Cloning of Genes for *Erwinia carotovora* subsp. *carotovora* Pectolytic Enzymes and Further Characterization of the Polygalacturonases

J. W. Willis, J. K. Engwall, and A. K. Chatterjee

Department of Plant Pathology, Throckmorton Hall, Kansas State University, Manhattan 66506. Present address of second and third authors: Department of Plant Pathology, 108 Waters Hall, University of Missouri, Columbia 65211.

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ABSTRACT

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The pectolytic enzymes produced by *Erwinia carotovora* subsp. *carotovora* strain Ecc71 were characterized by thin-layer isoelectric focusing (IEF), activity gel overlays, and qualitative enzyme assays. Ecc71 produced five pectate lyases (Pel1, pI ≥ 10.0; Pel2, pI 9.7; Pel3, pI 9.2; Pel4, pI 8.0; and Pel5, pI 6.6), one endo-polygalacturonase (Peh1, pI ≥ 10.0), and at least one exo-polygalacturonase (Peh2) in culture. The levels of pectolytic activity could be increased by induction with citrus pectin, but the enzyme profile remained constant. Four of the *pel* genes (*pel1, pel2, pel3,* and *pel4*) and the *peh1* gene were recovered from an Ecc71 gene library constructed in *E. coli* HB101. The simultaneous recovery of two or more of the pectolytic enzyme genes on single cosmid clones indicated clustering of these genes within the genome. The closely linked genes encoding Peh1 and Pel3 were resolved by Tn*5* mutagenesis. Lack of polar effects in the Tn*5* mutants demonstrated that the genes were distinct transcriptional units. Enzyme preparations from HB101(pAKC213::Tn*5*-2), which had *pel3* inactivated by Tn*5* insertion, were used to characterize Peh1. The enzyme had a pH optimum of 5.5, an inactivation temperature of 60 C, and was stimulated by Na^+, Li^+, K^+, and NH_4^+ ions. Peh1 activity resulted in loss of plant cell membrane integrity, as evidenced by release of polyphenol oxidase, and maceration of potato tuber tissue. Tn*5* mutagenesis followed by marker exchange was used to construct an Ecc71 *peh1*::Tn*5* mutant. This mutant maintained Peh2 activity and, like the parent Ecc71, was virulent under aerobic and anaerobic conditions.

Erwinia carotovora subsp. *carotovora* is the primary causal agent of bacterial soft rot of fruits, vegetables, and other plant tissues. It is a widespread, nonspecific pathogen as evidenced by its broad host range (8,34). *E. c.* subsp. *carotovora* produces a number of extracellular degradative enzymes including pectate lyases (Pel), polygalacturonases (Peh), protease, cellulase, phosphatidase, and pectin lyase (12,14,15). As demonstrated by biochemical tests and genetically modified strains, tissue maceration and cell death during pathogenesis are due primarily to the action of the extracellular enzymes (3,5,6,10,32).

Most *E. c.* subsp. *carotovora* strains elaborate a number of Pel species (15,19,27,31,39,41,46). Although a model has been proposed (39), the actual function of each enzyme in disease development and its importance to pathogen establishment and survival has not been determined. The multiplicity of isofunctional enzymes has precluded the isolation of structural gene mutants, because inactivation of one of the genes would be masked by expression of the others. Although avirulent mutants reduced in extracellular pectolytic, cellulolytic, and phosphatidase enzyme activities have been recovered (5,6), the genetic or biochemical bases of the pleiotropic phenotype remain unknown. However, because virulent revertants regained near-normal enzyme levels, the mutation probably affected enzyme export. We have isolated phenotypically similar mutants that are deficient in enzyme export; such mutants failed to macerate plant tissue (10).

Construction and recovery of pectolytic enzyme structural gene mutants have been made feasible by several recent developments: 1) separation of pectolytic enzymes using isoelectric focusing (IEF) gels, 2) detection of enzymes in the gel by activity assay (7,36), 3) cloning of the enzyme structural genes (27,38,44,46), and 4) construction of mutants by marker exchange (47). Such mutants would be invaluable in the elucidation of enzyme function in pathogenesis or pathogen survival.

As part of a systematic genetic analysis of *E. c.* subsp. *carotovora* pectolytic enzymes and studies of their roles in the host-pathogen interaction, we report: 1) the pectolytic enzyme profile of *E. c.* subsp. *carotovora* strain 71 (Ecc71) using IEF followed by activity gel overlay (IEF-AO), 2) cloning of a *peh* gene and its close linkage with a *pel* gene, 3) evidence for the clustering of pectolytic enzyme genes within the Ecc71 chromosome, 4) properties of Ecc71 endo-Peh including the ability to damage potato tuber tissue, 5) construction of an Ecc71 *peh1* mutant by marker exchange mutagenesis, and 6) the presence of an exo-Peh enzyme in Ecc71.

While this work was in progress, Lei et al (28) reported that Peh of *E. c.* subsp. *carotovora* strain EC was capable of causing tissue maceration. Our work confirms and extends their findings.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are described in Table 1.

Media. The following media were used: Luria broth (LB) (9), polygalacturonate-yeast extract agar (PYA) (11), PYA plus 1 mM EDTA (PYA-EDTA), and minimal salts medium (MM) (9). When required, antibiotics were added to the following concentrations: tetracycline (Tc), 10 μg/ml; kanamycin (Km), 50 μg/ml; ampicillin (Ap), 50 μg/ml; and chloramphenicol (Cm), 10 μg/ml. Citrus pectin (CP) (Sigma Chemical Co., St. Louis, MO) was used at a concentration of 0.5% (w/v) to induce pectolytic enzyme production in *E. c.* subsp. *carotovora*.

DNA-modifying enzymes and agarose gel electrophoresis. Restriction enzymes and T4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, IN) were used according to the supplier's directions. Horizontal gel electrophoresis was carried out in tris-borate buffer and 0.5 or 0.7% agarose according to Maniatis et al (30).

Construction of genomic library. A genomic library of Ecc71 was constructed by ligation of fragments 30–40 kb long from a partial *Sau*3A1 digest into the *Bam*HI site of pHC79 (21) followed by lambda packaging and transduction into *Escherichia coli* HB101 (46).

Plate assay for pectolytic activity. *E. coli* transductants carrying Ecc71 DNA were assayed for Pel and Peh activities on PYA plates as previously described (47). PYA-positive clones carrying *peh* and possibly carrying *pel* were positive on both PYA and PYA-EDTA. Because of the absolute requirement of Ca^{2+} for Pel activity, those clones carrying just *pel* genes were positive only on PYA.

Enzyme preparation and quantitative assays. Bacterial cultures were grown in the appropriate medium at 30 C for 16 hr. After removal of cells by centrifugation (5,000 *g*, 4 C, 10 min), the supernatant was concentrated 100-fold using an Amicon PM 10 filter (Amicon Corp., Danvers, MA). This concentration step was performed each time even though it was not essential for quantitating the pectolytic enzymes produced by Ecc71 or its mutants. The cells were then spheroplasted according to Witholt et al (45) and the periplasmic fluid concentrated by ultrafiltration as above. The bacterial spheroplasts were lysed by sonication (13) after resuspension in 10 mM Tris (pH 7.0). The sonicate was centrifuged (15,000 *g*, 4 C, 15 min) to remove cell debris. All fractions were dialyzed at least 8 hr against 10 mM Tris (pH 7.0).

Each fraction was assayed for Pel and Peh activities as described previously (47). One unit of Pel activity is defined as the amount of enzyme that produces 1 μmol of unsaturated digalacturonic acid (UDG) equivalent per minute at 30 C. One unit of Peh activity is defined as the amount of enzyme that produces 1 μmol of galacturonic acid (GTU) equivalent per minute at 30 C.

Isoelectric focusing and activity assay. Ultrathin-layer (1.0 mm) IEF was carried out in an acrylamide gel composed of 5% acrylamide, 0.13% *N,N'*-methylene-*bis*-acrylamide, 21% glycerol, 0.05% ampholyte (LKB, 3–10 pH range) (LKB Instruments, Inc., Rockville, MD), 0.55 mg/ml ammonium persulfate, and 1.0 μl/ml TEMED. Gels were cast and prefocused at 6 C for 45 min at a constant power of 12W using wicks soaked in 1 M NaOH for the cathode and 40 mM aspartic acid for the anode. One to 15 μl of enzyme preparation was loaded at the midpoint between the anodic and cathodic ends, and the gel was focused to equilibrium at 12W constant.

Bands containing pectolytic activity were visualized using a modification of the Bertheau et al procedure (7). Substrate gels (1 mm thick) containing 0.25% polygalacturonate (PGA) and 1% agarose in Pel assay buffer were cast on agarose gelbond plastic sheets (LKB or Pharmacia). The substrate gel was placed directly on the focused acrylamide gel, avoiding air bubbles, and incubated at 30 C for 1–4 hr. The substrate gel was then peeled off and submerged in a 1% aqueous solution of cetyltrimethylammonium bromide (CTAB) overnight. Areas of enzymatic activity appeared translucent against a white gel background. Electran isoelectric markers (range 4.7–10.6) (BDH Chemicals, Ltd., Gallard-Schlesinger, Carle Place, NY) were used to estimate relative pIs of Pel and Peh.

Transposon mutagenesis. Transposon (Tn*5*) mutagenesis of clones was carried out using λ467 as previously described (47). Transformants were screened for phenotypic changes caused by alterations in the *pel* or *peh* gene using the PYA plate assay.

Marker exchange mutagenesis. Ecc71 competent cells were transformed (43) with pAKC213::Tn*5*-1 and transformants selected on Luria agar (LA) plus Tc, Ap, and Km. Single colonies were inoculated to LB plus Km and grown 18 hr at 35 C to a Klett of 210 (about 1×10^9 cfu/ml). Dilutions of 10^{-6} were plated on LA plus Km, and resulting single colonies were patched to LA plus Km and LA plus Tc plates. Km^r Tc^s colonies were grown in LB for 18 hr at 30 C, and the supernatant and cell lysates were assayed for Peh activity.

Marker exchange recombination was further substantiated by Southern blot analysis (30) using ^{32}P-labeled pAKC213 and λ::Tn*5* as probes.

Determination of enzyme mode of action. Endo- or exo-cleavage of PGA by Peh was determined by examining the kinetics of viscosity reduction in comparison to the increase in reducing groups and by analyzing reaction products (4).

Changes in viscosity were determined at 25 C using a Cannon-Fenske Routine viscometer (size 300). Initial viscosity was determined with 10 ml of a reaction mixture consisting of 20 ml of 1.2% aqueous sodium polypectate and 10 ml of Peh buffer. One unit of enzyme was added to the remaining 20 ml, and viscosity was determined at 5-min intervals until equilibrium was reached. At corresponding time intervals, 0.05 ml was removed for determination of reducing groups (47).

Degradation products were determined using descending paper chromatography (20). Samples (30 μl) taken at 24- and 48-hr periods from a 1.5-ml reaction mixture consisting of 2% acid-insoluble PGA, 0.8 ml; 0.2 M phosphate buffer (pH 6.0), 0.6 ml; 20 mM EDTA, 0.05 ml; and 1 unit of enzyme were spotted on a sheet of No. 1 Whatman paper and developed for 24 hr at room temperature using butanol:acetic acid:water (5:2:3). Reaction mixture blanks without enzyme and also blanks without enzyme containing D-galacturonate, saturated digalacturonate, or saturated trigalacturonate in place of PGA were used as controls. After development, products were visualized using the method of Hough and Jones (22).

Effects of ions on enzymatic activity. The chloride salts of Ba^{2+}, Ca^{2+}, K^+, Li^+, Mg^{2+}, Mn^{2+}, Na^+, and NH_4^+ were added to final concentrations of 1, 50, 100, 150, and 200 mM in a Peh activity reaction mixture (see above), which contained no EDTA or NaCl. The effects of 1 and 2 mM EDTA were also tested. Peh activity was

TABLE 1. Bacterial strains, plasmids, and bacteriophage

Strain, plasmid, or bacteriophage	Relevant characteristics	Source or reference
Escherichia coli		
HB101	*pro leu thi lacY* Str^r *recA hsdR hsdM*	47
Erwinia carotovora subsp. *carotovora*		
71	Wild-type serogroup III	S. H. DeBoer
AC5213	Ecc71 *peh1*::Tn*5*	J. W. Willis et al
Phage		
λ467	λ*b*221 *rex*::Tn*5* c1857 *O*am8 *P*am29	N. Kleckner
λ::Tn*5*	Tn*5* contained within a 24-kb *Kpn*I fragment	E. W. Nester
Plasmid		
pHC79	$Cos^+Ap^rTc^r$	21
pBR329	$Ap^rTc^rCm^r$	17
pAKC211	Pel^+ cosmid clone of Ecc71	J. W. Willis et al
pAKC212	Peh^+Pel^+ cosmid clone of Ecc71	J. W. Willis et al
pAKC213	Subclone of pAKC212 in pBR329	J. W. Willis et al
pAKC218	Subclone of pAKC211 in pBR329	J. W. Willis et al
pAKC227	Pel^+ cosmid clone of Ecc71	J. W. Willis et al
pAKC231	Pel^+ cosmid clone of Ecc71	J. W. Willis et al
pAKC233	Pel^+ cosmid clone of Ecc71	J. W. Willis et al
pAKC240	Peh^+Pel^+ cosmid clone of Ecc71	J. W. Willis et al
pAKC245	Peh^+Pel^+ cosmid clone of Ecc71	J. W. Willis et al
pAKC247	Subclone of pAKC213 in pBR329	J. W. Willis et al
pAKC250	Peh^+Pel^+ cosmid clone of Ecc71	J. W. Willis et al

determined and compared with activity in a reaction to which no ions were added.

Temperature stability. Peh1 thermostability was ascertained by holding 0.5-ml samples of an enzyme preparation, diluted to 0.3 units per milliliter in 55 mM potassium phosphate buffer (pH 7.0), in a 1.5-ml polypropylene tube at given temperatures (0, 20, 30, 40, 50, 60, 70, 80, 90, or 100 C) for 10 min followed by a 30-min incubation on ice. Peh activity was determined and compared with activity of the 0 C sample.

pH optimum. Optimum pH was determined by assaying Peh activity of enzyme preparations using reaction mixtures of 0.5% PGA (w/v), 0.2 M NaCl, and 2 mM EDTA in a buffer solution [0.1 M sodium acetate; 0.1 M 2(*N*-morpholine) ethane sulfonic acid (MES); 0.1 M Trizma base; 0.1 M glycine] that had been adjusted to pH 3.9–10.0 in 0.5 unit increments using 10 N NaOH and glacial acetic acid.

Effects of Peh1 on potato tuber tissue. The ability of Peh1 to macerate potato tuber tissue was determined according to the method of Ishii (23) using 5 units of enzyme activity per milliliter of reaction mixture.

Damage to the potato tuber cellular membrane in a hypertonic solution was determined by measuring polyphenol oxidase (ECl.14.18.1) activity in the reaction supernatant. Polyphenol oxidase is compartmentalized in vesicles or plastids (26), and its presence in the supernatant after removing potato tuber cells and cell debris would result from membrane damage. Five milliliters of the maceration assay reaction mixture was withdrawn and centrifuged in graduated centrifuge tubes in a tabletop centrifuge for 10 min to pellet cells. The volume of macerated tissue was determined and the supernatant assayed for polyphenol oxidase activity as follows. An oxygen electrode was used to measure rates of decrease of dissolved oxygen in a stirred reaction mixture consisting of 2.2 ml 50 mM Tris (pH 7.5), 0.8 ml supernatant, and 0.2 ml 100 mM aqueous pyrocatechol. Polyphenol oxidase activity was compared with the activity present in a maceration reaction mixture lacking pectolytic enzyme, which had been blended in a Waring two-speed blender set on high for 15 sec and centrifuged to remove cell debris. The change in oxygen concentration over time caused by this control was considered as 100% release of polyphenol oxidase.

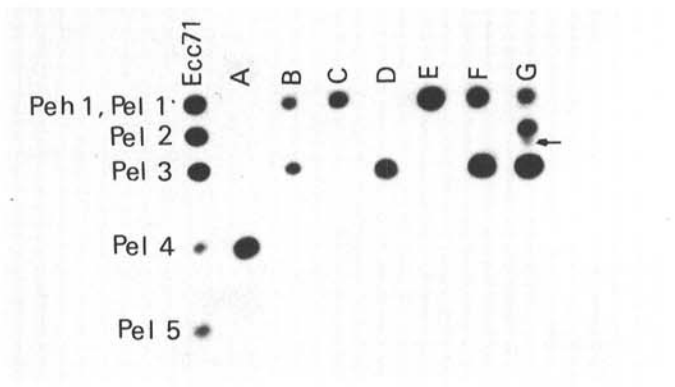


Fig. 1. Pectolytic enzyme IEF-AO (isoelectric focusing followed by activity gel overlays) profiles of Ecc71 and PYA$^+$ *Escherichia coli* clones. Ecc71 was grown in Luria broth plus 0.5% citrus pectin; *E. coli* clones were grown in Luria broth plus appropriate antibiotics. Ecc71 = Ecc71 periplasmic and extracellular fractions combined, A = HB101(pAKC211), B = HB101(pAKC212), C = HB101(pAKC213::Tn*5*-2), D = HB101 (pAKC213::Tn*5*-1), E = HB101 (pAKC227), F = HB101 (pAKC233), and G = HB101(pAKC240). Arrow indicates presence of an activity band in HB101(pAKC240) not found in Ecc71. This may represent a truncated version of one of the pectolytic enzymes.

Effect of *peh* mutation on virulence. Plant tissue was weighed and inoculated with Ecc71 and AC5213 by dipping toothpicks into a cell suspension (10^{10} cfu/ml) and puncturing the tissue to a depth of about 5 mm. Toothpicks dipped in sterile water were used as a control. Whole radish roots, carrot root pieces (about 5 cm long), celery petiole pieces (about 5 cm long), and potato tuber slices (about 2 cm thick) were inoculated at four points with each inoculum. Two trials of four replicates each were used. Inoculated plant parts and controls were then incubated in a moisture chamber at 28 C for 4 days. Visual observations were made daily. After 4 days, two replicates from each trial were rinsed to remove macerated tissue and weighed. Two replicates were also dried for 2 days at 80 C, and dry weights were taken. Percent loss in weight of each trial was determined and compared.

The same experiment was performed under anaerobic conditions using the BBL Gas Pak system (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD).

A separate experiment was performed using whole potato tubers. Small holes (about 1 cm deep) were made in whole tubers using the small end of a sterile Pasteur pipette and inoculated with 10 μl of Ecc71 or AC5213 (about 10^{10} cfu/ml) diluted 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} in 55 mM potassium phosphate buffer (pH 7.0). The wounds were sealed with petroleum jelly and the tubers incubated for 3 days at 28 C in a moisture chamber. The tubers were then sliced open and maceration visually rated.

RESULTS

Pectolytic enzyme profile of Ecc71. Enzyme preparations from culture filtrate and cells (periplasmic and cytoplasmic fluids) of Ecc71 grown under inducing and noninducing conditions were subjected to quantitative analysis and IEF-AO. With cultures grown in minimal salts plus 0.5% glycerol serving as the noninduced basal level, there was essentially no increase in Pel or Peh activity when Ecc71 was grown in LB. However, when grown in LB plus 0.5% CP, Pel activity increased eightfold and Peh activity increased 2.5-fold. When CP was added to minimal medium as the sole carbon source, there were 14-fold and threefold increases in Pel and Peh activities, respectively. Concomitant with the elevated enzyme activity, there was an increase in the quantity of enzyme exported; for example, Pel increased from 8 to 79% extracellular and Peh increased from 12 to 53% extracellular (Table 2).

Although the levels of pectolytic enzyme activity varied depending on the medium, the IEF-AO profiles were consistent with the typical Ecc71 pattern shown in Figure 1. Ecc71 produced five Pels (Pel1, Pel2, Pel3, Pel4, and Pel5) and one endo-Peh (Peh1) as determined using IEF and qualitative activity assays.

TABLE 2. Pectolytic enzyme activity and localization in *Erwinia carotovora* subsp. *carotovora* strain 71 grown under noninduced and induced conditions

Media	Pectate lyase		Polygalacturonase	
	Specific activity[a,b]	Percent extracellular	Specific activity[b,c]	Percent extracellular
Minimal medium (MM) plus 0.5% glycerol	0.5	8	1.1	12
MM plus 0.5% citrus pectin	7.2	79	3.1	53
Luria broth (LB)	0.6	10	1.4	13
LB plus 0.5% citrus pectin	3.9	75	2.8	48

[a] Specific activity expressed as micromoles of unsaturated digalacturonic acid equivalents produced per minute per milligram of protein. Protein concentration was determined by the method of Lowry et al (29).
[b] Enzyme activities given are total (intracellular plus extracellular) activities expressed in relationship to total protein.
[c] Specific activity expressed as micromoles of galacturonic acid equivalents produced per minute per milligram of protein.

Under the previously described culture conditions, Pel4 and Pel5 were primarily periplasmic, whereas Pel1, Pel2, Pel3, and Peh1 were primarily extracellular (data not shown). The pIs of Pel2, Pel3, Pel4, and Pel5 were estimated to be 9.7, 9.2, 8.0, and 6.6, respectively. Pel1 and Peh1 migrated to identical positions on a pH 3–10 IEF gel (pI $\geq$ 10). Ecc71 also produces at least one exo-Peh (Peh2), which was not detected with IEF-AO but which was resolved by constructing a Peh1-deficient strain (see below).

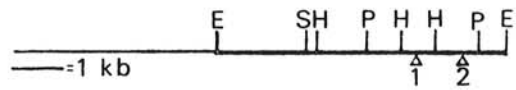
Cloning of pectolytic enzyme genes. Of 1,500 Ap^r transductants from the Ecc71 genomic library screened on PYA plates, eight PYA-positive clones were recovered. Four clones produced both Pel and Peh activity, and the remaining four only Pel; none were found to produce Peh alone (Table 3). Most enzymatic activity ($>$ 80%) was localized in the periplasmic space of the *E. coli* clones. Periplasmic fluids of the *E. coli* HB101 cosmid-carrying clones were subjected to IEF-AO. As shown in Figure 1, HB101(pAKC227) (lane E) and HB101(pAKC231) (not shown) produced only Pel1; HB101(pAKC211), only Pel4 (lane A); HB101(pAKC233), both Pel1 and Pel3 (lane F); HB101(pAKC212), both Pel3 and Peh1 (lane B); and HB101(pAKC240), Pel2, Pel3, and Peh1 and/or Pel1 (lane G). HB101 (pAKC245) and HB101 (pAKC250) also produced both Pel3 and Peh1 (IEF-AO profiles not shown). An additional band, indicated by arrow, with no corresponding band in the Ecc71 profile was also detected in HB101(pAKC240) (lane G).

Because plasmids from the three clones with Peh1 activity contained overlapping restriction fragments and had the same IEF-AO profiles, only one, HB101(pAKC212), was chosen for further characterization of Peh1 and the gene encoding it. Subcloning from pAKC212 by *Eco*RI digestion and ligation with *Eco*RI-digested pBR329 yielded pAKC213 (Fig. 2), a plasmid containing a 5.9-kb insert that encoded both Peh1 and Pel3. Further attempts at separation of activities by subcloning with other restriction enzymes (*Hin*dIII and *Pvu*I) were unsuccessful.

Transposon mutagenesis. To genetically separate *peh1* and Pel3, pAKC213 was mutagenized with Tn*5*. PYA-positive (those giving clear zones on PYA) and PYA-negative transformants were recovered. The PYA-negative mutants were *pel3*$^+$, *peh1*::Tn*5* when assayed quantitatively, indicating that Pel3 activity was too low to detect by this plate method. The PYA-positive transformants were of two types when assayed quantitatively: 1) *peh1*$^+$, *pel3*$^+$ and 2) *peh1*$^+$, *pel3*::Tn*5*. Two mutant plasmids (pAKC213::Tn*5*-1 and pAKC213::Tn*5*-2) were analyzed for site of Tn*5* insertion and enzymatic activity (Fig. 1, lanes C and D; Fig. 2). In pAKC213::Tn*5*-1, Tn*5* inserted in the small internal *Hin*dIII fragment, there was no detectable Peh activity, and the band of activity corresponding to Peh1 on IEF-AO was no longer present. In pAKC213::Tn*5*-2, Tn*5* inserted in the 0.9-kb *Hin*dIII *Pvu*I fragment. This insertion was less than 1 kb from the insertion in Tn*5*-1, yet Tn*5*-2 had normal Peh activity but no Pel activity, and the IEF-AO activity band corresponding to Pe13 was no longer present.

Characterization of Ecc71 Peh1. Enzyme preparations from the periplasmic space of HB101(pAKC213::Tn*5*-2) were used to biochemically characterize Peh1. Peh1 caused a rapid loss of viscosity of a PGA solution while maintaining a sustained increase in reducing groups over time (Fig. 3). Peh1 activity products were saturated dimer to multimer as determined by paper chromatography (data not shown). Therefore, Peh1 was classified as an endo-polygalacturonase. Peh1 was found to have an inactivation temperature of 60 C and a K_m of 0.982 mg/ml using PGA as a substrate. Peh1 was active in a pH range of 4.0–6.5 with an optimum pH of 5.5. Enhancement of enzymatic activity with K^+, Li^+, and Na^+ was observed at 50, 100, 150, and 200 mM concentrations; maximal enhancement occurred at 150 mM (Table 4). NH_4^+ was inhibitory at 1, 150, and 200 mM but stimulated activity at 50 and 100 mM. EDTA (1 and 2 mM) also increased activity (Table 4). Divalent cations (Ca^{2+}, Ba^{2+}, Mg^{2+}, and Mn^{2+}) used at 1 mM did not stimulate activity, and higher concentrations were inhibitory (data not shown).

The effect of Peh1 on potato tuber tissue was also analyzed (Fig. 4). Pel1 was used as a pectate lyase control for this assay. Although both Peh1 and Pel1 caused tissue maceration and leakage of polyphenol oxidase, Peh1 showed a lag period between onset of tissue maceration and release of polyphenol oxidase.



pAKC213::Tn5

Strain	PEH Sp. Act.[b]	PEL Sp. Act.[a]
HB101(pAKC213)	1.45	0.30
HB101(pAKC213::Tn5-1)	0	0.32
HB101(pAKC213::Tn5-2)	1.37	0

Fig. 2. Restriction map of pAKC213, Tn*5* insertion sites, and enzyme levels in *Escherichia coli* clones carrying pectate lyase and/or polygalacturonase-encoding plasmids. Bold line represents insert DNA. E = *Eco*RI, S = *Sal*I, H = *Hin*dIII, P = *Pvu*I, Δ = position of Tn*5* insertion, and a and b = specific activity expressed as micromoles unsaturated digalacturonic acid and galacturonic equivalents, respectively, produced per minute per milligram of protein.

TABLE 3. Characterization of pectolytic *Escherichia coli* cosmid clones of *Erwinia carotovora* subsp. *carotovora* strain 71 (qualitative and quantitative pectolytic enzyme analysis)

Clone	Specific activity[a]		Enzyme species[b]
	Pectate lyase	Polygalacturonase	
HB101(pAKC211)	1.16	ND[c]	Pel4
HB101(pAKC212)	0.05	1.11	Peh1 Pel3
HB101(pAKC227)	3.14	ND	Pel1
HB101(pAKC231)	4.77	ND	Pel1
HB101(pAKC233)	2.09	ND	Pel1 Pel3
HB101(pAKC240)	2.52	0.29	Pel2 Pel3 Pel?[d] Peh?[d]
HB101(pAKC245)	0.11	1.56	Peh1 Pel3
HB101(pAKC250)	0.12	1.10	Peh1 Pel3

[a] Specific activity expressed as micromoles of unsaturated digalacturonic acid equivalents produced per minute per milligram of protein for pectate lyase and as micromoles of galacturonic acid equivalents produced per minute per milligram of protein for polygalacturonase. Protein concentration was determined by the method of Lowry et al (29).

[b] Identity of enzyme species is based on isoelectric focusing and activity overlays (Fig. 1).

[c] ND = none detected.

[d] Identities of these two pectolytic enzymes cannot be determined from present data.

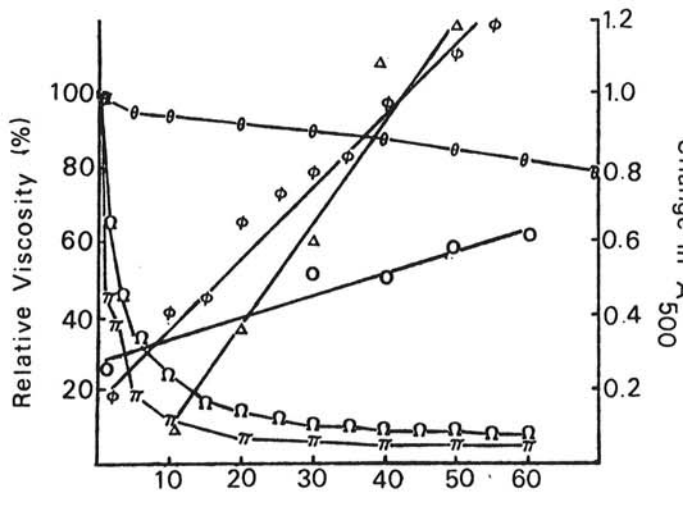


Fig. 3 Kinetics of viscosity reduction of a polygalacturonate solution and increase in reducing groups (A_{500}). Change in viscosity with enzyme preparations from: π = HB101(pAKC213::Tn*5*-2), Ω = Ecc71, and Θ = AC5213. Increase in reducing groups using enzyme preparations from: Δ = HB101(pAKC213::Tn*5*-2), ϕ = Ecc71, and o = AC5213.

Construction of an Ecc71 *pehI* mutant. After marker exchange mutagenesis of Ecc71 with pAKC213::Tn*5*-2, a Km^r Tc^s isolate (AC5213) was recovered that showed normal activity on PYA but no activity on PYA-EDTA, indicating loss of Peh1 activity. The fidelity of mutagenesis was substantiated by Southern blot analysis using nick-translated λ::Tn*5* and pAKC213 DNA to probe Ecc71 and AC5213 DNA digested with either *Eco*RI or *Sal*I (Fig. 5). The Tn*5* insertion occurred in a chromosomal *Eco*RI fragment that was originally about 5 kb (Fig. 5A, lane 1) and present in pAKC213, thereby increasing the size of the chromosomal fragment to about 11 kb (Fig. 5A, lane 3). *Sal*I-digested wild-type DNA showed two bands homologous to pAKC213, and AC5213 had three (Fig. 5A, lanes 2 and 4). This result confirmed the predicted pattern because *Sal*I cleaves Tn*5* and pAKC213 only once. As shown in Figure 5B, wild-type Ecc71 DNA (lanes 1 and 2) has no bands homologous to Tn*5*. However, AC5213 digested with *Eco*RI (lane 3) has a band carrying Tn*5* corresponding to the band hybridizing with pAKC213 (Fig. 5A, lane 3). Likewise, in AC5213 digested with *Sal*I, two of the three bands hybridizing with pAKC213 (Fig. 5A, lane 4) also hybridized with Tn*5* (Fig. 5B, lane 4). This pattern corresponds to that predicted from analysis of Figure 2 and indicates that AC5213 resulted from recombinational integration of the DNA segment in pAKC213 carrying Tn*5* and not from a transpositional event.

Quantitative analysis of enzyme preparations from AC5213 revealed normal levels of Pel activity while Peh activity was equal to about 8% of wild type. In the presence of 1 mM EDTA, which eliminated Pel activity, enzyme preparations of AC5213 reduced viscosity of a PGA solution at a rate much slower than similar preparations from Ecc71 or HB101(pAKC213::Tn*5*-2) (Fig. 3).

TABLE 4. Effects of ions on endopolygalacturonase (Peh1) activity

Ion	Percentage of activity[a] with ion concentrations (mM) of				
	1	50	100	150	200
K^+	100	140	196	196	180
Li^+	100	132	168	196	192
Na^+	100	128	180	200	188
NH_4^+	20	136	184	54	60
EDTA	122[b]	...	...	...	...

[a] Activity of Peh1 in buffer to which no ions had been previously added was used as the comparison standard.
[b] EDTA at 2 mM had 151% activity.

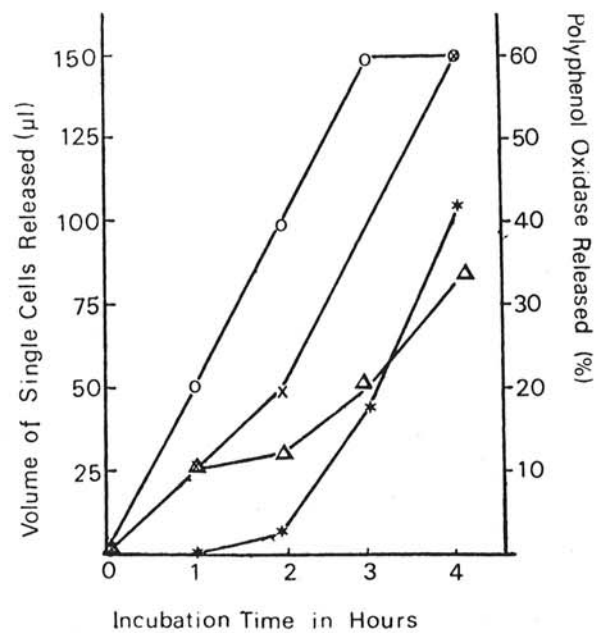


Fig. 4. Maceration of potato tuber tissue and release of polyphenol oxidase. Volume of cells released by: o = Peh1 and X = Pel1. Percent release of polyphenol oxidase by: Δ = Peh1 and * = Pel1.

The reaction products of enzymatic activity from AC5213 were determined to be dimer and trimer by paper chromatography (data not shown). Therefore, the 8% residual Peh activity in AC5213 was due to the presence of an exo-polygalacturonase (Peh2).

Effect of *pehI* mutation on pathogen virulence. Both Ecc71 and AC5213 caused extensive maceration of radish, celery, and carrot tissue under both aerobic and anaerobic environments. Potato tissue was only moderately macerated. No significant difference in weight loss (dry or wet weight) between samples infected with Ecc71 or AC5213 was detected in relation to weight loss of water-inoculated controls (data not shown). Similarly, there was no distinguishable difference in maceration of whole potato tubers. Additionally, Ecc71 and AC5213 caused identical symptoms at all inoculum levels, indicating that loss of Peh1 activity had no effect on infectivity. Thus, although Peh1 may contribute to disease symptoms, loss of Peh1 activity did not result in a detectable change in virulence on any of the hosts employed.

DISCUSSION

E. c. subsp. *carotovora* strain 71 produces at least five major Pels (Pel1, Pel2, Pel3, Pel4, and Pel5), one endo-Peh (Peh1) and at least one exo-Peh (Peh2). The number and identity of these enzymes is based on IEF-AO and on examination of an Ecc71 *pehI*::Tn*5* mutant. Our inability to detect Peh2 on IEF-AO may have been due either to the low level of activity or the exo-mode of action. We were also unable to separate Pel1 and Peh1 on IEF gels. Although their pIs may differ, there is no current method for separating proteins with pIs > 10.0 because this is the upper limit of available ampholytes. Comigration of these two enzymes could account for an earlier report of one enzyme with both Pel and Peh activities (39).

Although the enzyme levels, based on activity assays, increase when Ecc71 is grown in the presence of citrus pectin, the concomitant enzyme IEF-AO profile remains constant. Production of several pectolytic enzymes by Ecc71 follows the trend observed with other *E. c.* subsp. *carotovora* strains (27,31,37,39,41). Previous work reporting production of two Pels and one Peh by Ecc71 (46), based on nonequilibrium IEF fractionation of the enzymes, has now been extended in part because of the use of ultrathin-layer IEF and direct enzyme assays, which have higher resolution and sensitivity. The possibility still exists that some pectolytic enzymes may be produced exclusively in planta or in response to undescribed plant or environmental stimuli. Construction of Pel- and Peh-negative mutants will facilitate determination of the existence of such plant-specific responses.

Although the number and identity of pectolytic enzymes produced by Ecc71 was not affected by citrus pectin induction, the localization of activity was dramatically different. The increase in extracellular pectolytic activity may indicate that the transport

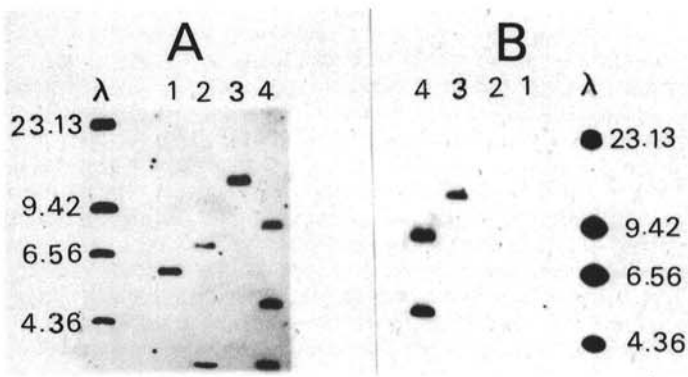


Fig. 5. Southern blot analysis of AC5213. **A,** Blot was probed with ^{32}P-labeled pAKC213 and *Hin*d III digested λDNA (BRL, Gaithersburg, MD). **B,** Blot was probed with ^{32}P-labeled λ::Tn*5* and *Hin*d III digested λ DNA. 1 = Ecc71 chromosomal DNA cut with *Eco*RI, 2 = Ecc71 chromosomal DNA cut with *Sal*I, 3 = AC5213 chromosomal cut with *Eco*RI, 4 = AC5213 chromosomal cut with *Sal*I, and λ = λDNA cut with *Hin*dIII.

system involved in export of pectolytic enzymes is also a regulated system that is coupled with enzyme production.

In Ecc71, Pel4 and Pel5 are primarily periplasmic, whereas Pel1, Pel2, and Pel3 are primarily extracellular. These results are consistent with those previously found with *E. c.* subsp. *carotovora* strain 14 (39). One interpretation of these results would be that the *E. c.* subsp. *carotovora* export system can differentiate between pectolytic enzymes. In contrast to *E. c.* subsp. *carotovora*, there is evidence that more than 90% of *Erwinia chrysanthemi* pectolytic activity is extracellular and all enzyme species have been identified in the supernatant (14,15,37).

From an Ecc71 cosmid gene bank, clones were recovered carrying pectolytic enzyme genes that were expressed in *E. coli* HB101. Upon IEF-AO analysis, one clone [HB101(pAKC240) (Fig. 1, lane G)] displayed a band of activity not found in Ecc71. This clone demonstrated Pel2, Pel3, and Peh activity and had an activity band (indicated by the arrow), which may be a truncated form of one of the pectolytic enzymes. Also, we have not ruled out the possibility that this additional enzyme is produced from a tightly regulated pectolytic enzyme gene that is not expressed in Ecc71 under the culture conditions we have employed but is constitutively expressed in the *E. coli* genomic background. Regardless of profile, the enzymes were localized in the periplasmic space. This observation is consistent with all reports of cloned pectolytic enzyme genes, whether from *E. c.* subsp. *carotovora* (27,46) or *E. chrysanthemi* (16,24,35,42). The pattern of gene recovery from our cosmid library indicates a clustering of some of the genes encoding pectolytic enzymes, as has been noted in several strains of *E. chrysanthemi* (14,15,25,42) and *E. c.* subsp. *carotovora* strains EC (27) and EC14 (38). These reports, however, have indicated linkage of Pels only. Our results demonstrated the close linkage between a *peh* and *pel* gene as well. Separation of these activities by Tn*5* mutagenesis establishes the existence of two independent transcriptional units.

To determine the importance of Peh1 to pathogenicity and disease development, the properties of the enzyme and the *peh1* mutants were studied. Biochemical characterization of the Ecc71 endo-Peh indicates it is similar to an endo-Peh from *E. c.* subsp. *carotovora* strain EC153 (33) because both have a pH optimum of about 5.5 and their activities are stimulated by addition of Na^+, K^+, and NH_4^+ ions. Because the endo-Peh of *E. c.* subsp. *carotovora* strain EC has not yet been characterized biochemically, a comparison of its properties with those of the endo-Pehs of Ecc71 and EC153 is not possible.

Lei et al (28) concluded that tissue maceration by endo-Peh of *E. c.* subsp. *carotovora* strain EC indicated its function as a virulence determinant in *E. c.* subsp. *carotovora*. In addition to tissue maceration, Ecc71 endo-Peh also caused the loss of cellular membrane integrity, leading to cytoplasmic leakage. This observation supports the hypothesis (2,40) that cell death and electrolyte loss resulting from pectolytic activity are due to loss of cell wall rigidity, which allows the cell membrane to be damaged in a hypertonic solution. The apparent lag period between onset of tissue maceration by Peh1 and release of host cytoplasmic enzymes (no lag was noted with Pel1) could be attributed to insufficient early cell wall damage by Peh1, although digestion of the middle lamella did result in cell separation. Subsequent digestion of cell wall components of the separated cells could then further weaken the wall and result in membrane rupture. It is also possible that the rate at which each enzyme releases toxic digestion products that cause membrane damage varies considerably. Though an earlier report (1) indicated that Pel digestion products were not toxic to plant cells, this study dealt with mixed products. Work by Davis et al (18) has indicated that oligosaccharides must be highly purified to function as elicitors of phytoalexins when applied to plants.

While in agreement with the basic assumption that endo-Peh is involved in disease symptoms, we also wished to determine whether endo-Peh was required for pathogenicity. Construction of an Ecc71 *peh1*::Tn*5* mutant by marker exchange mutagenesis allowed us to determine that loss of Ecc71 Peh1 activity does not result in a detectable change in virulence on carrot, radish, celery, or potato tissue under either an aerobic or an anaerobic environment. Whether Peh1 itself can function as a primary virulence determinant leading to tissue breakdown awaits construction of mutants lacking all Pel activity while maintaining a functional *peh1* gene.

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