonic acid (98% pure; Sigma) was dissolved in distilled water during adjustment to pH 8.5 with NaOH and centrifuged for 30 min at 20,000 × *g* to remove sedimenting materials. The increase in absorbance in the presence of PL was linear up to at least 1.0 absorbance unit at 232 nm, and 1.73 absorbance units min^{-1} was considered to represent the formation of 1 μmol of unsaturated uronide min^{-1} (25). In this paper, one unit of PL activity is defined as the liberation of 1 μmol of product min^{-1} under the conditions outlined above, and specific activity is defined as units $gram^{-1}$ of fresh bacterial cells.

Plant tissue maceration assays. In the first method, potato tuber slices (ca. 7 mm thick) were prepared, and wells (ca. 2 mm deep) were cut with a sterile no. 2 cork borer. The slices were washed with sterile water and then placed in petri dishes containing water in the bottom. The wells were filled with bacterial suspensions (ca. 50 μl; 10^7 cells ml^{-1}) in L broth, supplemented where appropriate with an antibiotic. The slices were incubated at 30 or 37°C and assessed at 12-h intervals for soft-rot symptoms.

In the second maceration assay, cells of EC16 or *E. coli* (ca. 10^8 cells ml^{-1}) in L broth with or without an antibiotic were injected ca. 1 cm into intact store-bought Russet potato tubers with a no. 18 needle and hypodermic syringe. Generally, 0.2 ml of cell suspension was injected at each of 3 places into each tuber. The wounds were then covered with vaseline, and the tubers were incubated at 30 or 37°C for 48 h and sliced for observation.

RESULTS

Isolation of PL clones. No trace of PL activity was observed in assays of culture fluids, periplasmic fractions, or cellular fractions of *E. coli* strains DH-1 or HB-101 or the same bacteria containing plasmid pHC79 or pBR329. Seven recombinant clones of plasmid pHC79 that formed halos on pectate agar plates were selected from the EC16 library (Fig. 1). The plasmids in these clones were provisionally referred to as pPL1, pPL2, etc. (Table 1). Clone PL3 produced the largest halo, whereas clones PL2 and PL6 gave very weak responses, and the others were intermediate. After several transfers at intervals of ca. 2 weeks on tetracycline-supplemented media and storage at 4°C, clones PL4 and PL5 produced smaller halos than they did when they were initially isolated, until halos were not observed at 9 months. Clones PL1, PL3, and PL7, however, gave consistent responses for the entire 9 months. After their initial isolation, the recombinant cosmids, as extracted by the rapid boiling method or purified by CsCl gradient centrifugation, all transformed DH-1 and HB-101 cells to tetracycline resistance, and these transformants all formed halos similar to those of the parent clones on pectate agar. Electrophoresis of the recombinant cosmids prepared by the rapid boiling method disclosed that all of the clones contained plasmids of ≥40 kb. Restriction of these plasmids with *Pst*I showed the presence of plasmid pHC79 and, in addition, all clones except pPL3 shared a common band at about 8.2 kb (Fig. 2). None of these other six clones yielded identical *Pst*I restriction patterns, however, indicating that siblings were not present. Clones PL3, PL4, and PL5 had overly intense bands at ca. 6.6 kb, the position of plasmid pHC79. This indicated that these clones either contained *Pst*I insertion fragments of a similar size to the vector or were polycosmids. Original clones PL4 and PL5 were probably polycosmids, since their progressively decreasing PL production corresponded to a loss of the *Pst*I band at ca. 8.2 kb.

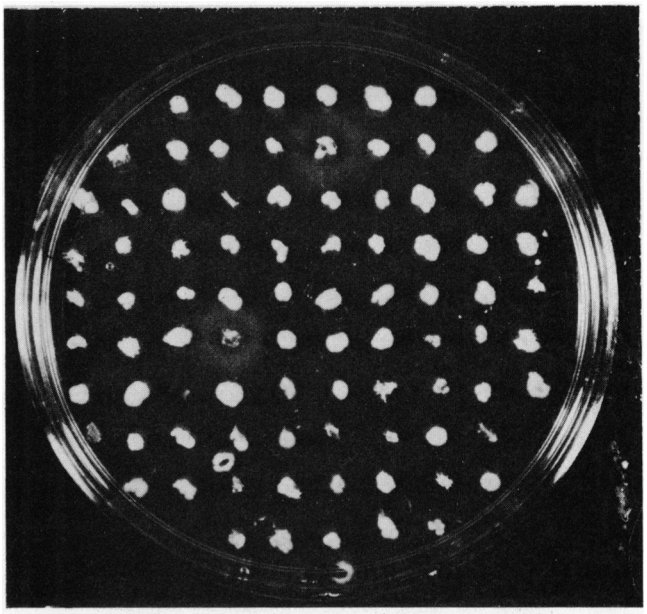

FIG. 1. Several randomly selected pHC79 library clones plated on L agar containing 0.7% sodium polypectate and 20 μg of tetracycline ml^{-1}. After growth for 24 h at 37°C, the plate was flooded with 5 ml of 1 M $CaCl_2$ and photographed after 30 min. Two putative PL-producing clones were identified by the halos surrounding the colonies.

PL activity in the *E. coli* clones. All seven putative PL cosmid clones exhibited PL activity in the culture fluids and periplasmic fractions, which was detectable by the spectrophotometric assay (Table 2). The PL activity exhibited by all clones was entirely abolished by addition of EDTA, as were the enzymes produced by *Erwinia chrysanthemi* (2). Strains HB-101 and DH-1 and the same strains harboring plasmid pHC79 or pBR329 did not exhibit detectable PL activity in any fraction. Unlike strain EC16, in which production of PL

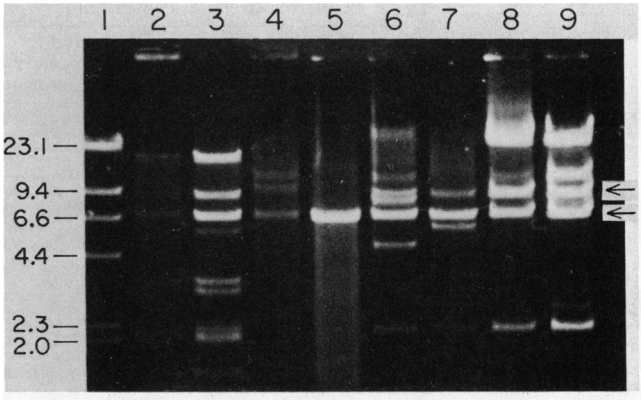

FIG. 2. Agarose gel (0.7%) showing the fragments generated from rapid-boil plasmid DNA with *Pst*I. Lanes: 1, phage λ *Hin*dIII standard with sizes of the fragments shown in kb pairs on the left; 2, pPL1; 3, pPL2; 4, pPL3; 5, pHC79; 6, pPL4; 7, pPL5; 8, pPL7; and 9, pPL6. Upper arrow denotes a common band at ca. 8.2 kb in all clones except PL3; lower area denotes pHC79.

TABLE 2. PL activity in seven EC16 cosmid clones selected on pectate agar and in strains DH-1 containing plasmid pHC79 and HB-101 containing plasmid pBR329

Strain or clone[a]	% Of total PL activity			Total activity (U/culture)[b]
	Culture fluid	Periplasmic fraction	Cell lysate	
E. coli				
DH-1(pHC79)	–	–	–	<0.01
HB-101(pBR329)	–	–	–	<0.01
Clone				
PL1	15	65	20	26
PL2	22	54	24	0.5
PL3	18	72	10	197
PL4	10	56	34	33
PL5	4	72	24	52
PL6	25	52	23	1.1
PL7	48	40	12	65

[a] All cosmid clones were in strain DH-1.
[b] Total activity is the summation of the activities in each cell fraction.

is strongly induced by pectic substances (1), inclusion of Sigma high-purity sodium polygalacturonate in the medium did not stimulate PL production by any of the *E. coli* clones (Table 3). Addition of Sigma sodium polygalacturonate (85% pure) caused considerable catabolite repression (data not shown), probably because of contaminating sugars. Repression of PL production was also observed when glucose was added to L broth cultures of various subclones of PPL3 and PL7 except pPL742 (Table 3).

Characterization of enzymes produced by the clones. During the course of the investigation, it was observed that the basic nature of the *Erwinia chrysanthemi* PL afforded a simple and efficient purification regime. This involved passing large volumes of dialyzed culture fluids or periplasmic fluids through a phosphocellulose cation-exchange column at alkaline pH and, after washing impurities through, eluting the enzymes with 0.25 M NaCl (Fig. 3A). After dialysis, phosphocellulose preparations from strain EC16 yielded a small activity peak at ca. pH 8.8 on the electrofocus column and a large peak at pH 9.8 (Fig. 3B). A small shoulder was consistently observed on the leading edge of the peak at pH 9.8, but it appeared that this was an artifact and not a third PL.

After partial purification by phosphocellulose chromatography, assays of the periplasmic PL of clones PL1, PL4, PL5, and PL7 all yielded single electrofocusing peaks at pH 9.8, and iterated experiments all exhibited the leading-edge asymmetry observed with the same peak from strain EC16. A representative elution profile is shown for clone PL1 (Fig. 3C). Assay of extracellular and periplasmic PL activity from clone PL3, however, yielded single symmetrical and coincident protein and activity peaks at pH 8.8 upon electrofocusing (Fig. 3D), with no detected activity at pH 9.8. Clones PL2 and PL6 were not investigated because of their low enzyme production.

Polyacrylamide gel electrophoresis of the proteins recovered from the electrofocusing column disclosed that the enzymes of pI 8.8 from *Erwinia chrysanthemi* and clone PL3 both had a mass of ca. 39 kilodaltons (Fig. 4). The apparent mass of the PL of pI 9.8 from *Erwinia chrysanthemi* was about 44 kilodaltons (Fig. 4), but the enzymes of pI 9.8 recovered from the periplasmic fractions of *E. coli* clones

TABLE 3. PL activity in *Erwinia chrysanthemi* EC16 and in various *E. coli* subclones and their parent cosmid clones on L broth, L broth plus 0.5% glucose, or L broth plus 0.5% sodium polygalacturonate

Strain or plasmid	PL activity (U g [fresh wt] of cells^{-1})[a]		
	L broth only	L broth plus glucose	L broth plus sodium polygalacturonate
Plasmid			
pPL3	35.2	2.48	37.5
pPL34	541	19.6	505
pPL7	13.3	4.8	15.0
pPL74	32.1	0.6	27.3
pPL742	60.5	57.1	65.8
pBR329	<0.1	<0.1	
Erwinia chrysanthemi EC16			585

[a] Data are averages of three replicates; all cultures were grown for 16 h on 200 ml of medium at 37°C without antibiotics; cell yields per flask varied between 0.5 and 0.9 g (fresh weight), and *E. coli* periplasmic fractions were prepared in a final volume of 16 ml; the data presented are for periplasmic fractions only. Garibaldi and Bateman medium containing sodium polypectate (8) was used for strain EC16 at 30°C, and PL activity was determined only in culture fluids. For growth of the *E. coli* clones, Sigma polygalacturonic acid (98% pure) was neutralized with NaOH and added to L broth after both were autoclaved at two times the final concentration.

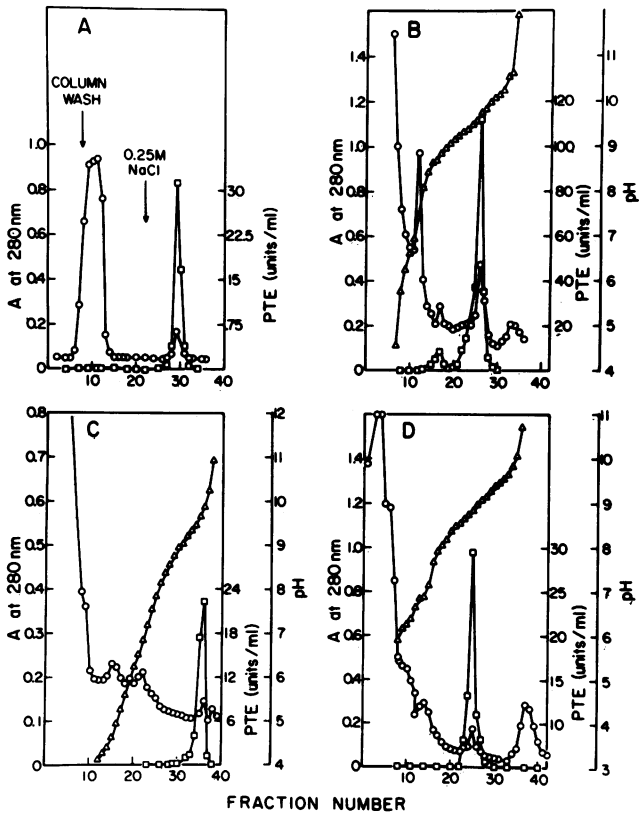

FIG. 3. Purification of PL produced by *Erwinia chrysanthemi* EC16 and two *E. coli* cosmid clones. (A) Phosphocellulose chromatography of the periplasmic fraction from clone PL3. (B) Electrofocusing of PL from strain EC16 after partial purification through the phosphocellulose step. (C) Electrofocusing of the periplasmic PL from clone PL1 partially purified through the phosphocellulose step. (D) Electrofocusing of the periplasmic PL from clone PL3 partially purified by phosphocellulose chromatography. Symbols: ○, absorbance at 280 nm; △, pH of the fractions; and □, PL activity.

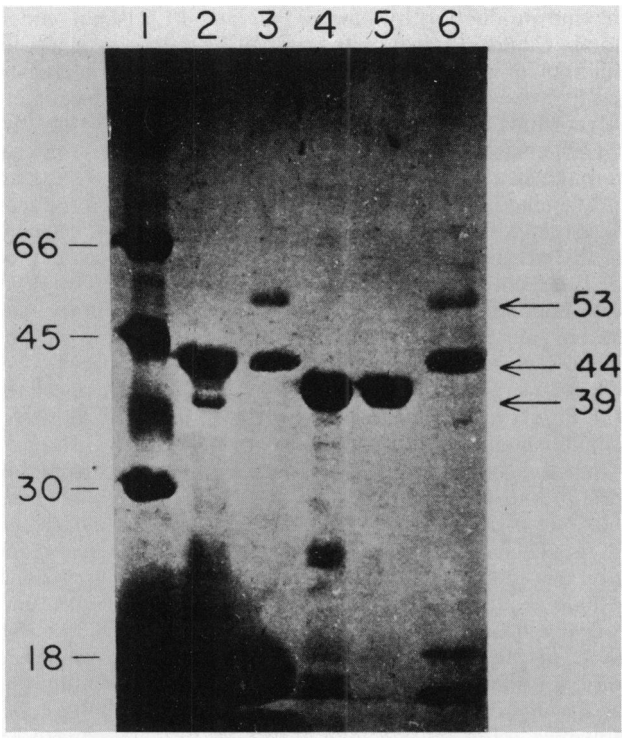

FIG. 4. SDS-polyacrylamide gel electrophoresis of proteins purified by electrofocusing; 12.5% slab gels (1 mm) were run at 5 W constant power for 4.5 h and silver stained; Lanes: 1, standards (bovine serum albumin, ovalbumin, carbonic anhydrase, lactoglobulin) with their masses in kilodaltons shown on the left; 2, mixed PL of pI 8.8 and 9.8 purified from *Erwinia chrysanthemi* EC16; 3, electrofocused PL of pI 9.8 from the periplasmic fraction of clone PL1; 4, extracellular PL of pI 8.8 purified from clone PL3 by electrofocusing; 5, periplasmic PL of pI 8.8 from clone PL3; and 6, electrofocused periplasmic PL of pI 9.8 from clone PL5. Arrows on the right denote masses of the designated bands in kilodaltons.

PL1, PL4, PL5, and PL7 all exhibited bands at 44 and 53 kilodaltons (Fig. 4). Although further tests were not made to determine whether the latter band represented a PL fusion protein, it was considered a probable contaminant.

Subcloning. Cosmid clones pPL3 and pPL7 were selected as producers of the enzymes of pI 8.8 and 9.8, respectively. The purified plasmids were restricted with *Pst*I, and the fragments were subcloned into plasmid pBR329 cut with the same enzyme. Tetracycline-resistant transformants were transferred to YC-sodium polygalacturonate plates, and PL-positive subclones were selected. These ampicillin-sensitive but chloramphenicol- and tetracycline-resistant clones were examined by gel electrophoresis. Several subclones obtained from cosmid pPL7 yielded only *Pst*I bands at 4.2 (pBR329) and 8.2 kb. One of these recombinant plasmids, pPL74, was selected for further work. Higher PL activity was exhibited in strain HB-101 with plasmid pPL74 than with the parent cosmid pPL7 (Table 3).

Several *Pst*I subclones of cosmid pPL3 in plasmid pBR329 were isolated that produced PL when transformed into strain HB-101. One of those which gave *Pst*I fragments at 4.2 (pBR329) and 6.6 kb was designated pPL34; this plasmid led to substantially higher PL production in strain HB-101 than the parent cosmid pPL3 (Table 3).

Restriction mapping of plasmids pPL34 and pPL74 disclosed no obvious similarities in their insertion sequences (Fig. 5). A further subclone of plasmid pPL74 was obtained by cutting it with *Eco*RI and ligating the various fragments into plasmid pBR329 cut with the same enzyme. Six PL-positive transformants were obtained that contained single 1.75-kb insertions at the *Eco*RI site of pBR329 as determined by mini-screens. All contained a single *Sal*I site in the insert DNA but none of the subclones was cut with *Bgl*II, *Kpn*I, or *Hin*dIII. This suggested that the 1.75-kb *Eco*RI fragment with a *Sal*I site (Fig. 5) contains the entire functional gene for the PL of pI 9.8. Furthermore, the *Sal*I site was found to be in the orientation furthest from the *Sal*I site of pBR329 in all six subclones analyzed. Electrophoresis of the plasmids from several PL-negative colonies detected three which contained 1.75-kb *Eco*RI insertions. However, all had the *Sal*I fragment in the orientation nearest to that in plasmid pBR329. These facts indicated that the promoter for the chloramphenicol resistance gene of the vector may be required for efficient enzyme production. Strain HB-101 carrying one of the positive subclones (pPL742) produced higher PL levels than did HB-101 with plasmid pPL74 and did not exhibit significant catabolite repression in the presence of glucose (Table 3).

The orientation requirements of subclone plasmid pPL742 and expression of the 1.75-kb insert in the tetracycline complex of plasmid pBR329 were further examined. The plasmid (50 μg) was cut with *Eco*RI; the 1.75-kb fragment was separated on a low-melting agarose gel, ligated with *Bam*HI-linkers (4), and again electrophoresed on low-melting agarose. The 1.75-kb fragment was then ligated with pBR329 cut with *Bam*HI and phosphatase treated before electrophoresis on the same gel. Several PL-positive and -negative transformants containing the 1.75-kb fragment were then selected and cut with *Sal*I to determine their orientation. Ten positive subclones all contained the *Sal*I

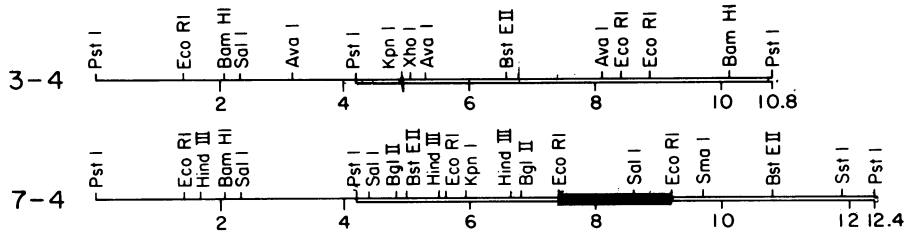

FIG. 5. Restriction maps of plasmids pPL34 and pPL74. Single lines represent pBR329 and the double lines are the inserted DNA segments. pPL34 and no *Apa*I, *Sac*I, *Nru*I, *Bgl*II, *Xba*I, or *Sst*I sites and no *Hin*dIII site in the insert DNA; pPL74 contained no *Apa*I, *Sac*I, *Xba*I, *Xho*I or *Nru*I sites and no *Bam*HI site in the insert DNA. The heavy line of pPL74 represents the *Eco*RI fragment which was subcloned into the *Eco*RI site of plasmid pBR329 to yield pPL742. The direction of transcription of the PL gene on pPL74 is left to right, based on studies with pPL742 (see the text). Sizes are in kilobases.

site in the nearest orientation to the endogenous *Sal*I site of plasmid pBR329. All five PL-negative recombinant plasmids were observed to have the *Sal*I site of the 1.75-kb fragment oriented furthest from the *Sal*I site of plasmid pBR329. These observations indicated that PL production was dependent on the tetracycline promoter.

Since the orientation of subclone plasmid pPL742 was reversed from that determined for the insertion of pPL74 (Fig. 5), the latter plasmid was cut with *Pst*I and recloned into the *Pst*I site of plasmid pBR329 as above. Again, the plasmids of several PL-positive and -negative colonies detected by the plate assay were examined by electrophoresis. All PL-negative colonies tested were found to contain only religated plasmid pBR329. Sixteen PL-positive clones all contained the 8.2-kb fragment when cut with *Pst*I. Of these plasmids, 6 had the 8.2-kb fragment in the same orientation as pPL74 when examined by restriction with *Eco*RI (fragments of 4.8, 4.1, 1.75, and 1.75 kb; Fig. 5); the other 10 PL-positive clones possessed the opposite orientation (*Eco*RI fragments of 6.0, 2.9, 1.75 and 1.75 kb). No significant differences were noted in the level of PL produced by the 16 clones, thus indicating that the 8.2-kb *Pst*I fragment carries an *Erwinia chrysanthemi* promoter sequence which is functional in *E. coli*.

The 6.6-kb *Pst*I insertion of plasmid pPL34 was also recloned in plasmid pBR329. Electrophoresis after digestion of the recombinant plasmids with *Eco*RI disclosed that PL production in *E. coli* was also not influenced by insert orientation.

Maceration of potato tuber tissue by the clones. Maceration tests with the cosmid clones disclosed that clones PL1, PL3, and PL7 in HB-101 or DH-1 consistently caused pronounced maceration of tuber slices after 24 h of incubation at 32 or 37°C. Slices incubated with *Erwinia chrysanthemi* EC16 generally showed more extensive maceration after 24 h at 32°C. Clones PL2 and PL6, along with both tested *E. coli* strains with or without plasmid pHC79 or pBR329, caused no detectable maceration in any test.

Injection of *E. coli* strain HB-101 carrying clones PL3, PL7, PL74, PL742, or PL34 into intact potato tubers caused the appearance, after 48 h at 30 or 37°C, of softened cavities from 0.5 to 2 cm in diameter with little or no discoloration at the site of injection. Injection of EC16 cells and incubation at 30°C resulted in somewhat larger cavities and slight discoloration of the macerated plant tissue. Again, no visible effect was noted when *E. coli* cells containing only plasmid pHC79 or pBR329 were injected.

DISCUSSION

Seven pHC79 clones that formed halos in the polygalacturonate agar assay were all confirmed to produce PL by spectrophotometric assay of their extracellular fluids and periplasmic fractions (Table 2). The plate assay was relatively sensitive, since no tested *E. coli* strain was observed to produce detectable PL in either the plate or spectrophotometric assays. Clones PL1, PL3, and PL7 but not the others have been stable in *E. coli* DH-1 for 14 months with transfer on L agar plates containing tetracycline at intervals of ca. 3 weeks. Initially, however, all seven PL cosmids uniformly transformed strain HB-101 to PL production. Two of the initial cosmid clones (PL2 and PL6) produced low levels of enzyme shortly after their isolation (Table 2). After three months, similar low levels were observed, but electrophoresis of these plasmids disclosed a common *Pst*I restriction fragment with the other clones producing an enzyme of pI 9.8, as will be discussed below (Table 2). Thus, the low enzyme production by clones PL2 and PL6 is not understood. Clones PL4 and PL5 initially produced significant amounts of the PL of pI 9.8, but production decreased greatly after 6 months, probably because these clones were polycosmids (Fig. 2).

The gene(s) coding for the PL of pI 9.8 must be separated from the structural gene for the enzyme of pI 8.8 by at least 40 kb, since no cosmid clone was isolated that produced more than a single PL in *E. coli*. We suspected that the gene for the enzyme of pI 9.8 might be nontandemly repeated on the EC16 chromosome because the clones with PL of pI 9.8 were obtained more frequently from the EC16 library, and the enzyme of pI 9.8 is the predominant PL produced by EC16. Hybridizations in which the inserts from pPL74 or pPL742 were used as probes against EC16 DNA restricted with *Bam*HI or *Pst*I did not support this possibility, however (unpublished data).

Subclones of plasmid pPL7 in *E. coli* exhibited from 5 to 15% of the fully induced PL activity of EC16, whereas subclone pPL34 exhibited nearly the same PL activity as EC16 (Table 3). This relatively efficient expression and secretion suggests that the translation signals and signal peptide sequences of the *Erwinia chrysanthemi* genes function well in *E. coli*. In addition, expression of the 8.2-kb *Pst*I insert of plasmid pPL74 and the 6.6-kb insert of pPL34 were not affected by orientation in pBR329, indicating that the *Erwinia chrysanthemi* promoters function well in *E. coli*. However, a further subclone of pPL74, pPL742, caused PL production with only one insertion orientation in the *Eco*RI and *Bam*HI sites of plasmid pBR329. It is therefore probable that the endogenous promoter of plasmid pPL74 was at least partly lost in the construction of pPL742. This is further indicated by the loss of catabolite repression in *E. coli* cells carrying pPL742 (Table 3). The occurrence of catabolite repression in all of the other clones and subclones (Table 3) suggests the presence of a recognition sequence for the *E. coli* catabolite activator protein within or near the promoter region (23).

Unlike *Erwinia chrysanthemi* EC16, both PL were produced constitutively in the *E. coli* clones, with no increased activity in response to polygalacturonate in the medium (Table 3). However, digalacturonate, not polygalacturonate, has been shown to be the actual inducer in *Erwinia chrysanthemi*; the production of digalacturonate is believed to be regulated by an exopolygalacturonase produced by the bacterium (2). Since the gene for this enzyme is not believed present in our *E. coli* PL clones, no conclusion may presently be made on the possible presence of regulatory elements controlling induction of PL.

Most of the PL was secreted into the periplasm or extracellular fluids of the *E. coli* clones (Table 2). This was especially true of clone PL7. *E. coli* cells are not generally thought to secrete proteins into the medium even if they are periplasmic (22), but EC16 cells secrete about 98% of the PL into the medium (1). These facts suggest that some feature of the PL themselves facilitates passage through the gram-negative outer membrane or that additional genes present in the cosmid clones enhance extracellular secretion. Mildenhall and Prior (17) recently reported that the water activity of the culture medium affected extracellular secretion of PL from *E. chrysanthemi*. We have not investigated whether a similar response occurs in *E. coli* cells containing the pPL plasmids.

The inserts of plasmids pPL34 and pPL74 showed no similarity of restriction maps (Fig. 5) and did not cross-hybridize at any tested stringency (unpublished observa-

tions). These indications that the cloned genes encoding PL of pI 8.8 and 9.8 are relatively dissimilar are not unexpected, since other workers have shown that *Erwinia* spp. PL with pI values below 9 are serologically distinct from those with pI values above 9 (15, 20). The production of such functionally similar but unrelated PL by these bacteria is not understood, but it may involve their differential activity or stability in various plant environments (2, 21).

Some of the PL cosmid clones and their pBR329 subclones conferred to *E. coli* the ability to macerate potato tuber tissue. These findings therefore extend and confirm the substantial evidence which indicates that these enzymes are important in plant tissue maceration by *Erwinia* spp. (2, 12).

ACKNOWLEDGMENTS

The research was supported by a grant of SCAR funds from the University of California to N.T.K.

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