Construction and Characterization of an *Erwinia chrysanthemi* Mutant with Directed Deletions in All of the Pectate Lyase Structural Genes

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Erwinia chrysanthemi EC16 produces four isozymes of pectate lyase (PL), an extracellular enzyme that macerates parenchymatous plant tissues. The *pelB* and *pelC* genes, which encode isozymes PLb and PLc, were deleted from the chromosome by marker exchange-eviction mutagenesis: an *nptI-sacB-sacR* cartridge, encoding kanamycin resistance and sucrose sensitivity, was inserted in a cloned *E. chrysanthemi* DNA fragment in place of the *pelB* and *pelC* genes; the marked deletion was then introduced into the chromosome by exchange recombination (selecting for kanamycin resistance) and subsequently evicted by a second recombinational exchange with an unmarked deletion derivative (selecting for sucrose tolerance). The resulting mutant, UM1003, was deficient in PLb and PLc and kanamycin-sensitive. The *pelA* and *pelE* genes, carried on adjacent *Eco*RI fragments in another *E. chrysanthemi* DNA clone, were replaced with an *nptI* cartridge and then exchanged into the UM1003 chromosome by selecting for kanamycin resistance. Mutant UM1005 was deficient in PLa, PLb, PLc, and PLe and produced less than 0.1% of the extracellular PL activity of the wild type. However, the specific growth rate of UM1005 was unchanged relative to the wild type in a minimal medium with polygalacturonic acid as the sole carbon source. Furthermore, although virulence was reduced 79–98% (depending on the assay) in potato tuber maceration tests, mutant UM1005 was still able to cause significant maceration in potato, carrot, and pepper tissues. These observations indicate that PL is not necessary for the utilization of pectate or the maceration of plant tissues by *E. chrysanthemi*.

Additional key words: maceration, marker exchange-eviction mutagenesis, *pel* genes

The enterobacterium *Erwinia chrysanthemi* causes diseases involving maceration of parenchymatous tissues in a wide variety of plants and excretes multiple isozymes of pectate lyase (PL; EC 4.2.2.2). PL is thought to play a major role in the soft-rot diseases caused by *E. chrysanthemi* and the related pathogen, *E. carotovora*, for several reasons: 1) pectic polymers (chains of 1,4-linked α-D-galacturonic acid and methoxylated derivatives), the substrate of PL, are structural constituents of the middle lamellae and primary cell walls of higher plants (McNeil *et al.* 1984); 2) purified PL can macerate parenchymatous plant tissues (Basham and Bateman 1975; Mount *et al.* 1970; Stephens and Wood 1975); and 3) *Escherichia coli* transformants containing highly expressed, cloned *E. chrysanthemi pel* (pectate lyase [PL]-encoding) genes are able to cause extensive maceration in potato tubers (Keen and Tamaki 1986; Payne *et al.* 1987). Although these observations indicate that PL is sufficient to enable an enterobacterium to cause soft-rot symptoms, they do not prove that PL production is actually necessary for maceration.

The genetic manipulations required to rigorously answer this question have been thwarted until now by the complexity of the *E. chrysanthemi* pectic enzyme system. Most strains of *E. chrysanthemi* produce five PL isozymes, one acidic (PLa), two neutral (PLb and PLc), and two alkaline (PLd and PLe), as distinguished by their isoelectric points (Bertheau *et al.* 1984; Ried and Collmer 1986; Van Gijsegem 1986). The *pelB* and *pelC* genes are clustered at

one chromosomal locus; the *pelA*, *pelD*, and *pelE* genes are clustered at another (Collmer *et al.* 1985; Keen *et al.* 1984; Kotoujansky *et al.* 1985; Reverchon *et al.* 1985; Van Gijsegem *et al.* 1985). Strain EC16 is unusual in that it produces only four isozymes of PL. These have been cloned, and sequences for two of the isozymes have been determined (Keen *et al.* 1984; Keen and Tamaki 1986). *E. chrysanthemi* also produces two other extracellular pectic enzymes: exo-poly-α-D-galacturonosidase (exoPG) and pectin lyase (PNL) (Collmer *et al.* 1982; Ried and Collmer 1986; Tsuyumu and Chatterjee 1984). ExoPG appears to be coordinately regulated with PL, whereas in many strains, PNL synthesis is induced by DNA-damaging agents that trigger the SOS response (in EC16, however, PNL is uninducible) (Tsuyumu and Chatterjee 1984).

Nonpathogenic mutants deficient in PL export (Out⁻) have been obtained by random mutagenesis but are unable to export other cell wall-degrading enzymes that could have a role in pathogenesis (Andro *et al.* 1984; Chatterjee and Starr 1977, 1978; Thurn and Chatterjee 1985). Strains with directed mutations in individual *pel* genes (*pelB*, *pelC*, or *pelE*) retain substantial virulence (Roeder and Collmer 1985, 1987; Payne *et al.* 1987). To more rigorously test the role of PL in soft-rot pathogenesis, we used marker exchange-eviction mutagenesis (Ried and Collmer 1987) to construct an EC16 mutant deficient in all four PL isozymes and evaluated the ability of the mutant to utilize pectate in culture and to cause symptoms in susceptible plant tissues. To our knowledge, this is the first report of an *E. chrysanthemi* mutant with directed deletions in all of the PL structural genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids are listed in Table 1. Except where noted, all bacteria were grown in Luria-Bertani (LB) medium (Maniatis et al. 1982) with appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml (50 μg/ml for broth); chloramphenicol, 10 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 50 μg/ml (20 μg/ml for broth); tetracycline, 20 μg/ml. LB-5% sucrose plates were made by adding a 50% solution (w/v) of filter-sterilized sucrose to autoclaved LB-agar medium before pouring. Pectate semisolid agar (Starr et al. 1977) was used to determine the pectolytic ability of E. coli containing various recombinant plasmids. Enzyme samples for isoelectric focusing analysis and inoculum for virulence assays were obtained from bacteria grown on King's Medium B (KB) (King et al. 1954). Growth rate with polygalacturonic acid as the sole carbon source was tested with the minimal medium of Zucker and Hankin (1970) supplemented with 0.5% (w/v) polygalacturonic acid (Pfaltz and Bauer, Inc.).

General DNA manipulations and bacterial transformations. Plasmid DNA was isolated and manipulated using standard techniques (Maniatis et al. 1982), except where noted, with restriction enzymes and related reagents from Bethesda Research Laboratories. E. coli was transformed by the calcium chloride procedure of Mandel and Higa (1970). A modification of the technique of Reverchon and Robert-Baudouy (1985) was used to transform E. chrysanthemi. Bacteria were grown to an OD$_{600}$ of approximately 1.0 in LB broth containing 10 mM MgCl$_2$, centrifuged, and resuspended in 0.5 volume of ice-cold 0.1 M MgCl$_2$, centrifuged again, and resuspended in 0.5 volume of ice-cold 0.1 M CaCl$_2$. After incubation on ice for 20 min, the cells were centrifuged and resuspended in 0.25 volume of ice-cold 0.1 M CaCl$_2$. Plasmid DNA (0.5–1.5 μg) in 0.1 ml of 15 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10 mM NaCl was added to 0.2 ml of these cells.

Bacterial matings. Transformants of HB101 (R64drd11, pLCV9) containing either pJR475 or pJR485 were grown in LB broth containing chloramphenicol, tetracycline, and either kanamycin or ampicillin, depending on the plasmid, to an OD$_{600}$ greater than 1.0. One-hundred microliters of these donor cells were spotted onto LB plates without antibiotics followed by 100 μl of the E. chrysanthemi recipient that had been grown in LB-nalidixic acid to an OD$_{600}$ greater than 1.0 before being centrifuged and resuspended in fresh LB without nalidixic acid. The mating mixture was incubated at 30° C for 8–16 hr, then the lawn of cells was resuspended in 10 mM MgSO$_4$ and spread on LB agar containing nalidixic acid plus either kanamycin or ampicillin to select for plasmid mobilization into E. chrysanthemi.

Marker exchange-eviction mutagenesis of pelB and pelC in E. chrysanthemi AC4150. Plasmid pCS3 was constructed by subcloning into pBR322 a 13-kb EcoRI fragment containing the E. chrysanthemi EC16 pelB and pelC genes that are carried on pPEL3 (Keen et al. 1984). pCS3 was partially digested with Sau3A, treated with calf intestinal alkaline phosphatase and subjected to electrophoresis in a low melting temperature agarose gel (FMC Corp.). Plasmid fragments approaching full length were excised and ligated by the method of Struhl (1985) with an nptI-sacB-sacR cartridge that had been similarly excised from a low melting temperature agarose gel following electrophoresis of BamHI-digested pUM24 (Ried and Collmer 1987). The

sacB gene in the cartridge is derived from Bacillus subtilis and encodes levansurase, an enzyme that accumulates in the periplasm of Gram-negative bacteria, resulting in lethal synthesis of levan in the presence of 5% sucrose (Gay et al. 1985). Kanamycin-resistant E. coli HB101 transformants were screened for loss of the ability to pit pectate semisolid agar. One plasmid, pJR475 was chosen for use in marker-exchange mutagenesis of pelB and pelC in E. chrysanthemi AC4150 after restriction mapping of the plasmids in several nonpectolytic transformants. The plasmid was mobilized into strain AC4150 with the two helper plasmids R64drd11 and pLCV9. The deletion mutation was exchanged into the E. chrysanthemi chromosome, to produce UM1002, by selecting for kanamycin-resistant recombinants following incubation of the culture in a phosphate-deficient medium that renders pBR322 derivatives unstable (Roeder and Collmer 1985).

The nptI-sacB-sacR cartridge was then removed from pJR475 by partial digestion with PstI, religation, and subsequent selection for ampicillin-resistant E. coli HB101 transformants. The resulting deletion derivative, pJR485, was mobilized into UM1002. Transconjugants were grown for 18 hr in LB broth shake cultures containing kanamycin and ampicillin, then 200 μl of the culture was diluted into 5 ml of LB broth lacking antibiotics. After 9 hr, portions of the culture were spread on LB-sucrose plates. A lawn of bacteria developed 15 hr later, and after an additional 17 hr, cream-colored colonies grew out of the thin, gray lawn. Mutant UM1003 was isolated from one these colonies to be used as a target for further mutagenesis of the pel genes.

Marker-exchange mutagenesis of pelA and pelE in E. chrysanthemi strains AC4150 and UM1003. All of the pelE gene and most of the pelA gene are contained on two adjacent, 1.75-kb EcoRI fragments in pPEL74 (N. T. Keen, personal communication). Deletion derivatives lacking these fragments were electrophoretically resolved from partial EcoRI digests of pPEL74 and then ligated with the 1.2-kb EcoRI fragment containing the nptI gene from pUC4K to produce plasmid pJR44K. The PstI fragment containing the cloned region with the nptI insert was transferred (following partial PstI digestion) to the PstI site of pBR322 to produce pJR51. Although pJR51 can be conjugated into E. chrysanthemi, transformation was used to introduce the plasmid into AC4150 and UM1003. The deletion mutation was then marker-exchanged into the E. chrysanthemi chromosome as described above.

Pectic enzyme assays. PL activity was determined by measuring the change in A$_{230}$ of a reaction mixture containing 0.07% (w/v) polygalacturonic acid, 30 mM Tris hydrochloride (pH 8.5), 0.1 mM CaCl$_2$, and 6.7% (v/v) enzyme sample. One unit of PL activity is defined as that amount of activity necessary to produce 1 μmol of product per minute at 25°. PNL was similarly assayed except that partially methylesterified citrus pectin (Sigma Chemical Co.) was used as a substrate and the reaction mixture in some assays was supplemented with 1.0 mM EDTA to inhibit PL activity.

Bacterial pectic enzyme production was also analyzed with activity-stained ultrathin-layer polyacrylamide isoelectric focusing gels. The 15 μl-samples applied to isoelectric focusing gels were obtained in three ways: 1) Bacteria in a 100-mm^2 area of cells grown 16 hr at 30° on KB agar were suspended in 40 μl of water and centrifuged for 2 min in an Eppendorf microfuge to yield a supernatant sample. 2) Bacterial cultures grown to stationary phase in KB broth were clarified by centrifugation and concentrated

with an immersible CX-10 membrane (Millipore Corp.). 3) Macerated potato tuber tissue was suspended in water (1.0 g in 1.0 ml) and clarified by centrifugation. Ultrathin-layer isoelectric focusing was performed as previously described (Ried and Collmer 1985) except that the electrofocusing was terminated as soon as the cytochrome C in the pI markers (FMC Corp.) was observed to band tightly. Also, to enhance visualization of PLa, the gel was incubated in 20 mM Tris hydrochloride (pH 8.5) for 2 min before application of the overlays used in the activity-staining procedure. The composition and preparation of the overlays was as described previously (Ried and Collmer 1985). Briefly, overlays for detecting PL were buffered at pH 8.5 and contained $CaCl_2$; those for detecting exoPG were buffered at pH 6.5 and contained EDTA.

Growth of *E. chrysanthemi* AC4150 and UM1005 in minimal medium containing pectate. The specific growth rate was determined from cultures exhibiting exponential growth in the minimal salts medium of Zucker and Hankin (1970) containing 0.5% polygalacturonic acid (pH 7.0) as the sole carbon source. Cultures were adjusted to an OD_{600} of 0.025 and incubated at 28° with shaking. Bacterial growth was monitored by OD_{600} determinations at 20-min intervals.

Potato tuber tissue maceration assays. Russet potato tubers obtained from R. W. Goth (USDA, Beltsville, MD) and from local retail market were prepared for inoculation by washing in running water, soaking for 20 min in 20% ethanol, soaking for 20 min in 0.5% NaOCl, rinsing in tap water, and air drying. Potatoes were then either cut into 10-mm slices and placed on water-saturated Whatman No. 1 filter paper in Petri dishes or left intact for injection inoculation. Inoculum was obtained from heavily streaked 16-hr KB plates. Tuber slices were inoculated by stabbing bacteria-laden toothpicks 4 mm into each slice. The slices were then incubated at 28° in a humid atmosphere. Inoculum for injection into whole tubers was obtained by washing the bacteria off of KB plates with 3 ml of sterile dilution buffer (15 mM potassium phosphate [pH 7.0], 17 mM NaCl, 0.1 mM $CaCO_3$), pelleting the bacteria in a clinical centrifuge, and then resuspending them in 3 ml of sterile dilution buffer. The bacterial suspension was then injected into tubers with disposable pipette tips by a modification of the procedure of Maher and Kelman (1983; Roeder and Collmer 1985). The concentration of the initial inoculum was determined by dilution plating. Inoculated tubers were maintained in a nitrogen atmosphere at 28°.
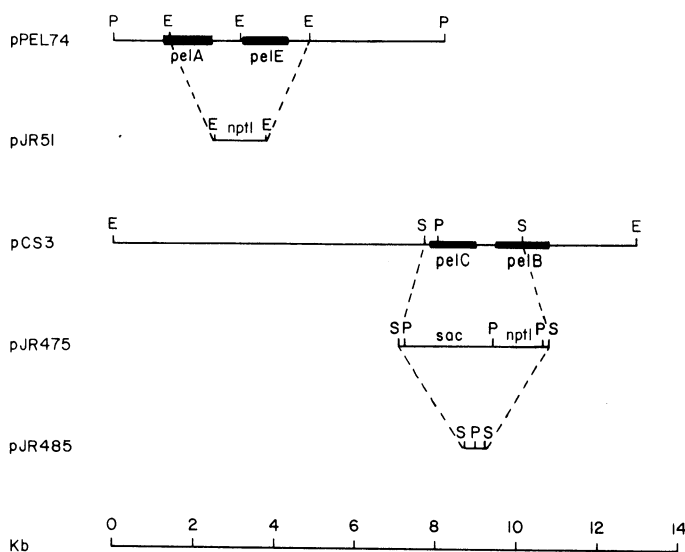
RESULTS

Construction of the Pel⁻ *E. chrysanthemi* mutant UM1005. Mutant UM1005 was constructed in three exchange recombination steps involving cloned sequences modified *in vitro* (see Fig. 1 and Table 1). The EC16 *pel* genes and flanking sequences had previously been cloned by Keen *et al.* (1984). The *pelB* and *pelC* genes were subcloned from pPEL3 into pBR322 to permit the conjugational transfer of deletion derivatives from *E. coli* to *E. chrysanthemi*.

In the first two mutant construction steps, the *pelB* and *pelC* genes were deleted from the *E. chrysanthemi* chromosome by a marker exchange-eviction mutagenesis

Fig. 1. Restriction map of the deletions in cloned *pel* genes used to construct *Erwinia chrysanthemi* Pel⁻ mutants. The cloned region of the *pel*-containing plasmids, including the location of specific *pel* genes and the relevant restriction sites, are shown for pPEL74 and pCS3. Dashed lines denote the replacement of sequences. These deletion derivatives were used to mutate AC4150 to UM1005 via a series of gene replacements: pJR475 (AC4150 to UM1002), pJR485 (UM1002 to UM1003), pJR51 (UM1003 to UM1005). Abbreviations: sac, *sacBsacR*; P, *Pst*I; E, *Eco*RI; S, *Sau*3A. The 28-bp fragment located in the place of the deleted *pelB* and *pelC* sequences in pJR485 is not drawn to scale.

Table 1. Bacterial strains and plasmids

Designation	Relevant characteristics	Origin
Escherichia coli		
HB101	*leu proA2 thi recA13 hsdS20*	Boyer and Roulland-Doussoix, 1969
Erwinia chrysanthemi		
AC4150	Nal^r derivative of EC16	Chatterjee *et al.*, 1983
UM1002	Δ(*pelB pelC*)::*nptI-sacB-sacR* derivative of AC4150	this work
UM1003	Δ(*pelB pelC*)::28-bp derivate of UM1002	this work
UM1004	Δ(*pelA pelE*)::*nptI* derivative of AC4150	this work
UM1005	Δ(*pelB pelC*)::28-bp, Δ(*pelA pelE*)::*nptI* derivative of UM1003	this work
Plasmids		
pBR322	Amp^r Tet^r	Bolivar *et al.*, 1977
pPEL3^a	Cosmid containing *pelB* and *pelC*	Keen *et al.*, 1984
pCS3	pBR322 derivative of pPEL3 containing *pelB pelC* at *Eco*RI site, Amp^r Tet^r	this work
pUM24	pUC4K derivative containing *nptI-sacB-sacR* cartridge, Kan^r Amp^r	Ried and Collmer, 1987
pJR475	pCS3 derivative containing Δ(*pelB pelC*)::*nptI-sacB-sacR*, Kan^r Amp^r Tet^r	this work
pJR485	pJR475 derivative containing Δ(*pelB pelC*)::28bp, Amp^r Tet^r	this work
pPEL74	pBR329 derivative of pPEL7 containing *pelA pelE*, Tet^r Cm^r	Keen *et al.*, 1985
pUC4K	pUC4 containing *nptI* cartridge	Vieira and Messing, 1982
pJR44K	pPEL74 derivative containing Δ(*pelA pelE*)::*nptI*	this work
pJR51	pBR322 derivative containing Δ(*pelA pelE*)::*nptI* fragment from pJR44K	this work
R64*drd*11	Transfer-derepressed derivative of R64, Tet^r	Van Haute *et al.*, 1983
pLVC9	pGJ28 derivative carrying ColE1 *mob*, Cm^r	G. Warren (unpublished)

^{a} This conforms with the revised designations of Keen and Tamaki (1986).

technique employing the *nptI-sacB-sacR* cartridge (Ried and Collmer 1987). Because the cartridge confers kanamycin resistance and sucrose sensitivity, exchange recombination events between unstable recombinant plasmids and a target chromosome involving both insertions and evictions can be selected. This facilitates the construction of complex mutants containing multiple, directed mutations without the introduction of an
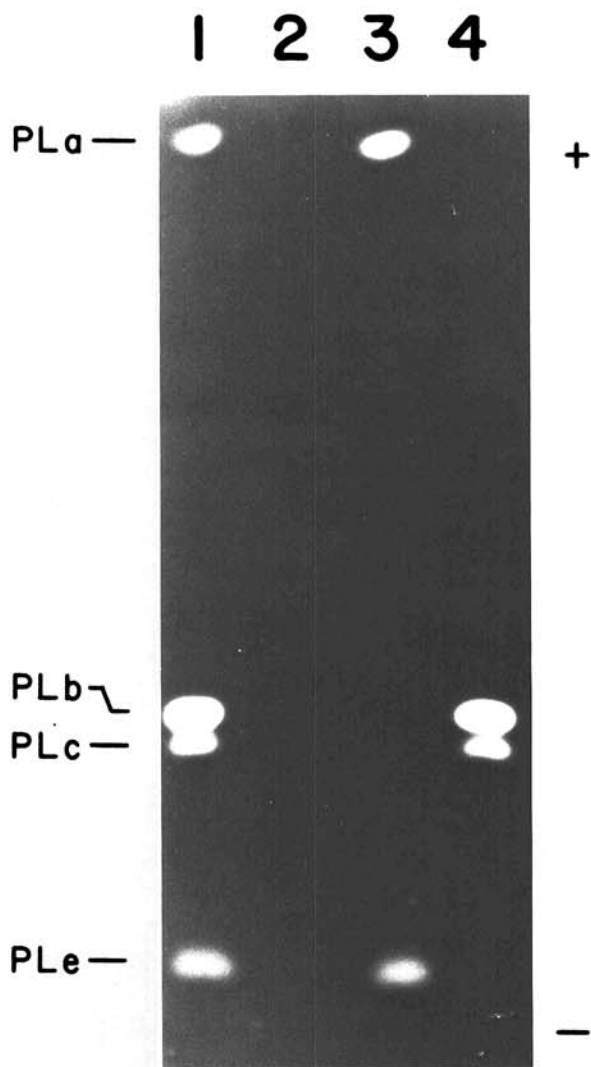

Fig. 2. Isoelectric focusing profiles of pectate lyase (PL) isozymes produced by *E. chrysanthemi* strains containing *pel* deletions. Extracellular PL isozymes were activity-stained with a pectate-agarose overlay after isoelectric focusing. Clear areas represent bands of activity where the substrate was degraded by PL and no longer stained with ruthenium red. Lanes: 1, AC4150 (wild type); 2, UM1005 (*pelA, pelB, pelC,* and *pelE* deleted); lane 3, UM1003 (*pelB* and *pelC* deleted); lane 4, UM1004 (*pelA* and *pelE* deleted). The anode and cathode are indicated by the + and −, respectively.

Table 2. Growth rate and pectate lyase (PL) production in pectate minimal medium of *Erwinia chrysanthemi* wild-type and Pel⁻ strains

Strains	Specific growth rate (hr^{-1})[a]	PL activity (U/ml)[b]
AC4150	0.41	5.3
UM1005	0.42	0.002

[a] The rate of increase in OD$_{600}$ was determined at 20-min intervals during logarithmic growth with polygalacturonic acid as the sole carbon source. Values represent the mean from three separate cultures.

[b] PL activity in culture supernatants was determined at the maximum OD$_{600}$. Values represent the mean from three separate cultures.

accompanying number of antibiotic resistance markers.

The cartridge was ligated in place of the deleted *pelB* and *pelC* genes in partial *Sau*3A-digested pCS3 to construct pJR475 and then excised from pJR475 by partial *Pst*I digestion to construct pJR485 (Fig. 1). Cartridge-containing sequences in pJR475 were exchanged into wild-type strain AC4150 by selecting for kanamycin resistance to produce UM1002 and then evicted from UM1002 by exchange substitution of the sequences in pJR485 (by selecting for sucrose tolerance) to produce UM1003. The *pelA* and *pelE* genes were then deleted from UM1003 by exchange recombination of the *nptI*-marked *pelA pelE* deletion in pJR51 to produce UM1005 (Fig. 1). The pJR51 deletion was also exchanged into AC4150 to produce mutant UM1004.

It is important to note that the sucrose-sensitive phenotype of *E. chrysanthemi* EC16 differs from that observed with strain CUCPB 1237 (Ried and Collmer 1987). The death of sucrose-sensitive EC16 cells appears to be delayed. Thus, in the isolation of UM1003, the colony that was the source of the mutant grew out of a lawn of apparently short-lived cells. When these colonies were picked to LB and LB-kanamycin plates, 56 of 63 were found to be kanamycin sensitive, indicating that the *nptI-sacB-sacR* cartridge had been evicted by reciprocal recombination between the deletion-derivative plasmid and the chromosome, followed by loss of the new plasmid. The deletion of appropriate *pel* genes was confirmed by the isoelectric focusing profiles of the PL isozymes produced by AC4150 and its derivatives (Fig. 2). UM1003 was deficient in PLb and PLc. Mutant UM1004 was constructed to provide evidence that the mutation introduced by pJR51 was specific for *pelA* and *pelE*, and it was found to be deficient in only PLa and PLe. UM1005 produced none of the major PL isozymes.

Characterization of the pectolytic phenotype of UM1005 in culture. *E. chrysanthemi* UM1005 was compared with AC4150 for its ability to grow and produce PL in a minimal medium containing soluble polygalacturonic acid as the sole carbon source (Table 2). The growth rate was unaffected by the mutations, but the mutant produced less than 0.1% of the PL activity of the wild type. Isoelectric focusing analysis of the extracellular pectic enzymes produced by UM1005 in KB broth showed that exoPG was still produced (Fig. 3). Two other weak bands of clearing were also detected in the overlay buffered for PL detection (Fig. 3). PNL assays confirmed that UM1005 produced only the low levels of activity previously reported for EC16 by Tsuyumu and Chatterjee (1984; data not shown).

Maceration of plant tissues by UM1005. The relative ability of AC4150 and UM1005 to macerate potato tuber tissues was assayed with aerobically incubated tuber slices inoculated with toothpicks and with anaerobically incubated whole tubers inoculated with pipette tips (Table 3). The amount of macerated tissue produced by the mutant in intact tubers was less than 2% of that produced by the wild type. However, in tuber slices, the amount of maceration produced by the mutant was 21% of that produced by the wild type. The significant maceration of potato tuber slices by UM1005 was observed with several batches of potatoes and also with carrot slices and pepper fruits (data not shown). Bacteria in macerated tissue from tuber slices that had been inoculated with UM1005 were streaked on nonselective KB and LB plates. Colonies that developed were then picked to LB and LB-kanamycin plates. All of these colonies had a colony morphology typical of *E.*

chrysanthemi and were kanamycin resistant.

Pectic enzymes extracted with water from macerated tissue in potato tuber slices inoculated with AC4150 and UM1005 were analyzed by activity-stained isoelectric focusing gels (Fig. 3). To facilitate comparison of enzymes produced by the two strains in infected tissue, equal amounts of macerated tissue were extracted for each. AC4150 appeared to produce all of the PL isozymes in tuber tissue (although PLb and PLc were not well resolved). UM1005 produced none of the major PL isozymes, although two weak bands in the alkaline region of the gel could be seen in the PL-detecting overlay. Both strains produced equivalent amounts of exoPG in macerated tissue.

Extracellular enzyme samples from AC4150 and UM1005 were tested for their ability to macerate potato tuber tissue. Thin, free-hand sections (about 1-mm thick) were incubated for 16 hr in water extracts from macerated potato tissue and in supernatants from cultures grown in KB broth. The samples from AC4150 caused substantial maceration; those from UM1005 caused no change in tissue coherence (data not shown).

DISCUSSION

By marker exchange-eviction mutagenesis, we constructed a mutant of *E. chrysanthemi* EC16 that contains deletions in the *pel* genes encoding the four extracellular PL isozymes produced by this bacterium. The Pel⁻ mutant, UM1005, produced less than 0.1% of the level of extracellular PL of the parental strain AC4150. Utilization of polygalacturonic acid in minimal medium was unaltered in the mutant. Surprisingly, although the virulence of the mutant in maceration assays was reduced several-fold (particularly in assays involving intact potato tubers) the mutant still

retained the ability to cause significant maceration in potato, carrot, and pepper tissues.

The ability of UM1005 to utilize soluble polygalacturonic acid in minimal medium can be attributed to exoPG activity. The *E. chrysanthemi* exoPG releases assimilable digalacturonate from the nonreducing end of pectic polymers and is active in phosphate-buffered media that poorly support PL activity because of divalent cation deficiencies (Collmer *et al.* 1982). Thus, the growth of UM1005 corroborates the previous observation that *E. chrysanthemi* can utilize pectate in media containing EDTA, an inhibitor of extracellular PL activity (Collmer *et al.* 1982). ExoPG activity may also account for the ability of mutant colonies to pit pectate semisolid agar media. Mutants deficient in this enzyme will now be sought to test these hypotheses.

The ability of the mutant to cause significant maceration in the tissues of three plants is puzzling. The possibility of

Table 3. Maceration of potato tuber tissues by *Erwinia chrysanthemi* wild-type and Pel⁻ strains

Strains	Wet weight (g) of macerated tissue per inoculation site	
	Tuber slices[a]	Whole tubers[b]
AC4150	1.26 ± 0.44	0.196 ± 0.068
UM1005	0.27 ± 0.14	0.003 ± 0.006

[a] Bacteria were stabbed 4 mm deep into tuber slices with a toothpick. Macerated tissue was gently scraped out and weighed after 28-hr incubation. Values represent mean and SD of six slices.
[b] Bacterial suspensions containing 7.5×10^8 (AC4150) and 5.8×10^8 (UM1005) colony forming units in 25 μl were injected into whole potato tubers. Macerated tissue was weighed after 68-hr anaerobic incubation. Values represent mean and SD of 11 inoculation sites.
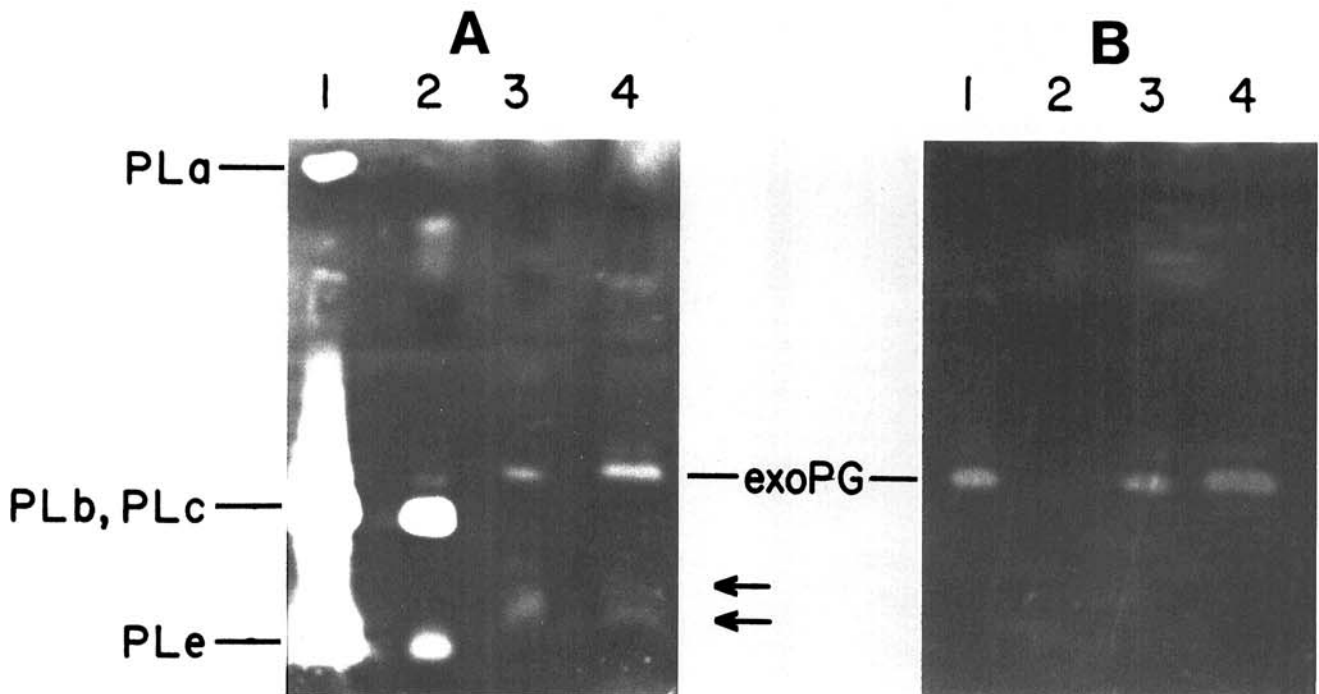
Fig. 3. Isoelectric focusing profiles of PL isozymes and exo-poly-α-D-galacturonosidase (exoPG) produced in macerated potato tissue by *E. chrysanthemi* AC4150 (wild type) and UM1005 (Pel⁻) and in culture by UM1005. Diagnostically buffered pectate-agarose overlays were used to preferentially detect either PL activity (**A**) or pectic hydrolase activity (**B**) following isoelectric focusing. Samples consisted of the supernatant from macerated tissue extracted with water (lanes 1–3) or a concentrated (20×) culture supernatant from a King's Medium B culture (lane 4). Lanes: 1, AC4150; 2, 10^{-2} dilution of sample in lane 1; 3, UM1005; 4, UM1005. The location of the PL isozymes and the exoPG is labeled. Arrows indicate the location of novel PL activity (panel A, lanes 3 and 4). The cathode was at the bottom of the gel.

the mutant reverting to Pel⁺ was eliminated by the construction of major deletions in the *pel* genes: all of *pelC* and *pelE* and most of *pelA* and *pelB* were deleted. The possibility that the maceration was caused by a pectolytic contaminant is diminished by the observation that macerated tissues yielded pure cultures of the mutant on nonselective media. Furthermore, exoPG, a useful marker for *E. chrysanthemi*, was the only major pectic enzyme detectable by isoelectric focusing analysis of macerate extracts.

The identity of the residual macerating factor produced by UM1005 is not known at this point. ExoPG would not be expected to macerate plant tissues because of its action pattern. PNL, although an effective macerating enzyme, is produced in only low levels by EC16. Similarly, the two novel bands of clearing produced by UM1005 (Fig. 3) are very faint, which suggests that the contributing enzyme activities are very low. Attempts to identify the macerating factor produced by UM1005 by biochemical fractionation have been frustrated so far by the lack of macerating activity in culture fluids of UM1005 or in water extracts of tissue macerated by the mutant. Numerous explanations can be postulated for this phenomenon. For example, the residual macerating factor may be bound to the bacterial surface or induced only *in planta* and then adsorbed to plant tissues.

Results obtained in the 1970s from studies involving the biochemical fractionation of culture extracts with maceration activity indicated that the PL produced by soft-rot erwinias (and the endocleaving pectic enzymes produced by pathogens in general) are important in causing the maceration associated with soft-rot diseases (Bateman and Basham 1976; Collmer and Keen 1986). Our results confirm the important contribution of PL to the macerating ability of *E. chrysanthemi*, but they also reveal that the tissue-degrading arsenal of *E. chrysanthemi* has another component. Mutant UM1005 will provide a key to the identification of the residual macerating factor, and the family of mutants associated with its construction will be important tools in further exploration of the role of the multiple PL isozymes in the overall biology of *E. chrysanthemi*.

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