JOURNAL OF BACTERIOLOGY, Dec. 1990, p. 6950–6958
0021-9193/90/126950-09$02.00/0

Vol. 172, No. 12

Molecular Cloning of the Structural Gene for Exopolygalacturonate Lyase from *Erwinia chrysanthemi* EC16 and Characterization of the Enzyme Product

ALAN D. BROOKS,[1] SHENG YANG HE,[3] SCOTT GOLD,[2] NOEL T. KEEN,[2] ALAN COLLMER,[3] AND STEVEN W. HUTCHESON[1]*

Department of Botany and the Center for Agricultural Biotechnology of the Maryland Biotechnology Institute, University of Maryland, College Park, Maryland 20742[1]; Department of Plant Pathology, University of California, Riverside, California 92521[2]; and Department of Plant Pathology, Cornell University, Ithaca, New York 14853[3]

Received 30 November 1989/Accepted 11 September 1990

The ability of *Erwinia chrysanthemi* to cause soft-rot diseases involving tissue maceration in many plants has been linked to the production of endo-pectate lyase. *E. chrysanthemi* EC16 mutant UM1005, however, contains deletions in the *pel* genes that encode the known endopectate lyases, yet still macerates plant tissues. In an attempt to identify the remaining macerating factor(s), a gene library of UM1005 was constructed in *Escherichia coli* and screened for pectolytic activity. A clone (pPNL5) was identified in this library that contained the structural gene for an exopolygalacturonate lyase (ExoPL). The gene for ExoPL was localized on a 3.3-kb *Eco*RV fragment which contained an open reading frame for a 79,500-Da polypeptide. ExoPL was purified to apparent homogeneity from *Escherichia coli* DH5α(pPNL5) and found to have an apparent molecular weight of 76,000 with an isoelectric point of 8.6. Purified ExoPL had optimal activity between pH 7.5 and 8.0 and could utilize pectate, citrus pectin, and highly methyl-esterified Link pectin as substrates. A PL$^-$ ExoPL$^-$ mutant of EC16 was constructed that exhibited reduced growth on pectate, but retained pathogenicity on chrysanthemum equivalent to that of UM1005. The results indicate that ExoPL does not contribute to the residual macerating activity of UM1005.

The major symptom of the bacterial soft rot caused by *Erwinia chrysanthemi* is the maceration of plant tissue that results from the destruction of the middle lamella and primary cell walls in colonized tissue (11, 26). The ability of *E. chrysanthemi* to macerate plant tissue has been linked to its ability to produce enzymes that degrade pectic polysaccharides, such as pectate lyase (PL; EC 4.2.2.2), pectin lyase (PNL), and exo-poly-α-D-galacturonosidase (ExoPG) (11, 26). PL appears to be the major pectolytic enzyme produced by this bacterium (11, 26). PL randomly cleaves the α-1,4-glycosidic bonds of unmethylated pectate by β-elimination to yield a series of oligomers that are 4,5-unsaturated at the nonreducing end. In contrast, hydrolytic enzymes, such as ExoPG, generate a saturated product. *E. chrysanthemi* EC16 produces four isozymes of PL: one acidic (PLa), two neutral (PLb and PLc), and one alkaline (PLe) (3). To establish the role of the individual PL isozymes in *E. chrysanthemi* EC16 pathogenesis, a series of mutants were constructed in which some or all of the *pel* genes encoding the known PL isozymes were deleted (35, 40). The virulence of *E. chrysanthemi* mutants lacking certain PL isozymes was reduced relative to the wild-type strain, but a mutant deficient in all four isozymes still retained the ability to cause significant maceration of plant tissues.

E. chrysanthemi, therefore, appears to produce additional factors that cause tissue maceration. In this paper, we present the first report of the molecular cloning from *E. chrysanthemi* of the structural gene for an exopolygalacturonate lyase (ExoPL), the characterization of the *pelX* gene and its enzymatic product, and construction and analysis of a *pelX* mutant. Preliminary reports of this work have been presented (10, 12; A. D. Brooks, A. Collmer, and S. W. Hutcheson, Phytopathology 79:1211, 1989).

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids are listed in Table 1. The nalidixic acid-resistant derivative of *E. chrysanthemi* EC16, AC4150, was used in these experiments. *Erwinia* and *Escherichia coli* strains were grown in King's medium B (KB) (25) except when noted. Growth rates with pectate as the sole carbon source were determined by using M9 broth (28) supplemented with 0.2% (wt/vol) pectate and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 7.5, but lacking NaCl (M9 pectate). Pectate semisolid agar (44) was used to determine the pectolytic ability of transformants. When indicated, antibiotics were added to the growth medium at the following concentrations: ampicillin (Ap), 100; chloramphenicol (Cm), 10; kanamycin (Kn), 50; nalidixic acid (Nx), 50 (20 for broth); and tetracycline (Tc), 20 μg/ml.

General DNA manipulations. Plasmid DNA was isolated and manipulated by using standard techniques (2, 28). Restriction enzymes, T4 DNA ligase, mung bean nuclease, and related reagents were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and used according to the manufacturer's instructions. Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim, Indianapolis, Ind.

Construction of genomic library of *E. chrysanthemi* UM1005. *E. chrysanthemi* UM1005 chromosomal DNA was partially digested with *Sau*3A, and 2- to 10-kb fragments were isolated by fractionation in a sucrose step gradient by the method of Silhavy et al. (43). The sized chromosomal DNA fragments were ligated into *Bam*HI-digested, calf intestine alkaline phosphatase-treated pUC19 and trans-

TABLE 1. Bacterial strains and plasmids

Designation	Relevant characteristics	Origin (reference)
Escherichia coli		
HB101	*leu proA2 thi recA13 hsdS20*	28
DH5α	F^- ϕ80d*lacZ*ΔM15Δ(*lacZYA argF*)U169 *recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ^- *thi-1 gyrA96 relA1*	Bethesda Research Laboratories, Gaithersburg, Md.
Erwinia chrysanthemi		
AC4150	Nx^r derivative of EC16	7
UM1005	Δ(*pelB pelC*)::28bp Δ(*pelA pelE*)::*nptI*	40
CUCPB5006	Δ(*pelB pelC*)::28bp Δ(*pelA pelE*)	19
CUCPB5007	Δ(*pelB pelC*)::28bp Δ(*pelA pelE*) *pelX*::*nptI-sacB-sacR*	This work
CUCPB5010	Δ(*pelB pelC*)::28bp Δ(*pelA pelE*) Δ(*pelX*) Δ4bp	This work
Plasmid		
pBR322	Ap^r Tc^r	5
pUC19	Ap^r	49
pPR328	Cm^r	36
pUM24	pUC4K derivative containing *nptI-sacB-sacR* cartridge, Ap^r Kn^r	39
pUM24CM	pPR328 derivative containing *nptI-sacB-sacR* cartridge from pUM24, Cm^r Ap^r	This work
pPNL5	pUC19 derivative containing cloned *pelX*	This work
pPNL5CM	pPR328 derivative of pPNL5 containing *pelX* on an *Eco*RV fragment in the *Sma*I site, Cm^r	This work
pPNL5B	pBR322 derivative of pPNL5 containing the entire insert in the *Eco*RI and *Sph*I sites, Ap^r	This work
pPNL5BK	Derivative of pPNL5B with *pelX*::*nptI-sacB-sacR*	This work
pPNL5B-4H	pPNL5 deletion derivative, *Hin*dIII site deleted	This work

formed into *Escherichia coli* HB101. To test for library completeness, transformants were screened on KB plates containing kanamycin to detect the presence of the *npt*I gene.

Sequence analysis of the *pelX* locus. Exonuclease III deletions were made from both ends of the insert of pPNL5 to localize the gene on the smallest DNA fragment. This resulted in a ca. 3.2-kb fragment that directed ExoPL activity by *Escherichia coli* DH5α cells on polypectate plates. Since the intact reading frame interfered with preparation of single-stranded DNA in *Escherichia coli* when oriented downstream of a vector promotor, the 3.2-kb DNA fragment was divided in half at the internal *Hin*dIII site (see Fig. 3). Additional *exo*III deletions were then performed and overlapping deletion clones were sequenced by methods published previously (47). Single-strand DNA was produced from most of the deletion clones for sequencing, but double-strand DNA was sequenced in some instances. Data were confirmed by sequencing both strands. An oligonucleotide was prepared to the complement of the sequence from bases 1629 to 1644 to confirm the sequence at the *Hin*dIII site at position 1546 (see Fig. 3).

Marker exchange mutagenesis of the *pelX* gene in *E. chrysanthemi* CUCPB5006. To facilitate mobilization into *E. chrysanthemi*, the insert DNA of pPNL5 was excised by *Sma*I and *Sph*I digestion and ligated into pBR322 to create pPNL5B. The *pelX* gene in pPNL5B was insertionally inactivated by ligating an *nptI-sacB-sacR* cartridge (39) into the *Hin*dIII site internal to the mapped *pelX* gene. The *nptI-sacB-sacR* cartridge was obtained from *Bam*HI-digested pUM24CM. This cartridge confers sensitivity to sucrose. The cartridge was treated with mung bean nuclease prior to ligation into *Hin*dIII-, mung bean nuclease-, calf intestine alkaline phosphatase-treated pPNL5B. The ligation mixture was transformed into *Escherichia coli* DH5α and transformants were selected on KB plates containing kanamycin and ampicillin. The resulting plasmid, pPNL5BK, was mobilized into *E. chrysanthemi* CUCPB5006 as described previously (42). Transconjugates were selected on KB agar containing kanamycin and nalidixic acid. Loss of pPNL5BK was induced by growth for 5 days in phosphate-deficient medium (42). One of the resulting Kn^r Ap^s sucrose-sensitive mutants was selected and designated CUCPB5007.

To construct an *E. chrysanthemi* mutant with an unmarked mutation in the *pelX* gene, *Hin*dIII-digested, mung bean nuclease-treated pPNL5B was religated to create a deletion derivative. The resulting plasmid was designated pPNL5B-4H. Plasmid pPNL5-4H was mobilized into CUCPB5007 by conjugation as described above, except that ampicillin selection was used. Marker eviction mutant selection was then performed (39). One of the resulting Kn^s Ap^s sucrose-tolerant mutants was selected and designated CUCPB5010. Mutagenesis was confirmed by Southern blot analysis.

Pectic enzyme assays. PL activity in extracts was determined by monitoring the formation of the unsaturated product spectrophotometrically (13). The reaction mixture contained 0.07% (wt/vol) pectate (polygalacturonic acid; product P21750 from Pfaltz and Bauer, Waterbury, Conn.), 30 mM Tris hydrochloride (pH 8.5), 0.1 mM $CaCl_2$, and 1.7% (vol/vol) enzyme sample, and product formed was estimated from the A_{232}. ExoPG activity was estimated by using the arsenomolybdate method of Nelson (33) to determine the increase in reducing groups generated by the hydrolytic activity of this enzyme (14). The assay reaction mixture was similar to that for the PL assay, except that 5.0 mM EDTA was added and $CaCl_2$ was omitted to inhibit any contaminating PLs. The activity of PNL, which acts specifically on methyl-esterified pectin, was estimated from the A_{232} of a reaction mixture containing 0.15% (wt/vol) citrus pectin (67% methyl esterified; Sigma Chemical Co., St. Louis,

Mo.), 30 mM HEPES (pH 8.5), 1 mM EDTA, and 0.33 to 6.7% (vol/vol) enzyme sample (50). PNL activity was confirmed by substituting 0.15% (wt/vol) Link pectin (>98% methyl esterified) for the citrus pectin in the above assay (48). Link pectin was prepared in acidified methanol by the method of Morell (31, 32). ExoPL activity was determined by monitoring the A_{232} of a reaction mixture containing 0.15% (wt/vol) Tris pectate (pectate titrated with Tris base to pH 7.5), 70 mM NaCl, 0.075 mM $CaCl_2$, and 0.1 mM $MnCl_2$ (17).

All spectrophotometric assays used a computer-assisted Hewlett-Packard model 8254A diode array spectrophotometer. One unit of activity was defined as the amount of enzyme required to produce 1 μmol of product per min and was calculated by using the extinction coefficient of the expected products as follows: PL and ExoPL, 4,600 M^{-1} cm^{-1}; PNL, 5,550 M^{-1} cm^{-1} (1, 13).

Virulence assay. The virulence of *E. chrysanthemi* CUCPB5010 was tested by inoculation of 8- to 10-cm apical stem cuttings of chrysanthemum (12). Surface-sterilized cuttings of chrysanthemum cv. Pert were placed in water suspensions containing 5×10^6 to 5×10^8 cells of the indicated *E. chrysanthemi* strain per ml. The cuttings were then transferred to tubes containing sterile distilled water and incubated at 30°C in a humid environment. After 36 h, stems were sectioned longitudinally and symptoms were scored. Alternatively, stems were cut into 1-cm segments and population levels were determined by dilution plating on KB medium (4). Representative bacterial colonies on dilution plates were picked to media containing appropriate antibiotics to confirm recovery of the inoculated strain.

Purification of ExoPL. *Escherichia coli* DH5α(pPNL5) was grown in KB broth at 33°C to stationary phase, and osmotic shock fluid was prepared by the method of Osborn and Munson (34). All subsequent steps were carried out at 4°C. Solid ammonium sulfate was added slowly to the shock fluid to 30% saturation. The turbid suspension was centrifuged at 10,000 × *g* for 30 min. The supernatant was recovered and solid ammonium sulfate was added to 95% saturation. After 1 h, the solution was centrifuged at 10,000 × *g* for 30 min. The precipitate was resuspended in water and dialyzed for 18 h against three changes of 5 mM HEPES buffer (pH 7.5). The dialysate was fractionated by preparative granulated bed isoelectric focusing in a Bio-Phoresis horizontal electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer's directions. After the isoelectric focusing was complete, the polyacrylamide gel matrix was sectioned with a 75-mm fractionating grid and each fraction was suspended in 3.0 ml of distilled water. The suspensions were filtered to remove the gel matrix and the pH was determined. Each fraction was then dialyzed against 5 mM HEPES buffer (pH 7.5) and ExoPL activity was determined.

Analytical procedures. Southern blot analysis was done with Gene Screen Plus membrane (Dupont, NEN Research Products, Wilmington, Del.) according to the manufacturer's instructions, except that 0.4 M NaOH was used as the carrier solvent and dextran sulfate was omitted from the hybridization mixture. Probes were labeled with [α-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.) by using a nick translation kit obtained from Bethesda Research Laboratories, Gaithersburg, Md. Autoradiographs were obtained by exposing blots to Kodak XAR-5 film at −70°C.

Thin-layer chromatography was performed as described previously (50). Fifteen microliters of each sample was applied to Chromagram cellulose sheets (catalog 13255; Kodak, Rochester, N.Y.). The chromatogram was developed with a 5:3:2 mixture of *n*-butanol-water-glacial acetic acid. The products were visualized by treating the dried chromatograms with 0.05% (wt/vol) bromphenol blue in ethanol.

The amino-terminal amino acid sequence was determined by using an Applied Biosystems 470A Protein Sequencer. Ampholytes were removed from the purified ExoPL preparation prior to sequence analysis by ultrafiltration in Centricon tubes (molecular weight cutoff, 10,000; Amicon Division, W. R. Grace and Co.).

Protein concentrations were estimated by the method of Bradford (6). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (27). Protein was visualized by silver staining by using the method of Heukeshoven and Dernick (21).

RESULTS

Isolation of pPNL5 from an *E. chrysanthemi* UM1005 genomic library. To facilitate characterization of the remaining macerating activity in *E. chrysanthemi* UM1005, a genomic library containing an average insert of 7 kb was constructed in pUC19 and screened for pectolytic activity. The library appeared to be complete: the *nptI* gene, which was present as a single copy in the UM1005 chromosome, was detected in 5 of the 2,000 colonies screened. Pectolytic transformants were identified on pectate semisolid agar plates. After incubating for 2 weeks, eight colonies were observed to cause pitting. Plasmids of each of these transformants were isolated and mapped for *Hin*dIII, *Eco*RI, and *Pst*I sites. The inserts of three clones shared overlapping restriction patterns. One, pPNL5, was chosen for further analysis.

Identification of ExoPL activity produced by clones containing pPNL5. To determine the nature of the pectolytic activity associated with pPNL5, culture filtrates from *Escherichia coli* DH5α(pPNL5) were screened for PL, PNL, and ExoPG activity. Culture filtrates contained 2.54 U of PL activity per ml, but exhibited PNL activity as well. PNL activity was 0.26 U/ml when Link pectin (97% methyl esterified) was used as the substrate, whereas 0.75 U/ml of activity was observed with citrus pectin (67% methyl esterified). No exoPG activity could be detected in the culture filtrates of *Escherichia coli* DH5α(pPNL5). PL activity in culture filtrates was dependent on culture age. Activity was highest in culture filtrates of stationary-phase cultures (data not shown).

To determine whether the enzyme was an exo- or an endo-PL, the product of the enzyme was characterized by thin-layer chromatography. A single product was produced from purified polygalacturonate, irrespective of the enzyme concentration and reaction time (Fig. 1).

Purification and physical properties of ExoPL. Culture filtrates from *E. chrysanthemi* CUCPB5006 (a Kn^s Δ*pelABCE* derivative of EC16) exhibited only low levels of PL activity. *E. chrysanthemi* CUCPB5006 culture supernatants contained 4.0×10^{-4} U of PL activity per ml with specific activity of 0.25 U/mg of protein. Osmotic shock fluids of *Escherichia coli* DH5α(pPNL5) contained 2.54 U of PL activity per ml with a specific activity of 158 U/mg of protein. Osmotic shock fluids of DH5α(pPNL5) were used as the source for purification of this enzyme. By a combination of ammonium sulfate precipitation (30 to 95%) and preparative isoelectric focusing, ExoPL was purified to apparent homogeneity. The resulting preparation was homogeneous for a polypeptide with an apparent mass of 76,000 Da (data not shown). The isoelectric point was determined by isoelectric focusing to be 8.6 ± 0.35.

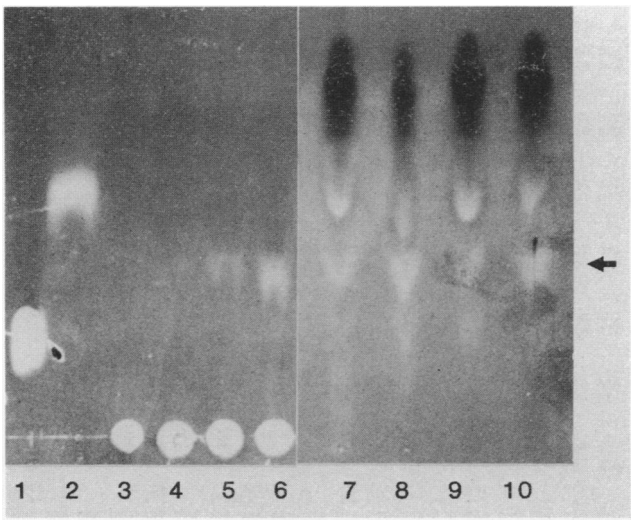


FIG. 1. Thin-layer chromatography of the product produced by ExoPL activity on pectate. The reaction mixture contained 0.15% (wt/vol) Tris-pectate, 70 mM NaCl, 0.075 mM $CaCl_2$, and 0.1 mM $MnCl_2$. Osmotic shock fluids of *Escherichia coli* DH5α(pPNL5) were used as the source of ExoPL. Reactions were continued for 30 min and stopped by the addition of 5 μmol of EDTA. Thin-layer chromatography was performed as described in Materials and Methods. Lanes: 1, saturated digalacturonate; 2, saturated galacturonate; 3 to 6, 0, 0.005, 0.01, and 0.02 U of ExoPL added to the reaction mixture, respectively; 7 to 10, 0.0025, 0.005, 0.01, and 0.02 U of PLc, an endo-PL, added to the reaction mixture, respectively. Arrow, Unsaturated digalacturonate.

Enzymatic properties of ExoPL. The purified enzyme was found to have optimal activity between pH 7.0 and 8.0 when pectate was the substrate. A more alkaline pH optimum was observed when pectin was the substrate. Optimal activity was observed at pH 8.0 with citrus pectin, whereas the optimum was pH 8.5 when Link pectin was used as the substrate. The enzyme activity on pectate was enhanced by the addition of Na^+ and Ca^{2+} (Fig. 2). ExoPL activity on pectate was nearly completely inhibited by the addition of 1 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), 1 mM EDTA, or 5 mM nitrilotriacetic acid

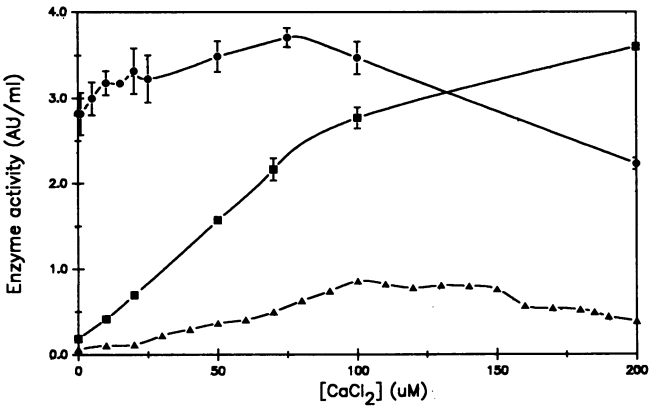


FIG. 2. Effect of Na^+ and Ca^{2+} levels on ExoPL activity. The reaction mixture contained 0.15% (wt/vol) Tris-pectate either alone (triangles) or supplemented with 70 mM NaCl (circles) or 70 mM NaCl and 1 mM EGTA (boxes). Assay conditions were as described in Materials and Methods.

TABLE 2. Effect of chelating agents on ExoPL activity[a]

Chelator added	ExoPL activity (U/ml)		
	Link pectin	Citrus pectin	Pectate
None	0.09	0.75	3.70
EDTA, 1 mM	0.26	0.48	0.03
EGTA, 1 mM	0.15	0.90	0.14
NTA, 5 mM	0.26	0.64	0.04

[a] Assay conditions were as described in Materials and Methods, except that the specified amount of each chelating agent was added to the substrate. Enzyme purified from *Escherichia coli* DH5α(pPNL5) osmotic shock fluids was used to initiate the reaction.

(NTA). Activity could be specifically restored by the addition of 0.2 mM $CaCl_2$. Neither magnesium nor manganese could substitute for calcium.

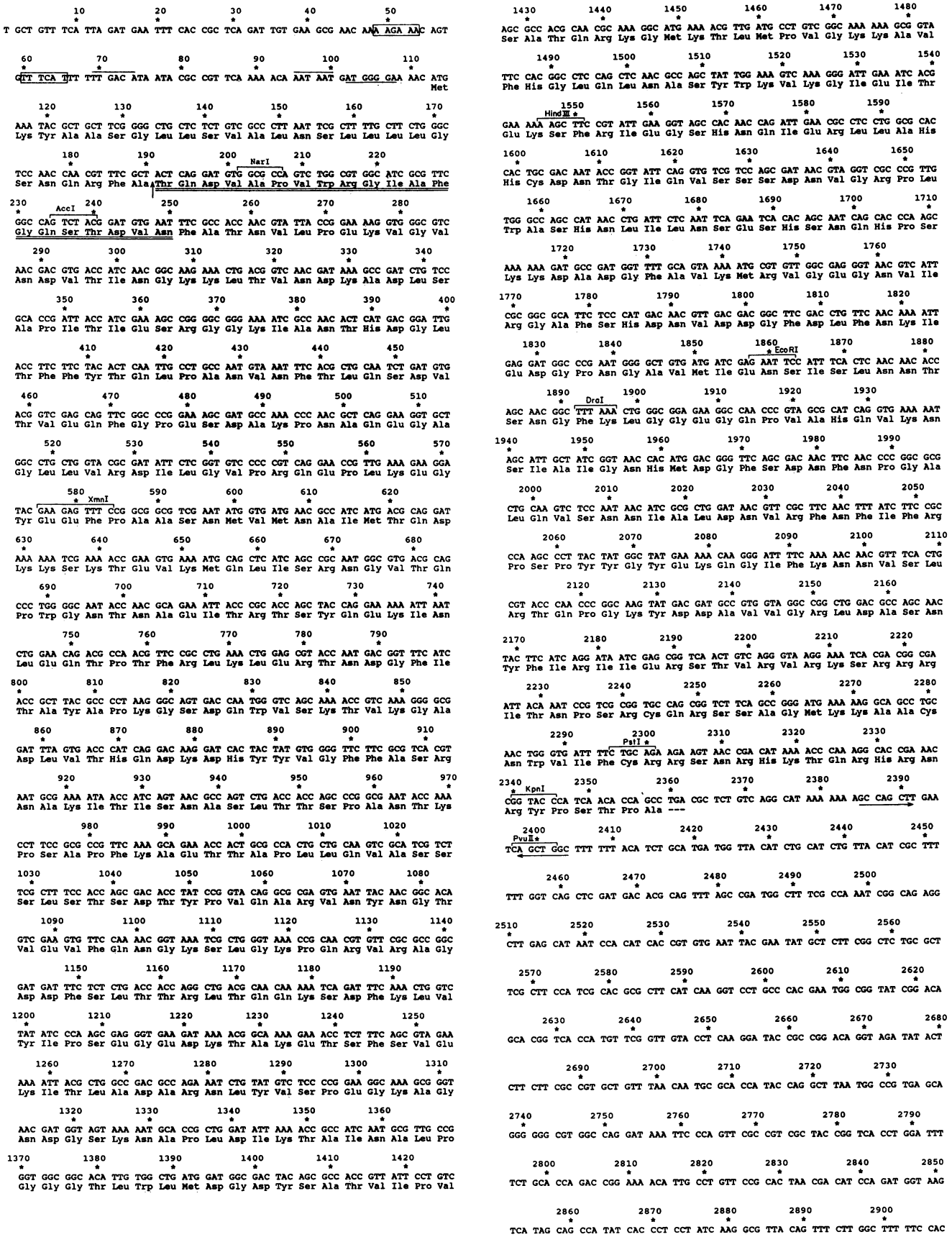
In contrast to activity observed on pectate, ExoPL activity on Link pectin was stimulated by EDTA, EGTA, and NTA (Table 2). Maximal activity on Link pectin was observed when either 5 mM NTA or 1 mM EDTA was present. The same levels of chelating agents, however, had little effect on the ExoPL activity on citrus pectin. The reason for the different behavior of ExoPL on pectate and pectin has not been established, but may be due to the interaction of Ca^{2+} with the substrate.

The ExoPL product, after digestion of pectate under optimal conditions, was purified by ethanol precipitation, followed by Cu^{2+}-ethanol precipitation. The absorbance of the isolated product was found to have a sharp peak at 232 nm, indicating the presence of an unsaturated residue that is consistent with lyase activity. The purified product was compared with the crude product by thin-layer chromatography. Both preparations of the product exhibited the same relative mobility and comigrated with unsaturated digalacturonic acid produced by complete digestion of pectate with PLc (Fig. 1; unpublished observations).

Localization of the *pelX* gene and sequence analysis. Deletion analysis of pPNL5 indicated that the *pelX* gene was located on a 3.3-kb *Eco*RV fragment. Only pPNL5 derivatives containing this *Eco*RV fragment conferred PL activity. This 3.3-kb *Eco*RV fragment was subcloned to produce pPNL5CM. Osmotic shock fluids of *Escherichia coli* DH5α (pPNL5CM) exhibited ExoPL activity (data not shown).

A 3.2-kb DNA fragment internal to the above *Eco*RV fragment was sequenced. A long open reading frame was identified on one strand (Fig. 3), but no open reading frames of significant size were identified on the other reading frames on either strand. Compressions occurred on sequencing gels at bases 203 to 206 which compromised sequence determination in this region. However, the deduced base sequence constitutes a unique *Nar*I restriction site whose presence was confirmed by gel electrophoresis. Furthermore, the deduced base sequence in this region was consistent with subsequent peptide sequencing data, as discussed below. Deletion of DNA upstream from the unique *Xmn*I site at position 575 destroyed ExoPL activity, but a deletion at the unique *Acc*I site at position 234 yielded ExoPL activity in *Escherichia coli* cells when the resultant DNA fragment was recloned into the *Cla*I site of pUC129. This construct, however, may have generated a translational fusion protein with the 5′ end of the vector *lacZ* gene which retained PL activity.

The best candidate for a translational initiation codon was the ATG located at base 113, which was preceded by a sequence that would be expected to function as a Shine-


NarI
AccI
XmnI
HindIII
EcoRI
DraI
PstI
KpnI
PvuII

```
2910          2920          2930          2940          2950          2960
 *             *             *             *             *             *
TAC TTC ATC AAT GGA ACC AAC CAT GTA GAA CGC CTG TTC CGG TAG GTG GTC GTA TTC

   2970          2980          2990          3000          3010          3020
    *             *             *             *             *             *
GCC GTC CAT AAT GCC TTT GAA GCC ACG GAT GGT GTC TTT CAA CGA CAC ATA TTT ACC

        3030          3040          3050          3060          3070
         *             *             *             *             *
CGG AGA ACC GGT AAA TAC TTC AGC TAC GAA GAA CGG CTG AGA CAG GAA GCG TTG GAT

3080          3090          3100          3110          3120          3130
 *             *             *             *             *             *
CTT ACG CGC ACG GGA TAC CAC CAG CTT GTC TTC TTC AGA CAG CTC GTC CAT ACC CAG

   3140          3150          3160          3170
    *        EcoRV *             *          * PstI
AAT CGC GAT GAT ATC CTT CAG TTC CTG ATA GCG CTG CAG
```

FIG. 3. DNA sequence of the *pelX* gene and flanking DNA from *E. chrysanthemi* EC16. The putative KdgR binding site beginning at base 49 is denoted by the boxes, and the potential promoter sequences beginning at base 69 are indicated by overlining. A putative Shine-Dalgarno sequence beginning at base 102 is underscored, and inverted repeats occurring after the translational stop are denoted by arrows. The N-terminal sequence of the mature, secreted protein is denoted by the double underlining, and the deduced cleavage site of the preprotein is indicated with a vertical arrow at base 190. Selected restriction enzyme sites are indicated.

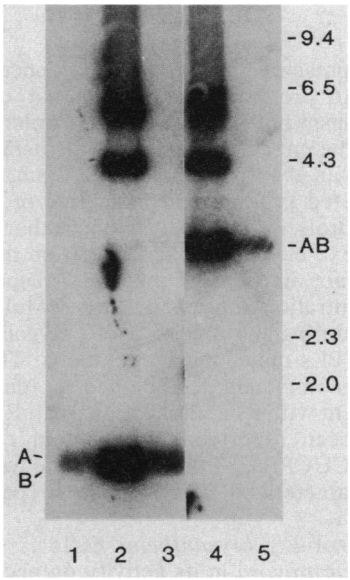


FIG. 4. Southern blot analysis of the *pelX* gene in *E. chrysanthemi* CUCPB5010. Genomic or plasmid DNA was digested with *Eco*RV and *Hin*dIII and fractionated by electrophoresis in a 0.8% agarose gel. DNA was transferred by capillary action to Gene Screen Plus (DuPont) and hybridized with ^{32}P-labeled pPNL5CM. Lanes: 1, *E. chrysanthemi* UM1005 genomic DNA; 2, pPNL5; 3, *E. chrysanthemi* CUCPB5006 genomic DNA; 4, pPNL5B-4H; 5, *E. chrysanthemi* CUCPB5010 genomic DNA. Bands A and B represent fragments containing portions of the wild-type *pelX* gene. Band AB represents the *pelX* gene with the internal *Hin*dIII site deleted.

Dalgarno box (Fig. 3). The amino terminus of the resulting peptide shows strong resemblance to a signal peptide sequence. To test whether this was the case and to confirm the translational initiation site, the N terminus of the secreted ExoPL was sequenced. These data confirmed the deduced peptide sequence shown in Fig. 3 and indicated that the amino terminus of the secreted protein had been cleaved between the alanine and threonine residues at position 190 (Fig. 3). These data accordingly confirmed the amino terminus of the secreted *pelX* gene product and support the localization of the translational start at base 113.

Occurrence of the translational stop at base 2360 was supported by the finding that a deletion 3′ at the *Pst*I site at 2296 abolished ExoPL activity in *Escherichia coli* cells, but full activity was exhibited by a 3′ deletion to the *Pvu*II site at base 2397 (Fig. 3). The presence of a putative *kdgR* binding site from bases 49 to 65 suggests that the expression of *pelX* may be controlled by KdgR, a negative regulator of several genes involved in pectate catabolism (37). Consensus promoter sequences, TTGACA and AATAAT, begin at bases 69 and 95, respectively. This putative promoter is located unusually close to the ribosome-binding site. Following the translational stop was a G+C-rich inverted repeat sequence at base 2385 followed by a poly(T) sequence. This region may, therefore, function as a Rho-independent transcriptional terminator. Several codons that would be considered unusual in *Escherichia coli* were present, for example, five AGG arginine codons (16). Codon usage was 52% G+C for the coding region but 61% on the third position, again somewhat unusual for genes in enteric bacteria.

The entire open reading frame of *pelX* contained 749 amino acids and yielded a computer-calculated molecular weight of 82,200 and a pI of 10.3. The secreted *pelX* protein contained 723 amino acids and yielded a computer calculated molecular weight of 79,500 and a pI of 10.2. This molecular weight was in good agreement with that determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified ExoPL, but the calculated pI was considerably higher than that determined by electrofocusing of the secreted enzyme. Data base searching in the PIR library did not disclose significant homology of the deduced protein product with known peptides.

Role of ExoPL in the virulence of *E. chrysanthemi* EC16 and in pectate metabolism. To determine the role of ExoPL in *E. chrysanthemi* pathogenicity, *pelX* mutants of *E. chrysanthemi* were generated. Plasmids pPNL5BK and pPNL5B-4H were used to create the marker exchange mutant CUCPB5007 and the deletion mutant, CUCPB5010, respectively. No ExoPL activity could be detected from culture filtrates of *Escherichia coli* DH5α containing either pPNL5BK or pPNL5B-4H. Southern blot analysis revealed that this procedure had mutated the *pelX* gene (Fig. 4). CUCPB5010 chromosomal DNA had the same hybridization pattern as the insert of pPNL5B-4H when both were digested with *Hin*dIII and *Eco*RV. Both constructs lacked the internal *Hin*dIII site of the *pelX* gene.

Growth of each strain on M9 pectate was monitored to determine whether the mutation affected pectate metabolism. *E. chrysanthemi* CUCPB5010 grew more slowly in M9 pectate than strain CUCPB5006. The doubling time for log growth on M9 pectate was 2.4 h for CUCPB5006, while the doubling time for CUCPB5010 was 4.4 h. Both strains grew equally well in KB broth. This suggests that mutations in the *pelX* gene adversely affect the ability of *E. chrysanthemi* to use pectate as a carbon source.

To determine whether the *pelX* mutation affects the pathogenicity of *E. chrysanthemi*, chrysanthemum cuttings were inoculated with CUCPB5010 and CUCPB5006. Inocula of $>5 \times 10^6$ cells per ml caused visible tissue maceration within 48 h. Tissue maceration, however, was equivalent for both strains. When the initial inoculum concentration was $<5 \times 10^6$ per ml, both strains caused water soaking, but tissue remained turgid and intact. Both bacterial strains multiplied to the same levels in tissue. Recovery of both strains was 10^8 cells per ml at 3 cm from the base of the stem 36 h after inoculation.

DISCUSSION

The results presented here provide evidence that *E. chrysanthemi* EC16 produces an ExoPL that contributes to pectate catabolism but not to bacterial virulence. Digestion of pectate by the enzyme encoded by the *pelX* gene yielded a product with an absorbance peak at 232 nm, characteristic of an unsaturated product and indicative of lyase activity rather than hydrolytic cleavage. Evidence that the *pelX* gene product is an exo-cleaving lyase stems from the presence of a single product in pectate digests, irrespective of the enzyme concentration and reaction time. Multiple products are observed during the digestion of polygalacturonate by endo-cleaving PLs under these conditions. The inability of the enzyme to completely degrade pectate (data not shown) is also consistent with its identification as an ExoPL. ExoPL activity had been reported previously in extracts of *E. chrysanthemi* CUCPB1237, but the enzyme was not purified or further characterized and its role in pathogenesis is unknown (9).

The ExoPL of *E. chrysanthemi* EC16 is similar to the ExoPL of *E. carotovora* in its activity on pectate. Like the *E. carotovora* enzyme, *E. chrysanthemi* ExoPL requires an alkaline pH and Na^+ for activity under these conditions (cf references 18, 23, and 41). *E. carotovora* ExoPL activity on pectate was strongly inhibited by EDTA and weakly inhibited by NTA, but the activity could be restored by the addition of almost any divalent cation (cf reference 23). In contrast, EDTA, EGTA, and NTA strongly inhibited *E. chrysanthemi* ExoPL activity on pectate and activity could be specifically restored by the addition of Ca^{2+}. This suggests that the *E. chrysanthemi* EC16 ExoPL activity on pectate is Ca^{2+} dependent. Based on its mobility during thin-layer chromatography and preliminary mass spectrometry data, the product of the *E. chrysanthemi* EC16 ExoPL appears to be 4,5-unsaturated digalacturonate. Unsaturated digalacturonic acid has been reported to be the product of an ExoPL identified in *E. chrysanthemi* CUCPB1237 (9).

The ability of *E. chrysanthemi* EC16 ExoPL to cleave highly methylated Link pectin is unique for an ExoPL. The significance of this ability has not been determined. Neither strain CUCPB5006 nor strain CUCPB5010 was able to grow on minimal medium with Link pectin as the sole carbon source. This suggests that the ability of this ExoPL to cleave pectin in vitro may be of little significance in vivo.

This paper is the first report of the molecular cloning and characterization of the *pelX* gene from *E. chrysanthemi*. The nucleotide sequence of the *E. chrysanthemi* EC16 *pelX* gene lacked homology with that of other pectic enzymes, including the periplasmic family of PLs described by Hinton et al. (22). This suggests that this enzyme is different from other PLs. Like the other sequenced *pel* genes of this bacterium (46), the deduced gene product contained a candidate signal sequence. This is consistent with isolation of the enzyme from osmotic shock fluids of *Escherichia coli* DH5α (pPNL5). Also in common with other *pel* genes, translational termination in the *pelX* open reading frame is followed by a putative Rho-independent transcription stop sequence. The function of this sequence in *E. chrysanthemi*, however, has not been established.

The *E. chrysanthemi pelX* mutant CUCPB5010 was not able to grow as well as the *pelX*$^+$ strain CUCPB5006 on pectate as the sole carbon source. This suggests that ExoPL functions in pectate metabolism in *E. chrysanthemi* EC16. *E. chrysanthemi* pathogenicity on chrysanthemum, however, did not appear to be affected by mutations in the *pelX* gene. It therefore appears that the contribution of ExoPL to symptom formation may be minor.

Although *Escherichia coli* clones carrying the *pelX* gene caused pitting in pectate semisolid agar and ExoPL could be conveniently purified from osmotic shock fluids of such clones, the localization of the enzyme has not been rigorously determined in either *Escherichia coli* or *E. chrysanthemi*. The presence of a signal peptide suggests that the enzyme is exported at least to the periplasm in *E. chrysanthemi*. A periplasmic ExoPL would be expected to cleave oligogalacturonates in addition to polygalacturonate, as reported previously for the ExoPL produced by *E. carotovora* (17) and for the ExoPL produced by *E. chrysanthemi* CUCPB1237 (9). The enzyme would thus complement the activity of cytoplasmic oligogalacturonide lyase in the catabolism of oligogalacturonates. Oligogalacturonide lyase preferentially cleaves 4,5-unsaturated digalacturonate and has decreasing activity on larger oligomers (30). Degradation of 4,5-unsaturated oligogalacturonates to 4-deoxy-L-*threo*-5-hexosulose uronate is necessary for both PL induction and bacterial growth in pectate minimal media (8, 9). The presence of a potential KdgR binding site in the 5′ flanking sequence would suggest that *ogl* and *pelX* are coordinately regulated (37). The cell-bound activity of ExoPL in cleaving 4,5-unsaturated trigalacturonic acid to 4,5-unsaturated digalacturonic acid and 4-deoxy-L-*threo*-5-hexosulose uronic acid in *E. chrysanthemi* CUCPB1237 was postulated to account for the ability of oligogalacturonide lyase-deficient mutants of this strain to be induced by unsaturated oligogalacturonate trimers but not dimers or tetramers (9). Mutants deficient in both oligogalacturonide lyase and ExoPL are needed to test further the role of the ExoPL in PL induction and pectate catabolism.

The identity of the residual enzymes produced by CUCPB5010 that are responsible for maceration of plant tissue, pitting of pectate semisolid agar, and degradation of extracellular pectic polymers remains a puzzle. Although mutants deficient in ExoPG demonstrate that this extracellular enzyme can have a significant role in pectate catabolism in the absence of extracellular PL activity, the mutants also reveal that ExoPG has little direct role in maceration (19). We hope that the contribution of ExoPL to the pectolytic capacity of *E. chrysanthemi* will become clearer when all of the enzymes in this remarkably complex enzyme system have been identified.

ACKNOWLEDGMENTS

This work was supported in part by grant 87-CRCR-1-2352 from the U.S. Department of Agriculture Competitive Research Grants Office to A.C. and by funds made available to S.W.H. from the Center for Agricultural Biotechnology.

We thank J. Cohen for assistance with the mass spectrometry. We are also thankful to Ted Thannhauser (Analytical and Synthetic Lab, Biotechnology Program, Cornell University) for helping us determine the N-terminal sequence of ExoPL.

LITERATURE CITED

1. **Albersheim, P.** 1966. Pectin lyase from fungi. Methods Enzymol. **8**:628–631.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. Wiley-Interscience, New York.
3. **Barras, F., K. K. Thurn, and A. K. Chatterjee.** 1987. Resolution of four pectate lyase structural genes of *Erwinia chrysanthemi* (EC16) and characterization of the enzymes produced in *Escherichia coli*. Mol. Gen. Genet. **209**:319–325.
4. **Bertoni, G., and D. Mills.** 1987. A simple method to monitor

growth of bacterial populations in leaf tissue. Phytopathology 77:832.
5. **Bolivar, F., R. Rodriguez, P. J. Green, M. Betach, H. L. Heyneker, H. W. Boyer, J. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
7. **Chatterjee, A. K., K. K. Thurn, and D. A. Feese.** 1983. Tn*5*-induced mutations in the enterobacterial phytopathogen, *Erwinia chrysanthemi*. Appl. Environ. Microbiol. 45:644–650.
8. **Chatterjee, A. K., K. K. Thurn, and D. J. Tyrell.** 1985. Isolation and characterization of Tn*5* insertion mutants of *Erwinia chrysanthemi* that are deficient in polygalacturonate catabolic enzymes oligogalacturonate lyase and 3-deoxy-D-glycero-2,5-hexodiulosonate dehydrogenase. J. Bacteriol. 162:708–714.
9. **Collmer, A., and D. F. Bateman.** 1981. Impaired induction and self-catabolite repression of extracellular pectate lyase in *Erwinia chrysanthemi* mutants deficient in oligogalacturonide lyase. Proc. Natl. Acad. Sci. USA 78:3920–3924.
10. **Collmer, A., A. D. Brooks, S. Y. He, and G. L. Cleveland.** 1988. Construction and characterization of *Erwinia chrysanthemi* mutants containing mutations in genes encoding extracellular pectic enzymes, p. 356–361. *In* R. Palacios and D. P. S. Verma (ed.), Molecular genetics of plant-microbe interactions 1988. American Phytopathology Society Press, St. Paul.
11. **Collmer, A., and N. T. Keen.** 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. 24:383–409.
12. **Collmer, A., J. L. Ried, G. L. Cleveland, S. Y. He, and A. D. Brooks.** 1988. Mutational analysis of *Erwinia chrysanthemi* mutants containing mutations in genes encoding extracellular pectic enzymes, p. 35–48. *In* B. Staskawicz, P. Ahlquist, and O. Yoder (ed.), Molecular biology of plant-pathogen interactions, New Ser., vol. 101. Alan R. Liss, Inc., New York.
13. **Collmer, A., J. L. Ried, and M. S. Mount.** 1988. Assay methods for pectic enzymes. Methods Enzymol. 161:329–334.
14. **Collmer, A., C. H. Whalen, S. V. Beer, and D. F. Bateman.** 1982. An exo-poly-α-D-galacturonosidase implicated in the regulation of extracellular pectate lyase production in *Erwinia chrysanthemi*. J. Bacteriol. 149:626–634.
15. **Gold, L., and G. Stormo.** 1987. Translational initiation, p. 1302–1307. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
16. **Gouy, M., and C. Gautier.** 1982. Codon usage in bacteria. Correlation with gene expressivity. Nucleic Acids Res. 10:7055–7074.
17. **Hatanaka, C., and J. Ozawa.** 1972. Exopectic acid transeliminase of *Erwinia*. Agric. Biol. Chem. 36:2307–2313.
18. **Hatanaka, C., and J. Ozawa.** 1973. Effect of metal ions on activity of exopectic acid transeliminase of *Erwinia*. Agric. Biol. Chem. 37:593–597.
19. **He, S. Y., and A. Collmer.** 1990. Molecular cloning, nucleotide sequence, and marker exchange mutagenesis of the exo-poly-α-D-galacturonosidase-encoding *pehX* gene of *Erwinia chrysanthemi* EC16. J. Bacteriol. 172:4988–4995.
20. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 210: 294–298.
21. **Heukeshoven, J., and R. Dernick.** 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis 6:103–112.
22. **Hinton, J. C. D., J. M. Sidebotham, D. R. Gill, and G. P. C. Salmond.** 1989. Extracellular and periplasmic isoenzymes of pectate lyase from *Erwinia carotovora* subspecies *carotovora* belong to different gene families. Mol. Microbiol. 3:1785–1795.
23. **Ikeda, S., Y. Kegoya, and C. Hatanaka.** 1984. Effect of chelating agents on exopolygalactuonate lyase of *Erwinia carotovora* subsp. *carotovora*. Agric. Biol. Chem. 48:2777–2782.
24. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram negative bacteria. Gene 70:191–197.
25. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Med. 44:301–307.
26. **Kotoujansky, A.** 1987. Molecular genetics of pathogenesis by soft-rot erwinias. Annu. Rev. Phytopathol. 25:405–430.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Min, K. T., M.-H. Kim, and S.-S. Lee.** 1988. Search for the optimal sequence of the ribosome binding site by random oligonucleotide-directed mutagenesis. Nucleic Acids Res. 16: 5075–5079.
30. **Moran, F., S. Nasuno, and M. P. Starr.** 1968. Oligogalacturonide trans-eliminase of *Erwinia carotovora*. Arch. Biochem. Biophys. 125:734–741.
31. **Morell, S., L. Baur, and K. P. Link.** 1934. The methylglycosides of the naturally occurring ring hexuronic acids. J. Biol. Chem. 105:1–13.
32. **Morell, S., and K. P. Link.** 1933. The methylglycosides of the naturally occurring hexuronic acids. J. Biol. Chem. 100:385–396.
33. **Nelson, N.** 1984. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375–380.
34. **Osborn, M. J., and R. Munson.** 1974. Separation of the inner (cytoplasmic) and outer membranes of gram negative bacteria. Methods Enzymol. 31:642–653.
35. **Payne, J. H., C. Schoedel, N. T. Keen, and A. Collmer.** 1987. Multiplication and virulence in plant tissues of *Escherichia coli* clones producing pectate lyase at high levels and *Erwinia chrysanthemi* mutants deficient in pectate lyase production. Appl. Environ. Microbiol. 53:2315–2320.
36. **Quigley, N. B., and P. R. Reeves.** 1987. Chloramphenicol resistant cloning vector based on pUC9. Plasmid 17:54–57.
37. **Reverchon, S., Y. Huang, C. Bourson, and J. Rober-Baudouy.** 1989. Nucleotide sequences of the *Erwinia chrysanthemi ogl* and *pelE* genes, negatively regulated by the *Kdg*R gene product. Gene 85:125–134.
38. **Ried, J. L., and A. Collmer.** 1986. Comparison of pectic enzymes produced by *Erwinia chrysanthemi*, *Erwinia carotovora* subsp. *carotovora*, and *Erwinia carotovora* subsp. *atroseptica*. Appl. Environ. Microbiol. 52:305–310.
39. **Ried, J. L., and A. Collmer.** 1987. An *nptI-sacB-sacR* cartridge for constructing directed, unmarked mutations in gram-negative bacteria by marker exchange-eviction mutagenesis. Gene 57: 239–246.
40. **Ried, J. L., and A. Collmer.** 1988. Construction and characterization of an *Erwinia chrysanthemi* mutant with directed deletions in all the pectate lyase structural genes. Mol. Plant Microbe Interact. 1:32–38.
41. **Roberts, D. P., P. M. Berman, C. Allen, V. K. Stromberg, G. H. Lacy, and M. S. Mount.** 1986. *Erwinia carotovora*: molecular cloning of a 3.4 kilobase DNA fragment mediating production of pectate lyases. Can. J. Plant Pathol. 8:17–27.
42. **Roeder, D. L., and A. Collmer.** 1985. Marker-exchange mutagenesis of a pectate lyase isozyme gene in *Erwinia chrysanthemi*. J. Bacteriol. 164:51–56.
43. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Starr, M. P., A. K. Chatterjee, P. B. Starr, and G. E. Buchanan.** 1977. Enzymatic degradation of polygalacturonic acid by *Yersinia* and *Klebsiella* species in relation to clinical laboratory procedures. J. Clin. Microbiol. 6:379–386.
45. **Starr, M. P., and F. Moran.** 1962. Eliminative split of pectic substances by phytopathogenic soft-rot bacteria. Science 135: 920–921.
46. **Tamaki, S. J., S. Gold, M. Robeson, S. Manulis, and N. T. Keen.** 1988. Structure and organization of the *pel* genes from *Erwinia*

chrysanthemi EC16. J. Bacteriol. **170**:3468–3478.
47. **Trollinger, D., S. Berry, W. Belser, and N. T. Keen.** 1989. Cloning and characterization of a pectate lyase gene from *Erwinia carotovora* EC153. Mol. Plant Microbe Interact. **2**:17–25.
48. **Tsuyumu, S., and A. K. Chatterjee.** 1984. Pectin lyase production in *Erwinia chrysanthemi* and other soft-rot *Erwinia* species. Physiol. Plant Pathol. **24**:291–302.
49. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
50. **Zink, R. T., J. K. Engwall, J. L. McEvoy, and A. K. Chatterjee.** 1985. *recA* is required in the induction of pectin lyase and carotovoricin in *Erwinia carotovora* subsp. *carotovora*. J. Bacteriol. **164**:390–396.