JOURNAL OF BACTERIOLOGY, Sept. 1990, p. 4988–4995
0021-9193/90/094988-08$02.00/0

Vol. 172, No. 9

Molecular Cloning, Nucleotide Sequence, and Marker Exchange Mutagenesis of the Exo-Poly-α-D-Galacturonosidase-Encoding *pehX* Gene of *Erwinia chrysanthemi* EC16

SHENG YANG HE AND ALAN COLLMER*

Department of Plant Pathology, Cornell University, Ithaca, New York 14853

Received 6 February 1990/Accepted 18 June 1990

The *pehX* gene encoding extracellular exo-poly-α-D-galacturonosidase (exoPG; EC 3.2.1.82) was isolated from a genomic library of the pectate lyase-deficient *Erwinia chrysanthemi* mutant UM1005 (a Nal^r Kan^r Δ*pelABCE* derivative of EC16) by immunoscreening 2,800 *Escherichia coli* HB101 transformants with an antibody against exoPG protein. The cloned *pehX* gene was expressed highly from its own promoter in *E. coli*, and most of the enzyme was localized in the periplasm. The nucleotide sequence of *pehX* revealed the presence of an amino-terminal signal peptide and an open reading frame encoding a preprotein of 64,608 daltons. The cloned *pehX* gene was insertionally inactivated with Tn*phoA* and used to mutate the chromosomal *pehX* gene of *E. chrysanthemi* AC4150 (Nal^r) and CUCPB5006 (Nal^r Kan^s Δ*pelABCE*) by marker exchange mutagenesis. Analysis of the resulting mutants, CUCPB5008 (Pel$^+$ Peh$^-$) and CUCPB5009 (Pel$^-$ Peh$^-$), indicated that exoPG can contribute significantly to bacterial utilization of polygalacturonate and the induction of pectate lyase in the presence of extracellular pectic polymers. CUCPB5009 retained a slight ability to pit polygalacturonate semisolid agar and macerated chrysanthemum pith tissues when large numbers of bacteria were inoculated.

The enterobacterium *Erwinia chrysanthemi* causes diseases involving maceration and killing of parenchymatous tissues (soft rots) in a wide variety of plant hosts (26). Pectic enzymes produced by the bacterium have been proposed to account for the soft rot symptoms (9). These enzymes include extracellular exo-poly-α-D-galacturonosidase (exo PG; EC 3.2.1.82), pectin methylesterase, and multiple isozymes of pectate lyase (9). In strain EC16, the *pel* genes encoding the four major isozymes of pectate lyase have been cloned (1, 18) and sequenced (19, 34). Their predominant role in maceration was demonstrated by constructing *E. chrysanthemi* mutant UM1005, in which all four genes were deleted from the bacterial genome, and observing that maceration ability was significantly reduced (31). UM1005, however, retained the potential to macerate appropriate plant tissues when high levels of inoculum were used, and the mutant could utilize polygalacturonate as a sole carbon source (31). It thus became evident that mutations in additional pectic enzyme genes would be required to fully understand the basis for the pathogenic and pectolytic capacities of this bacterium.

ExoPG was first isolated from cell extracts of *E. aroideae* (*E. carotovora*) by Hatanaka et al. (16) and was found to cleave polygalacturonate at the penultimate glycosidic bond, generating exclusively digalacturonic acid. In *E. chrysanthemi*, exoPG was purified and characterized biochemically in strain CUCPB1237 (14). ExoPG is the largest of several proteins secreted to the medium by *E. chrysanthemi* EC16 and is present in the culture supernatants of many, but not all, strains of *E. chrysanthemi* (29, 30). Previous physiological experiments had suggested that exoPG could play an important role in the bacterial utilization of polygalacturonate and in the induction of pectate lyase synthesis (14). Although the action pattern of the enzyme on polygalacturonate argues against a direct role in tissue maceration, exoPG was the only pectic depolymerase clearly demonstrable either in culture supernatants of UM1005 or in potato tuber tissue macerated by the Pel$^-$ mutant (31).

In order to better understand the activities of exoPG in the plant pathogenicity of *E. chrysanthemi*, we have now cloned and characterized the *pehX* gene from *E. chrysanthemi* UM1005 and constructed and analyzed exoPG-deficient mutants.

(Portions of this work have appeared elsewhere [8, 10, 11].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids are listed in Table 1. *E. chrysanthemi* EC16 was originally isolated from chrysanthemum, in which it causes bacterial blight characterized by maceration of the pith and collapse of the stem (4, 25). All *E. chrysanthemi* strains used in this study are derivatives of EC16. Bacteria were routinely grown in King medium B (KB) (20) or Luria-Bertani (LB) medium (22) with appropriate antibiotics. *E. chrysanthemi* strains were also grown in Zucker and Hankin minimal medium (39) or basal minimal medium A-P (22) supplemented with appropriate concentrations of potassium phosphate (pH 7.6), 0.2% (vol/vol) glycerol, or 0.2% (wt/vol) polygalacturonate (Pfaltz and Bauer, Inc.) for marker exchange mutagenesis and analysis of polygalacturonate utilization.

General DNA manipulations. Plasmid DNA was isolated and manipulated by standard techniques (22). Except where noted, DNA-modifying enzymes were purchased from Bethesda Research Laboratories, International Biotechnology, Inc., or Boehringer Mannheim. Chromosomal DNA was isolated by the method of Silhavy et al. (33). Plasmids were introduced into *E. coli* by transformation and into *E. chrysanthemi* either by conjugations with pBR322 derivatives mobilized by the helper plasmids pLCV9 and R64*drd*11 (36)

* Corresponding author.

TABLE 1. Bacteria and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
HB101	F⁻ *hsdS20 (hsdR hsdM) recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44* λ	22
CC118	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA*v20 *galE galK thi rpsE rpoB argE*(Am) *recA1*	23
DH5α	F⁻ *endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA96 relA1* φ80d *lacZ*ΔM15	Bethesda Research Laboratories
E. chyrsanthemi		
AC4150	Nal^r derivative of EC16	5
UM1005	Δ(*pelB pelC*)::28 bp Δ(*pelA pelE*)::*nptI* derivative of AC4150	31
BC501	Δ(*pelB pelC*)::28 bp Δ*pelE*::*nptI-sacB-sacR* derivative of AC4150	This work
CUCPB5006	Δ(*pelB pelC*)::28 bp Δ(*pelA pelE*) derivative of AC4150	This work
CUCPB5008	*pehX*::Tn*phoA* derivative of AC4150	This work
CUCPB5009	*pehX*::Tn*phoA* derivative of CUCPB5006	This work
Plasmids		
pBluescript SK	Amp^r	Strategene
pBR322	Amp^r Tet^r	3
pUC19	Amp^r	38
R64*drd*11	Transfer-derepressed derivative of R64, Tet^r	36
pLVC9	pGJ28 derivative carrying ColE1 *mob*, Cm^r	G. Warren (36)
pPEL74	pBR329 derivative of pPEL7 containing *pelA pelE*; Tet^r Cm^r	18
pPEL748	pINIII containing *pelE*	18
pJR44	*Eco*RI deletion derivative of pPEL74	This work
pSH44B	*Pst*I fragment from pJR44 in *Eco*RV site of pBR322	This work
pPEH1	pUC19 containing 8.5-kb UM1005 chromosomal DNA, exoPG⁺	This work
pPEH4	pBR322 containing 7.4-kb *Eco*RI fragment from pPEH1, exoPG⁺	This work
pPEH9	pPEH4 derivative containing *pehX*::Tn*phoA*, exoPG⁻	This work
pPEH10	pUC19 carrying 2.3-kb *Sal*I fragment from pPEH1, exoPG⁺	This work
pPEH11	pUC19 containing 4.7-kb *Kpn*I fragment from pPEH1, exoPG⁺	This work

or by electroporation. For electroporation, the following conditions were used: bacteria were grown to late logarithmic phase (OD_{600} of 0.8 to 1.0) in 5 ml of KB broth at 37°C. Cells were collected after centrifugation and washed twice in ice-cold electroporation buffer, which consisted of 7 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4), 272 mM sucrose, and 1 mM $MgCl_2$. The washed cells were incubated with the desired amount of plasmid DNA in 0.4 ml of electroporation buffer on ice for 10 min and electroporated at 2,500 V with the capacitor set at 25 μFD. The electroporated cells were immediately incubated in 5 ml of warm KB for 1 h before being spread on appropriate plates. Under these conditions, 200 to 400 transformants per microgram of plasmid (10 to 20 kilobases [kb]) DNA could be obtained.

ExoPG antibody preparation. ExoPG antigen was prepared from *E. chrysanthemi* CUCPB1273. The culture supernatant of CUCPB1237 grown in Zucker and Hankin minimal medium supplemented with 0.5% polygalacturonate was concentrated and desalted repeatedly by using an immersible CX-10 membrane (Millipore Corp.) to give a 1.5-ml solution containing 5 mg of protein per ml. Proteins in this preparation were resolved on a 10% preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel by electrophoresis under conditions described previously (13). The band corresponding to exoPG (67 kilodaltons [kDa]) was excised from the gel, and approximately 600 μg of exoPG protein in 2.0 ml of Tris-EDTA buffer (pH 7.5) was recovered from the homogenized gel after centrifugation. A New Zealand White rabbit was injected twice with the concentrated CUCPB1237 supernatant containing 5 mg of protein per ml (250 μl plus 250 μl of Freund incomplete adjuvant) 5 days apart. The following three injections were with purified exoPG (500 μl of enzyme sample plus 500 μl of Freund incomplete adjuvant) and were carried out 6 days later and at 2-week intervals. Two weeks after the last injection, approximately 50 ml of antiserum was collected, passed through a filter (0.22-μm pore size), and stored at −70°C for later use.

Immunoblot and ultrathin-layer polyacrylamide isoelectric focusing gels. All bacterial cultures for protein analyses were grown at 30°C to late logarithmic phase in KB broth. Proteins in the bacterial periplasmic fraction were released by the method of Witholt et al. (37). The remaining proteins in the bacterial spheroplasts were obtained by sonication as described previously (24). Concentrated protein samples were obtained either by ultrafiltration in Centricon tubes (molecular weight cutoff of 10,000; Amicon Division, W. R. Grace & Co.) or by precipitation with ice-cold trichloroacetic acid solution at a final concentration of 5.0%, followed by suspension in an appropriate volume of 10 mM Tris hydrochloride buffer (pH 7.0). Ultrathin-layer isoelectric focusing and activity staining were done under conditions described previously (29) except that a solution of 1.0% hexadecyltrimethylammonium bromide was used for overlay staining. The same samples were subjected to SDS-polyacrylamide gel analyses under conditions described before (13), and the immunoblot was done with GeneSceen (Du Pont) according to the manufacturer's recommendations. The enzyme-linked second antibody was a goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase. Nonspecific antibodies were removed by incubation of exoPG antiserum with *E. coli* HB101 cell lysate overnight, followed by a high-speed spin at 4°C for 10 min in an Eppendorf microcentrifuge. Supernatant containing exoPG antibody was kept at 4°C with 0.02% (wt/vol) sodium azide added.

Cloning of the *pehX* gene from UM1005. A partial *Sau*3A

digest of *E. chrysanthemi* UM1005 chromosomal DNA was fractionated on a 0.7% agarose (genetic biotechnology grade; FMC Corp.) gel. A slice of agarose gel containing DNA fragments (7 to 9 kb in size) was cut out and electrophoretically eluted in an Elutrap apparatus (Schleicher & Schuell). These *Sau*3A fragments were cloned into the *Bam*HI site of pUC19 as described previously (13).

E. coli HB101 was then transformed with ligated, recombinant plasmid DNA and spread on LB-ampicillin plates. Approximately 2,800 transformants were screened with exo PG antibody by the following procedure. Two hundred colonies were picked to each LB-ampicillin plate, which was then incubated at 37°C overnight. For bacterial cell lysis, the plates were first exposed to chloroform for 10 min and then covered by a piece of nylon membrane (GeneSceen Plus) saturated with a lysis solution containing 0.10% SDS and 0.25 mg of lysozyme per ml. Thirty minutes later, the membrane was transferred to a new petri dish, followed by the immunoblot steps described above. The exoPG-positive colonies were picked to both LB-ampicillin and polygalacturonate semisolid agar plates for further analysis and pitting assays.

Enzymatic assays. Pectate lyase and exoPG activity were measured as described before (12). One unit of activity is defined as releasing 1 μmol of product per min. Samples for the enzymatic assays were from either KB culture supernatants or osmotic shock fluids.

Mutagenesis of the *pehX* gene with Tn*phoA*. pPEH4 (pBR322 carrying the 7.4-kb *Eco*RI fragment from pPEH1) was first transformed into *E. coli* CC118. Infection with λ::Tn*phoA* and the subsequent selection step essentially followed the procedure described by Manoil et al. (23) except that 450 μg of kanamycin per ml was used. Transductants were then grown in KB broth containing ampicillin (50 μg/ml) and kanamycin (450 μg/ml) overnight, and the supernatants were assayed for loss of exoPG activity. Plasmids were also isolated from Kan^r transductants to determine the loci of the Tn*phoA* insertions by restriction mapping. Marker exchange mutagenesis of the *E. chrysanthemi* genome with a *pehX*::Tn*phoA* construct was done as described previously (32).

Construction of CUCPB5006. *E. chrysanthemi* UM1005 is not useful for further mutagenesis with transposons or cartridges conferring kanamycin resistance because it contains the *nptI* gene at the chromosomal locus of the *pelA-pelE* cluster (31). Consequently, a Kan^s Pel$^-$ strain was constructed to permit mutagenesis of the *pehX* gene with Tn*phoA*. pPEL74, carrying *pelA-pelE*, was partially digested with *Sau*3A and ligated with the *Bam*HI-cut *nptI-sacB-sacR* cartridge (31) to produce pJR501. This plasmid, in which only *pelE* was inactivated, was transformed into *E. chrysanthemi* UM1003 (Kan^s Δ*pelBC*; 31), and the inactivated *pelE* was marker-exchanged into the UM1003 chromosome to generate BC501, which was PelBCE deficient, Kan^r, and sucrose sensitive.

The following plasmid was then constructed to permit deletion of *pelA-pelE* and eviction of the *nptI-sacB-sacR* cartridge. First, the adjacent *Eco*RI fragments in pPEL74, containing most of *pelA* and *pelE*, were deleted to produce pJR44. The resulting 4.7-kb *Pst*I fragment was excised, treated with mung bean nuclease, and ligated into the *Eco*RV site of pBR322. The resulting recombinant plasmid, pSH44B, was conjugated into BC501. Sequences containing the *pelA-pelE* deletion were introduced into the chromosome in exchange for *pelA* and *pelE*::*nptI-sacB-sacR* by selecting sucrose-tolerant colonies on LB plates containing 5.0% sucrose and then picking these colonies to LB-kanamycin, LB-tetracycline, LB-nalidixic acid, and polygalacturonate semisolid agar replicate plates. One of the colonies which was Nal^r Tet^s Kan^s Pit$^+$ (pitting positive) was chosen for further study and was designated CUCPB5006.

Sequencing of the *pehX* gene. The *Sal*I fragment of pPEH10 was cloned into sequencing vector pBluescript SK. A series of exonuclease III deletions were generated from both directions, and the sequences of both strands were determined by using the T7 Sequencing Kit from Pharmacia LKB Biotechnology. Data were analyzed with the Microgenie 6.0 system. The *pehX* nucleotide sequence has been submitted to the GenBank data bank with the accession number M31308. The gene designation (pectic enzyme hydrolase) follows the proposed nomenclature for genes of degradative-enzyme production developed at the EMBO Workshop on Soft-Rot Erwiniae, 23–27 July 1984, Marseille-Luminy, France.

N-terminal sequencing of exoPG. ExoPG of strain EC16 was purified to homogeneity from the osmotic shock fluid of *E. coli* DH5α containing pPEH10 by preparative granulated-gel bed isoelectric focusing (100 ml of Bio-Lyte electofocusing gel containing 0.2% ampholyte with a pH range of 3 to 10). All operations followed the manufacturer's instruction manual for the Bio-Phoresis horizontal electrophoresis cell (Bio-Rad Laboratories). The N-terminal amino acid sequence (first 13 amino acids) was determined by using an Applied Biosystems 470A protein sequencer.

Assays of the effects of enzymes and bacteria on plant tissues. Two methods were used to study the in planta virulence of Peh$^-$ mutants and the macerating capacity of the isolated exoPG. (i) Chrysanthemum shoot cuttings (cv. Pert) were inoculated by applying to a fresh cut at the base of the stem 10 μl of sterile water containing 10^2, 10^4, or 10^6 bacteria. After 20 min, all of the bacterial suspension was absorbed, and the cuttings were transferred to sterile plastic tubes containing sterile water and incubated at 30°C in a growth chamber for 48 h. The cuttings were then sliced open with a razor blade, and the length of maceration in the pith tissue was recorded. (ii) The effects of isolated exoPG on plant tissues were analyzed with thinly sliced potato tuber and chrysanthemum shoot disks. Surface-sterilized potato tubers and chrysanthemum shoots (4 mm in diameter) were cut into disks (8 by 1 mm and 4 by 1.5 mm, respectively). Both potato tuber and chrysanthemum shoot disks were treated with 1 mM $CaCl_2$ before addition of the enzyme, to increase tissue viability (2). Two potato tuber disks or six chrysanthemum shoot disks were put into a sterile plastic tube containing 3 ml of solution consisting of either 100 mM Tris hydrochloride (pH 8.5) and 1 mM $CaCl_2$ (for pectate lyase isozyme PelE) or 100 mM phosphate buffer (pH 6.5) and 10 mM EDTA (for exoPG). Ten units of PelE or exoPG in the concentrated osmotic shock fluids from *E. coli* strains containing cloned *pelE* (pPEL748) or *pehX* (pPEH1) was added to each tube. The same amounts of heat-inactivated (boiled for 10 min) PelE and exoPG were used as controls. The plastic tubes were then shaken (120 rpm) at 25°C for 12 h before the tissue was examined for maceration.

RESULTS

Cloning and analysis of the *pehX* gene. Our previous experiences with exoPG suggested that *pehX*$^+$ *E. coli* transformants would be difficult to identify in enzymatic screens. ExoPG antibodies were thus used to immunoscreen the *E. chrysanthemi* UM1005 DNA library. Mutant UM1005 was

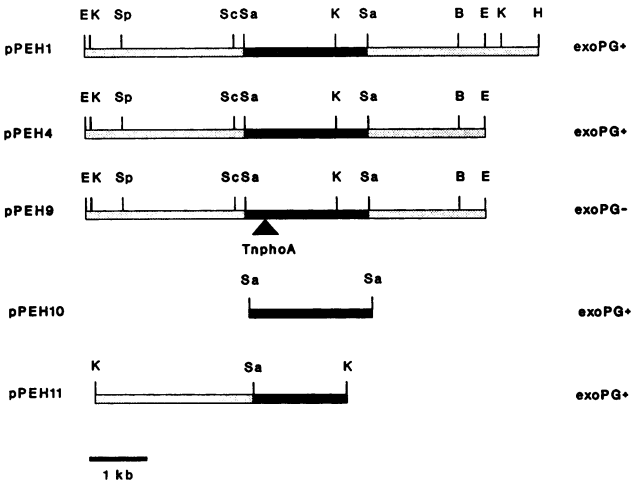

FIG. 1. Physical map of *pehX* clones and mutants. The restriction enzymes are abbreviated as follows: B, *Bam*HI; K, *Kpn*I; Sa, *Sal*I; Sc, *Sca*I; Sp, *Sph*I; E, *Eco*RI; H, *Hin*dIII. The arrow indicates the locus of Tn*phoA* insertion. All subcloning was done in pUC19. Overnight KB culture supernatants were assayed for exoPG activity as described in Materials and Methods. The orientation of the DNA insertion in pPEH1 is indicated by the orientation of *Eco*RI and *Hin*dIII sites, which are in the multiple cloning site of pUC19. The insertions in pPEH10 and pPEH11 have the same orientation as in pPEH1.

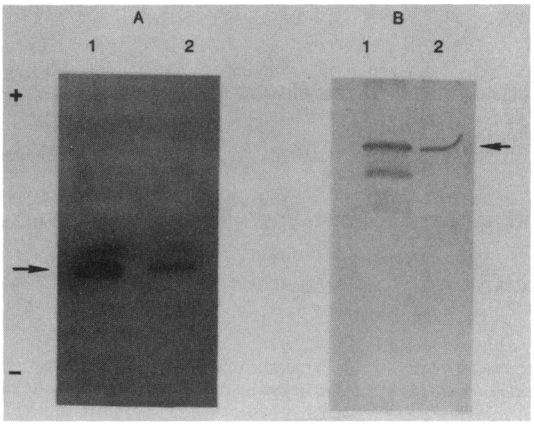

FIG. 2. Activity-stained isoelectric focusing gel and immunoblotted SDS gel of the exoPG from HB101(pPEH1) and UM1005. Ten microliters of a 10-fold-concentrated KB culture supernatant was loaded onto each lane for both the isoelectric focusing gel and the SDS gel. Other conditions are described in Materials and Methods. (A) Arrow indicates the clearing due to exoPG activity. Lanes: 1, UM1005; 2, HB101(pPEH1). (B) Arrow indicates the exoPG protein band corresponding to a molecular mass of 67 kDa. Lanes: 1, UM1005; 2, HB101(pPEH1).

used as the source of the library because the *pel* deletions would diminish interference from the pectate lyase isozymes in identification of *pehX*$^+$. In the primary immunoscreen of 2,800 HB101 colonies containing recombinant pUC19 with UM1005 chromosomal DNA (7 to 9 kb), 11 positive colonies were identified. These positive colonies were then subjected to a second immunoblot with preabsorbed antibody; one clone retained strong reactivity with this antibody. The plasmid was isolated from this clone, found to include an 8.5-kb insertion in pUC19, and designated pPEH1. The restriction map is shown in Fig. 1. Two subclones of pPEH1, pPEH10 and pPEH11, retained exoPG activity. Addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to the LB broth at a final concentration of 1 mM at the beginning of the incubation did not affect the expression of *pehX* in *E. coli* DH5α carrying either pPEH10 or pPEH11, indicating that in both of the subclones, the native *pehX* promoter was still directing *pehX* expression in *E. coli*.

Characterization of the *pehX* gene product. Samples from osmotic shock fluids of *E. coli* HB101 containing pPEH1 were subjected to isoelectric focusing coupled with exoPG activity staining. A cleared band was found at a pI corresponding to that caused by the exoPG from UM1005 (pI 8.3, Fig. 2). An immunoblotted SDS gel of the same samples with exoPG antibody also revealed a protein band which comigrated with the exoPG (67 kDa, Fig. 2) from the culture supernatant of UM1005. These two observations confirmed that the exoPG activity of HB101 containing pPEH1 was produced by the cloned *pehX* gene from UM1005. Fractionation of *E. coli* HB101(pPEH1) into supernatant, periplasm (osmotic shock fluid), and cytoplasm plus membranes (sonicated spheroplast mixture) after its growth into early stationary phase in LB broth showed that most of the exoPG activity resided in the periplasmic fraction (>80%), about 15% of the activity was found in the medium, and a trace amount of activity was in the cytoplasm plus membrane fraction (<5%). A higher proportion of activity was detected in the medium during stationary growth phase, apparently due to cell lysis. The *pehX* gene was expressed quite well from its native promoter in *E. coli* (about 18.5 U/mg of bacterial protein) in an overnight KB culture. Under similar conditions (in the absence of polygalacturonate), *E. chrysanthemi* produced about 1.3 U of exoPG per mg of bacterial protein. The temperature (30 versus 37°C) during incubation of *E. coli* strains containing pPEH1 did not seem to significantly affect the total activity of exoPG detected, in contrast to the case of *pel* genes reported by Keen et al. (19).

Nucleotide sequence of the *pehX* gene. The DNA sequence of the 2.3-kb *Sal*I fragment in pPEHI was determined (Fig. 3). The coding sequence for exoPG was located from positions 214 to 2028. A putative amino-terminal signal peptide (27 amino residues), with a relatively long charged region resembling that of PelE (19), was identified and was consequently confirmed by N-terminal sequencing of the mature exoPG protein (Fig. 3). A typical ribosome-binding site, consisting of AGGA, was located 7 bases upstream of the translational initiation codon. A putative promoter region (from 148 to 177) was identified which shared 75% homology with the consensus *E. coli* promoter and 100% homology with the *E. chrysanthemi pelB* promoter (19). There was only one open reading frame downstream from the promoter region, coding for a preprotein of 64,608 daltons, about 2 kDa smaller than that of mature exoPG estimated from SDS-polyacrylamide gel electrophoresis. The reason for this discrepancy is not known, although similar discrepancies between molecular masses predicted from nucleotide sequences and those estimated from SDS-polyacrylamide gels were also reported for two extracellular pectate lyase isozymes, PelE and PelA (19).

The coding region of exoPG was terminated by two successive stop codons, TGA CGG TAA, located between positions 2020 and 2028, which were followed by a G+C-rich, near-perfect inverted repeat from positions 2038 to 2059. However, this inverted repeat was not followed by a stretch of T's, which is the typical constituent of a rho-independent terminator. A putative binding site of catabolic activator protein (CAP) was also identified, beginning with

```
       10         20         30         40         50         60
CGACGCCAACGGCCATCTTAACCGCAAGAAGGGGTAACAATCGGTTAGGGGTAACAATTG

       70         80         90        100        110        120
GTTACCCCTTTCTTTTATCCCCTCGATTGATTTGTGATCCCTTCCGGGGAAAAAAGAAAT

      130        140        150        160        170        180
ACGGGATGTATTTTTATAAAATAGTATTTTGAAAAAAACATCCCACACGGATAAAACACC

      190        200        210        220        230        240
GCCATCAATTTAAAAGAGAACTAAGGAACAAGGATGAAAGTCATCACATTTTCACGTCGT
                                        MetLysValIleThrPheSerArgArg

      250        260        270        280        290        300
TCGGCCCTTGCCTCGATAGTAGCCACATGCCTGATGAGTACACCAGCACTGGCGGCAACG
SerAlaLeuAlaSerIleValAlaThrCysLeuMetSerThrProAlaLeuAlaAlaThr
                                                          * *
      310        320        330        340        350        360
GCTCAGGCACCGCAAAAACTCCAGATTCCCACACTGTCTTACGACGACCATAGCGTCATG
AlaGlnAlaProGlnLysLeuGlnIleProThrLeuSerTyrAspAspHisSerValMet
 * * * * * * * * * * *

      370        380        390        400        410        420
CTGGTGTGGGACACGCCGGAAGACACGTCCAACATTACCGACTATCAGATTTACCAAAAT
LeuValTrpAspThrProGluAspThrSerAsnIleThrAspTyrGlnIleTyrGlnAsn

      430        440        450        460        470        480
GGCCAGTTGATTGGACTCGCCAGCCAAAATAATGACAAAAACTCGCCGGCCAAACCGTAC
GlyGlnLeuIleGlyLeuAlaSerGlnAsnAsnAspLysAsnSerProAlaLysProTyr

      490        500        510        520        530        540
ATCAGTGCGTTCTACAAAAGTGACGCCGCGAATTTTCACCACCGAATTGTCCTGCAAAAC
IleSerAlaPheTyrLysSerAspAlaAlaAsnPheHisHisArgIleValLeuGlnAsn

      550        560        570        580        590        600
GCTAAAGTCGATGGTCTGAAAGCCGGCACCGATTATCAGTTTACCGTTCGTACAGTCTAT
AlaLysValAspGlyLeuLysAlaGlyThrAspTyrGlnPheThrValArgThrValTyr

      610        620        630        640        650        660
GCCGATGGCACAACTTCGAACGACAGTAACACAGTTACCACCACCACCACCGCTGTACCC
AlaAspGlyThrThrSerAsnAspSerAsnThrValThrThrThrThrThrAlaValPro

      670        680        690        700        710        720
AAAGTCATCAACATCACCCAGTACGGCGCCAAAGGCGACGGCACTACGCTCAATACCAGC
LysValIleAsnIleThrGlnTyrGlyAlaLysGlyAspGlyThrThrLeuAsnThrSer

      730        740        750        760        770        780
GCGATTCAGAAAGCGATTGACGCCTGCCCAACCGGTTGCCGTATCGACGTTCCGGCCGGG
AlaIleGlnLysAlaIleAspAlaCysProThrGlyCysArgIleAspValProAlaGly

      790        800        810        820        830        840
GTGTTCAAAACCGGCGCTCTGTGGCTGAAAAGCGATATGACGCTGAATCTGTTGCAAGGT
ValPheLysThrGlyAlaLeuTrpLeuLysSerAspMetThrLeuAsnLeuLeuGlnGly

      850        860        870        880        890        900
GCAACGCTGCTGGGGTCGGATAATGCGGCCGACTATCCCGATGCGTATAAAATTTATAGC
AlaThrLeuLeuGlySerAspAsnAlaAlaAspTyrProAspAlaTyrLysIleTyrSer

      910        920        930        940        950        960
TACGTCTCGCAGGTACGCCCTGCTTCACTGCTGAACGCTATCGATAAAAACAGCTCGGCT
TyrValSerGlnValArgProAlaSerLeuLeuAsnAlaIleAspLysAsnSerSerAla

      970        980        990       1000       1010       1020
GTGGGCACCTTTAAAAACATCCGTATCGTCGGTAAAGGCATTATCGACGGCAACGGCTGG
ValGlyThrPheLysAsnIleArgIleValGlyLysGlyIleIleAspGlyAsnGlyTrp

     1030       1040       1050       1060       1070       1080
AAACGCAGTGCGGATGCCAAAGATGAACTGGGCAATACCCTGCCGCAGTACGTGAAGAGT
LysArgSerAlaAspAlaLysAspGluLeuGlyAsnThrLeuProGlnTyrValLysSer

     1090       1100       1110       1120       1130       1140
GATAACAGCAAAGTGAGCAAAGATGGCATTCTGGCGAAAAACCAGGTTGCGGCGGCCGTG
AspAsnSerLysValSerLysAspGlyIleLeuAlaLysAsnGlnValAlaAlaAlaVal

     1150       1160       1170       1180       1190       1200
GCTACCGGCATGGATACCAAAACCGCCTACAGCCAGCGCCGCTCCAGTTTGGTCACCCTA
AlaThrGlyMetAspThrLysThrAlaTyrSerGlnArgArgSerSerLeuValThrLeu

     1210       1220       1230       1240       1250       1260
CGCGGCGTCCAAAACGCTTATATCGCCGATGTCACCATCCGCAACCCAGCTAACCACGGC
ArgGlyValGlnAsnAlaTyrIleAlaAspValThrIleArgAsnProAlaAsnHisGly

     1270       1280       1290       1300       1310       1320
ATCATGTTCCTGGAAAGCGAGAATGTGGTGGAAAACAGCGTTATCCACCAGACGTTCAAT
IleMetPheLeuGluSerGluAsnValValGluAsnSerValIleHisGlnThrPheAsn

     1330       1340       1350       1360       1370       1380
GCCAACAATGGCGATGGCGTCGAATTTGGCAACAGCCAAAACATCATGGTATTCAACAGC
AlaAsnAsnGlyAspGlyValGluPheGlyAsnSerGlnAsnIleMetValPheAsnSer

     1390       1400       1410       1420       1430       1440
GTGTTCGATACCGGGGACGATAGCATCAACTTCGCAGCAGGGATGGGCCAGGACGCACAG
ValPheAspThrGlyAspAspSerIleAsnPheAlaAlaGlyMetGlyGlnAspAlaGln

     1450       1460       1470       1480       1490       1500
AAGCAAGAGCCTTCGCAAAACGCCTGGTTGTTCAACAACTTTTTCCGTCACGGCCACGGC
LysGlnGluProSerGlnAsnAlaTrpLeuPheAsnAsnPhePheArgHisGlyHisGly

     1510       1520       1530       1540       1550       1560
GCTGTGGTGTTAGGCAGCCACACCGGCGCAGGGATTGTCGATGTTCTGGCGGAAAACAAC
AlaValValLeuGlySerHisThrGlyAlaGlyIleValAspValLeuAlaGluAsnAsn

     1570       1580       1590       1600       1610       1620
GTGATAACCCAGAACGATGTGGGTCTGCGCGCCAAGAGCGCCCCGGCCATCGGCGGCGGC
ValIleThrGlnAsnAspValGlyLeuArgAlaLysSerAlaProAlaIleGlyGlyGly

     1630       1640       1650       1660       1670       1680
GCACATGGCATCGTGTTCCGCAACAGCGCGATGAAAAACCTGGCCAAACAGGCCGTCATC
AlaHisGlyIleValPheArgAsnSerAlaMetLysAsnLeuAlaLysGlnAlaValIle

     1690       1700       1710       1720       1730       1740
GTGACGTTAAGTTACGCCGACAACAACGGCACCATCGACTATACCCCGGCCAAAGTGCCC
ValThrLeuSerTyrAlaAspAsnAsnGlyThrIleAspTyrThrProAlaLysValPro

     1750       1760       1770       1780       1790       1800
GCACGATTCTATGACTTCACCGTCAAAAACGTCACCGTGCAGGACAGTACCGGCTCAAAC
AlaArgPheTyrAspPheThrValLysAsnValThrValGlnAspSerThrGlySerAsn

     1810       1820       1830       1840       1850       1860
CCGGCGATTGAGATCACCGGCGACAGCAGCAAAGATATCTGGCATAGCCAGTTTATCTTC
ProAlaIleGluIleThrGlyAspSerSerLysAspIleTrpHisSerGlnPheIlePhe

     1870       1880       1890       1900       1910       1920
AGCAACATGAAACTCAGCGGCGTAAGCCCGACGTCAATCAGCGACCTGAGCGACAGCCAG
SerAsnMetLysLeuSerGlyValSerProThrSerIleSerAspLeuSerAspSerGln

     1930       1940       1950       1960       1970       1980
TTCAACAACCTGACGTTCAGCAATCTGCGCAGCGGCTCTTCACCGTGGAAATTCGGTACC
PheAsnAsnLeuThrPheSerAsnLeuArgSerGlySerSerProTrpLysPheGlyThr

     1990       2000       2010       2020       2030       2040
GTGAAAAACGTGACCGTAGACGGTAAAACCGTCACCCCCTGACGGTAAGCCTCAACACGC
ValLysAsnValThrValAspGlyLysThrValThrProEndArgEnd

     2050       2060       2070       2080       2090       2100
CGGGGCATGGCGCCCCGCGGTATCCGCTTCCCGTGTTAGCCTTTGTCTCTGGCCTTCGCCA

     2110       2120       2130       2140       2150       2160
ATTGAGCGCGTAGGTTCGCCAGATGGTTCTGCCCTTTCTGCATCGCTCTGCGGGCTGACG

     2170       2180       2190       2200       2210       2220
ACTTTGCGTTCGGTTTCCCACATCACATCATCTGCGGCAGTTCCAGCAAAAAACGACTGG

     2230       2240       2250       2260       2270       2280
GCTCCGGGCGGATCAACTCGCCGTATTCGCGGCTCGCGGCACAGGGTGAACGTTAACTCT

     2290       2300       2310       2320       2330
TTCTGAGCCCGGGTGATACCGACGTAGGCCAGACGGCGCTCCTCGTCGACGGTCCCGAT
```

FIG. 3. Nucleotide sequence of 2.3-kb *E. chrysanthemi* EC16 chromosomal DNA containing *pehX* gene. The sequence of the noncoding strand is shown. All possible transcriptional and translational control elements are underlined and discussed in the text. Asterisks mark the N-terminal amino acids of the mature exoPG protein determined by protein sequencing.

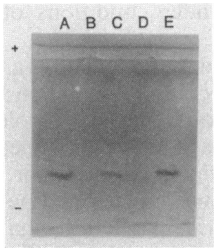

FIG. 4. Isoelectric focusing and exoPG activity staining of CUCPB5006, CUCPB5008, CUCPB5009, and AC4150 culture supernatants. Ten microliters of 10-fold-concentrated overnight KB broth cultures was applied to each lane. Bands in the activity-stained overlay indicate the location of exoPG in the electrofocused gel. Lanes: A and E, AC4150; C, CUCPB5006; B, CUCPB5008; D, CUCPB5009.

the *E. coli* conserved CAP-binding sequence TGTGA at position 93. No sequence similar to the putative KdgR-binding site was present in the sequenced *pehX* region. A possible binding site for the KdgR repressor has been reported for other genes involved in pectate catabolism (28). Sequence analysis revealed that pPEH11 should encode a truncated form of exoPG lacking the carboxy terminus which nevertheless retained enzymatic activity. This was confirmed by immunoblotting *E. coli* DH5α harboring either pPEH10 or pPEH11; a truncated protein was detected from the latter strain by using exoPG antibody (data not shown).

Sequence comparison between *pehX* and all four *pel* genes of *E. chrysanthemi* EC16 (34) at either the nucleotide or amino acid level revealed only limited homologies: eight amino acid residues of exoPG, IDKNSSAV, positioned between 243 and 250 shared high homology to PelB (156-IDNSPNV) and PelE (168-IDNSTNV), but was less homologous to PelC (157-VDDSPNV) and PelA (180-ITNG AHHV). No significant homology was found between *pehX* and *celZ* (15) or *pme* (27), which encode two other extracellular enzymes secreted by *E. chrysanthemi*.

The *pehX* gene in *E. chrysanthemi* appears to be closely linked to the *rep* locus. Sequences downstream of *pehX*, from positions 2112 to 2329 (223 bases), shared significant homology (78.2%) with the *E. coli rep* gene (from positions 2184 to 1403), which is involved in DNA replication.

Construction of *E. chrysanthemi* mutants deficient in *pehX* gene. Tn*phoA* was used to mutate the *pehX* gene because of its potential to generate PehX-PhoA hybrids (23), of use in future studies on the mechanism of exoPG secretion. λ::Tn*phoA* was transduced into CC118 containing pPEH4. One Kan^r transductant was confirmed to be Peh$^-$ by exoPG assays of sonicated cells. Plasmid was isolated, and the insertion of Tn*phoA* was mapped within the *Sal*I-*Kpn*I fragment as shown in Fig. 1. This plasmid, pPEH9, was then mobilized into both AC4150 and CUCPB5006, and subsequent marker exchange mutagenesis resulted in the construction of CUCPB5008 (Pel$^+$ Peh$^-$) and CUCPB5009 (Pel$^-$ Peh$^-$), respectively. Disruption of *pehX* function was confirmed by isoelectric focusing gel analysis of 20-fold-concentrated KB culture supernatants coupled with exoPG activity staining (Fig. 4). The exoPG bands disappeared from lanes of both CUCPB5008 and CUCPB5009, and exoPG activity was no longer present in the supernatants of these mutants (data not shown). There was no indication that the Tn*phoA* insertion in pPEH9 produced a hybrid protein. However, there were several blue colonies on later transduction plates supplemented with 5-bromo-4-chloro-3-indolyl-phosphate, which indicated the formation of exoPG-PhoA hybrid proteins.

TABLE 2. Utilization of polygalacturonate by *E. chrysanthemi* AC4150 and its mutant derivatives[a]

Strain (phenotype)	Medium buffer	Final OD_{600} (mean ± SD)
AC4150 (Pel$^+$ Peh$^+$)	Tris	1.10 ± 0.03
	Phosphate	0.82 ± 0.02
CUCPB5008 (Pel$^+$ Peh$^-$)	Tris	1.05 ± 0.02
	Phosphate	0.08 ± 0.00
CUCPB5006 (Pel$^-$ Peh$^+$)	Tris	0.18 ± 0.01
	Phosphate	0.96 ± 0.02
CUCPB5009 (Pel$^-$ Peh$^-$)	Tris	0.18 ± 0.02
	Phosphate	0.08 ± 0.02

[a] Bacteria were first grown overnight in Tris-buffered medium (basal medium A-P) supplemented with 10 mM phosphate, 5 mM $CaCl_2$, and 0.2% glycerol or in phosphate-buffered Zucker and Hankin medium supplemented with 0.2% glycerol and then transferred to the same media with 0.2% polygalacturonate as the sole carbon source at an initial OD_{600} of 0.10. The bacteria were then shaken at 30°C for 36 h before determination of OD_{600}.

Role of exoPG in the utilization of polygalacturonate. The contribution of exoPG to polygalacturonate catabolism was investigated by culturing *E. chrysanthemi* AC4150 and its mutant derivatives in different minimal media containing polygalacturonate as the sole carbon source. Media containing different buffers were used to favor the activity of either pectate lyase or exoPG. ExoPG with 1 U of activity in standard assay buffer produced 0.86 U of activity in phosphate-buffered Zucker and Hankin medium, but only 0.12 U in Tris-buffered basal A-P medium. One unit of pectate lyase in standard assay buffer produced 0.75 U in Tris-buffered medium but had no detectable activity in phosphate-buffered medium. The data in Table 2 demonstrate the importance of exoPG in polygalacturonate utilization in cultures that are deficient in pectate lyase activity because of either mutations or unfavorable reaction conditions.

Involvement of exoPG in the induction of pectate lyase in the presence of polygalacturonate. To determine the role of exoPG in the induction of pectate lyase when *E. chrysanthemi* is cultured in medium containing polygalacturonate, pectate lyase production was compared in wild-type and Peh$^-$ mutant strains. The two media described above were modified by the addition of 0.2% glycerol and 0.2% polygalacturonate to support equivalent bacterial growth in all cultures. The data support the involvement of exoPG in pectate lyase induction. There was a nearly fourfold increase in pectate lyase synthesis in AC4150 relative to CUCPB5008 in phosphate-buffered medium (8.6 ± 0.9 versus 2.2 ± 0.3 U/mg, respectively; mean ± SD, $n = 3$). Although there was no difference between the two strains in the final level of pectate lyase synthesis in Tris-buffered medium (15.2 ± 1.5 versus 14.6 ± 1.2 U/mg for AC4150 and CUCPB5008, respectively), there was a 2-h delay in the induction of pectate lyase in CUCPB5008 relative to AC4150 (data not shown).

Contribution of exoPG to the maceration capacity of *E. chrysanthemi*. The ability of the pectate lyase-deficient *E. chrysanthemi* mutant UM1005 to pit polygalacturonate semisolid agar plates and to cause at least limited plant tissue maceration raised the possibility that exoPG contributes to these two phenotypes. The contribution of exoPG to the pectolytic phenotype in plate assays was confirmed. *E. coli* strains containing the *pehX* gene were found to cause weak pitting in polygalacturonate semisolid agar plates when the pH of the medium was reduced below 7.0. Furthermore, inactivation of *pehX* from pectate lyase-deficient strains significantly reduced, but did not abolish, the ability of *E. chrysanthemi* to pit polygalacturonate semisolid agar me-

TABLE 3. Virulence of AC4150 and its mutants on chrysanthemum shoot cuttings

Strain (phenotype)	No. of bacteria inoculated	Length of pith tissue macerated[a] (mm)
AC4150 (Pel^+ Peh^+)	4.3×10^2	2.7 ± 0.6
	4.3×10^4	12.5 ± 2.5
	4.3×10^6	26.3 ± 4.3
CUCPB5008 (Pel^+ Peh^-)	4.1×10^2	2.9 ± 0.4
	4.1×10^4	12.3 ± 2.3
	4.1×10^6	23.0 ± 8.0
CUCPB5006 (Pel^- Peh^+)	7.4×10^2	0.0
	7.4×10^4	0.0
	7.4×10^6	1.5 ± 0.5
CUCPB5009 (Pel^- Peh^-)	6.1×10^2	0.0
	6.1×10^4	0.0
	6.1×10^6	1.5 ± 0.4

[a] Values are the mean ± standard deviation for four shoot cuttings.

dium. CUCPB5009 showed only slight pitting after 36 h of incubation, while other mutants (UM1005, CUCPB5006, and CUCPB5008) and wild-type AC4150 caused obvious pitting within 10 h after fresh colonies were picked onto polygalacturonate semisolid agar plates (data not shown).

No evidence was found for a role of exoPG in maceration. Osmotic shock fluid containing 10 U of exoPG activity from DH5α(pPEH10) and HB101(pPEH1) per ml was used to test the maceration ability of exoPG in vitro. The enzyme preparation caused no visible maceration in whole potato tuber, potato slices, or chrysanthemum shoot cutting assays (data not shown). The same amount of PelE caused extensive maceration of both potato tuber disks and chrysanthemum pith tissue. Furthermore, no significant differences in the maceration of chrysanthemum shoot cuttings were found between either CUCPB5006 (Pel^- Peh^+) and CUCPB5009 (Pel^- Peh^-) or AC4150 (Pel^+ Peh^+) and CUCPB5008 (Pel^+ Peh^-), as shown in Table 3. However, the differences in virulence were obvious between AC4150 and its Pel^- or Pel^- Peh^- mutants. CUCPB5006 and CUCPB5009 needed at least a 10^4-fold-higher concentration of bacteria to cause comparable rotting of chrysanthemum.

DISCUSSION

As an enterobacterium with diverse metabolic abilities related to plant pathogenicity, *E. chrysanthemi* has become an important experimental organism (21). The elaborate pectic enzyme system of the bacterium has been the focus of initial studies because the enzymes (i) are involved in systemic invasion and maceration of plant tissues, (ii) permit the aggressive catabolism of pectic polymers which is characteristic of soft rot erwinias, (iii) are efficiently secreted to the medium, and (iv) are self-induced in the presence of an extracellular substrate (9, 21). ExoPG is the largest of the pectic enzymes known to be secreted by *E. chrysanthemi*, and it is the only extracellular enzyme which attacks the termini of pectic polymers and cleaves by hydrolysis. As shown by the mutants we have constructed, exoPG makes an important contribution to some, but not all, of these abilities.

The continued utilization of polygalacturonate by pectate lyase-deficient *E. chrysanthemi* mutants or by wild-type strains cultured in media inhibitory to pectate lyase had previously suggested that exoPG activity could be sufficient for bacterial utilization of polygalacturonate under certain conditions (14). Through the use of *pehX* mutants, we have now demonstrated that exoPG is actually necessary for pectate utilization under conditions of pectate lyase deficiency (Table 2).

We have also found that exoPG has a demonstrable role in the induction of pectate lyase when *E. chrysanthemi* is cultured in media containing high-molecular-weight pectic polymers. Pectate lyase induction appears to be an autocatalytic process in which extracellular degradation of the substrate by basal levels of secreted pectic enzymes results in the release of assimilable oligogalacturonates, which signal the presence of the polymer (6, 7). The induction of pectate lyase by the Peh^- mutant CUCPB5008 was significantly reduced relative to the wild type.

Our results suggest that exoPG may play a crucial role in both bacterial nutrition and induction of pectate lyase under environmental conditions in which pectate lyase is less active. Although the relative activities of the two enzymes in plants or in plant debris remain poorly defined, their complementary reaction requirements could explain why most soft rot erwinias secrete a pectic hydrolase along with multiple isozymes of pectate lyase (9, 30). Interestingly, the only pectic hydrolase demonstrated to be secreted by *E. carotovora* is an endo-cleaving polygalacturonase (9).

The nucleotide sequence of *pehX* is consistent with that expected for an extracellular, catabolic enzyme; it possesses both a signal peptide region and a typical CAP-binding site. Although the pectate lyase isozymes and exoPG have distinct enzymatic properties, they bind to the same polymer and are both secreted into the medium by the same Out machinery in *E. chrysanthemi* EC16 (35). Our analysis of *pehX* failed to identify any amino acid sequences that were common to all of these proteins and is thus consistent with the report of Hinton et al. (17), in which analysis of the sequences of depolymerases secreted by erwinias failed to reveal consensus sequences associated with protein export.

The residual tissue maceration ability of *E. chrysanthemi* UM1005 had suggested that another enzyme(s) still produced by the Pel^- mutant must be responsible for this phenotype. Since exoPG was the only clearly identifiable pectolytic enzyme produced in culture or in rotted tissue by the mutant (31), its possible role had to be assessed. However, we found that inactivation of the *pehX* gene had no effect on the maceration ability of either Pel^- or Pel^+ *E. chrysanthemi* strains. Furthermore, concentrated *E. coli* osmotic shock fluids containing exoPG caused no visible maceration in the susceptible tissues of either potato or chrysanthemum. We thus conclude that exoPG cannot account for the residual maceration ability of Pel^- mutants of EC16 and that any contributions of this enzyme to the maceration abilities of the bacterium are indirect.

We have recently detected the production of novel pectic lyases in the supernatants of Pel^- *E. chrysanthemi* cultures grown on isolated chrysanthemum cell walls (Kelemu and Collmer, unpublished) and are now pursuing the possibility that the residual maceration and pectolytic capacities of this bacterium result from the production of these enzymes.

ACKNOWLEDGMENTS

We are grateful to Jeffrey L. Ried for preparing antibodies against exoPG and for constructing strain BC501 and plasmid pJR44. We thank Yoder Brothers, Inc., Barberton, Ohio, for supplying us with fresh chrysanthemum cuttings. We are also thankful to Ted Thannhauser (Analytical and Synthetic Lab, Biotechnology Program, Cornell University) for helping us determine the N-terminal sequence of exoPG.

This work was supported by grant 87-CRCR-1-2352 from the Competitive Research Grants Office of the U.S. Department of Agriculture.

LITERATURE CITED

1. **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.
2. **Basham, H. G., and D. F. Bateman.** 1975. Killing of plant cells by pectic enzymes: the lack of direct injurious interaction between pectic enzymes or their soluble reaction products and plant cells. Phytopathology **65**:141–153.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
4. **Burkholder, W. H., L. A. McFadden, and A. W. Dimock.** 1953. A bacterial blight of chrysanthemums. Phytopathology **43**:522–526.
5. **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.
6. **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.
7. **Collmer, A., and D. F. Bateman.** 1982. Regulation of extracellular pectate lyase in *Erwinia chrysanthemi*: evidence that reaction products of pectate lyase and exo-poly-α-D-galacturonosidase mediate induction on D-galacturonan. Physiol. Plant Pathol. **21**:127–139.
8. **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. APS Press, St. Paul, Minn.
9. **Collmer, A., and N. T. Keen.** 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. **24**:383–409.
10. **Collmer, A., J. L. Ried, A. D. Brooks, and S. Y. He.** 1988. Pectic enzyme production and *Erwinia chrysanthemi* pathogenicity, p. 76–82. *In* N. T. Keen, T. Kosuge, and L. L. Walling (ed.), Physiology and biochemistry of plant-microbial interactions. Waverly Press, Inc., Baltimore.
11. **Collmer, A., J. L. Ried, G. L. Cleveland, S. Y. He, and A. D. Brooks.** 1988. Mutational analysis of the role of pectic enzymes in the virulence of *Erwinia chrysanthemi*, p. 35–48. *In* B. Staskawicz, P. Ahlquist, and O. Yoder (ed.), Molecular biology of plant-pathogen interactions. UCLA Symposia on Molecular and Cellular Biology, New Series, vol. 101. Alan R. Liss, Inc., New York.
12. **Collmer, A., J. L. Ried, and M. S. Mount.** 1988. Assay methods for pectic enzymes. Methods Enzymol. **161**:329–335.
13. **Collmer, A., C. Schoedel, D. L. Roeder, J. L. Ried, and J. F. Rissler.** 1985. Molecular cloning in *Escherichia coli* of *Erwinia chrysanthemi* genes encoding multiple forms of pectate lyase. J. Bacteriol. **161**:913–920.
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. **Guiseppi, A., B. Cami, J. L. Aymeric, G. Ball, and N. Creuzet.** 1988. Homology between endoglucanase Z of *Erwinia chrysanthemi* and endoglucanase of *Bacillus subtilis* and alkalophilic *Bacillus*. Mol. Microbiol. **2**:159–164.
16. **Hatanaka, C., and J. Ozawa.** 1969. Isolation of a new exo-polygalacturonase producing digalacturonic acid from pectic acid. Agric. Biol. Chem. **33**:116–118.
17. **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.
18. **Keen, N. T., D. Dahlbeck, B. Staskawicz, and W. Belser.** 1984. Molecular cloning of pectate lyase genes from *Erwinia chrysanthemi* and their expression in *Escherichia coli*. J. Bacteriol. **159**:825–831.
19. **Keen, N. T., and S. Tamaki.** 1986. Structure of two pectate lyase genes from *Erwinia chrysanthemi* EC16 and their high-level expression in *Escherichia coli*. J. Bacteriol. **168**:595–606.
20. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Med. **22**:301–307.
21. **Kotoujansky, A.** 1987. Molecular genetics of pathogenesis by soft-rot erwinias. Annu. Rev. Phytopathol. **25**:405–430.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**:8129–8133.
24. **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 isozymes PLb and PLe at high levels and of an *Erwinia chrysanthemi* mutant deficient in PLe. Appl. Environ. Microbiol. **53**:2315–2320.
25. **Pennypacker, B. W., P. E. Nelson, and R. S. Dickey.** 1974. Histopathology of chrysanthemum stems artificially inoculated with *Erwinia chrysanthemi*. Phytopathology **64**:1344–1353.
26. **Perombelon, M. C. M., and A. Kelman.** 1980. Ecology of the soft rot erwinias. Annu. Rev. Phytopathol. **18**:361–387.
27. **Plastow, G. S.** 1988. Molecular cloning and nucleotide sequence of the pectin methyl esterase gene of *Erwinia chrysanthemi* B374. Mol. Microbiol. **2**:247–254.
28. **Reverchon, S., Y. Huang, C. Bourson, and J. Robert-Baudouy.** 1989. Nucleotide sequences of the *Erwinia chrysanthemi ogl* and *pelE* genes, negatively regulated by the *kdgR* gene product. Gene **85**:125–134.
29. **Ried, J. L., and A. Collmer.** 1985. An activity stain for the rapid characterization of pectic enzymes in isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gels. Appl. Environ. Microbiol. **50**:615–622.
30. **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.
31. **Ried, J. L., and A. Collmer.** 1988. Construction and characterization of an *Erwinia chrysanthemi* mutant with directed deletions in all of the pectate lyase structural genes. Mol. Plant-Microbe Interact. **1**:32–38.
32. **Roeder, D. L., and A. Collmer.** 1985. Marker exchange mutagenesis of a pectate lyase isozyme gene in *Erwinia chrysanthemi*. J. Bacteriol. **164**:51–56.
33. **Silhavy, T., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **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.
35. **Thurn, K. K., and A. K. Chatterjee.** 1985. Single-site chromosomal Tn*5* insertions affect the export of pectolytic and cellulolytic enzymes in *Erwinia chrysanthemi*. Appl. Environ. Microbiol. **50**:894–898.
36. **Van Haute, E., H. Joos, M. Maes, G. Warren, M. Van Montagu, and J. Schell.** 1983. Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for the reversed genetics of the Ti plasmids of *Agrobacterium tumefaciens*. EMBO J. **2**:411–417.
37. **Witholt, B., M. Boekhout, M. Brock, J. Kingma, H. van Heerikhuizen, and L. deLeij.** 1976. An efficient and reproducible procedure for the formation of spheroplasts from variously grown *Escherichia coli*. Anal. Biochem. **74**:160–170.
38. **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.
39. **Zucker, M., and L. Hankin.** 1970. Regulation of pectate lyase synthesis in *Pseudomonas fluorescens* and *Erwinia carotovora*. J. Bacteriol. **104**:13–18.