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Marker-Exchange Mutagenesis of a Pectate Lyase Isozyme Gene in *Erwinia chrysanthemi*†

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The phytopathogenic enterobacterium *Erwinia chrysanthemi* contains *pel* genes encoding several different isozymes of the plant-tissue-disintegrating enzyme pectate lyase (PL). The *pelC* gene, encoding an isozyme with an approximate isoelectric point of 8.0, was mutagenized by a three-step procedure involving (i) insertional inactivation of the cloned gene by ligation of a *kan*-containing *Bam*HI fragment from pUC4K with a partial *Sau*3A digest of *E. chrysanthemi pelC* DNA in pBR322; (ii) mobilization of the pBR322 derivative from *Escherichia coli* to *E. chrysanthemi* by the helper plasmids R64*drd*11 and pLVC9; and (iii) exchange recombination of the *pelC*::*kan* mutation into the *E. chrysanthemi* chromosome by selection for kanamycin resistance in transconjugants cultured in phosphate-limited medium (which renders pBR322 unstable). The resulting *E. chrysanthemi* mutant was Kan^r Amp^s, lacked pBR322 sequences, and was deficient in only one of the four major PL isozymes, PLc, as determined by activity-stained isoelectric-focusing polyacrylamide gels. The rates of PL induction and cell growth in a medium containing polygalacturonic acid as the sole carbon source were not significantly reduced in the mutant. No difference was detected in the ability of the mutant to macerate potato tuber tissue. The evidence suggests that this isozyme is not necessary for soft-rot pathogenesis.

The enterobacterium *Erwinia chrysanthemi* causes diseases involving maceration and killing of parenchymatous tissues (soft-rots) in a wide variety of plant hosts (30). The bacterium excretes multiple isozymes of pectate lyase (EC 4.2.2.2; PL), an enzyme that cleaves internal glycosidic linkages in pectic polymers by β-elimination and which has been implicated as an important factor in soft-rot pathogenesis. Pectic polymers (chains of 1,4-linked α-D-galacturonic acid and methoxylated derivatives) are structural constituents of the middle lamellae and primary cell walls of higher plants (27). Highly purified PL, by its action on these polymers, is able to kill and macerate plant tissues (1, 29). The capacity to produce total extracellular PL has been shown to transfer conjugationally with the capacity for macerating activity (6). Recently, *E. chrysanthemi* genes encoding PL have been cloned into *Escherichia coli* (9, 18, 19, 39). The pectolytic *E. coli* clones possess a limited ability to macerate potato tuber tissues (9, 18), thus providing further evidence for the importance of PL as a disease factor.

Although several gene transfer systems have been developed in *E. chrysanthemi* (4–6, 20, 33), mutations in PL structural genes have remained elusive, and the role of the PL isozymes in pathogenesis awaits genetic exploration. The difficulty in isolating PL structural gene mutations by screening populations of random mutants is now understandable in light of the demonstration that *E. chrysanthemi* contains several PL isozyme structural genes, each of which can confer the pectolytic phenotype to recombinant *E. coli* clones (9, 18, 19, 39). Site-directed mutagenesis by marker exchange of a mutated cloned gene (34) provides a method for generating specific mutations that are difficult to screen for, e.g., because of phenotypic masking by multiple isozymes. To facilitate manipulation of individual PL isozyme structural genes in *E. chrysanthemi*, we developed an efficient system for exchanging in vitro-mutagenized PL isozyme genes carried on pBR322 into the *E. chrysanthemi* chromosome. In this report we describe the construction and phenotypic properties of an *E. chrysanthemi* strain lacking a single PL isozyme.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains and plasmids are listed in Table 1. Cultures were maintained on LB agar (25) containing appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml (50 μg/ml in broth); chloramphenicol, 10 μg/ml; kanamycin, 50 μg/ml; tetracycline, 20 μg/ml.

DNA analysis. Plasmid DNA was isolated by a modification of the Holmes and Quigley procedure (16). Chromosomal DNA was isolated by the method of Matthysse (26). Restriction mapping was done by comparing single and double restriction endonuclease digests (*Eco*RV, *Ava*I) of the recombinant plasmids with the known restriction map of pCSR1 (9). Hybridization analysis was done by the blotting technique of Southern (35). The hybridization probe was labeled with Biotin-11-dUTP (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), using the Bethesda Research Laboratories nick translation system. DNA was hybridized by the procedure of Leary et al. (22). Hybridization with the probe was detected with the Bethesda Research Laboratories DNA detection system.

In vitro mutagenesis. pCSR1 was partially digested with *Sau*3A and electrophoresed in a 0.8% agarose gel. The band containing linearized plasmid of approximately full length was extracted from the gel by the DEAE membrane procedure (13) according to the manufacturer's instructions (Schleicher & Schuell, Inc., Keene, N.H.). The DNA was then dephosphorylated with calf intestine alkaline phosphatase. pUC4K was digested with *Bam*HI and electrophoresed, and the 1.2-kilobase (kb) band containing the kanamycin resistance gene was extracted. A ligation mixture containing 90 ng of the linearized pCSR1 DNA, 90 ng of the *kan* gene, and 2 U of T4 DNA ligase (Bethesda Research

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TABLE 1. Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Origin
E. coli		
HB101	*leu proA2 thi recA13 hsdS20*	3
JC2926	*recA* derivative of AB1157 (40)	Gift from G. Warren
E. chrysanthemi		
CUCPB 1237[a]	Rif^r Str^r prototrophic	7
1237-K3	*pelC*::*kan* derivative of CUCPB 1237	This work
Plasmids		
pCSR1	Tc^s *pelC*$^+$ derivative of pBR322	9
pDR103	*pelC*::*kan* derivative of pCSR1	This work
pUC4K	Km^r derivative of pUC4	41
R64*drd*11	Transfer-derepressed derivative of R64 (40)	Gift from G. Warren
pLVC9	Cm^r Km^s derivative of pGJ28 (40) carrying ColE1 *mob*	Gift from G. Warren

[a] Cornell University Collection of Phytopathogenic Bacteria.

Laboratories) in a total volume of 16 μl of ligase buffer was incubated for 16 h at 12°C. The recombinant plasmid was then transformed into *E. coli* HB101 by the calcium chloride procedure of Mandel and Higa (24), and cells were spread on LB agar containing ampicillin and kanamycin. Amp^r Kan^r transformants were screened on pectate semisolid agar medium (36) for loss of pectolytic ability.

Bacterial matings. Matings between *E. coli* HB101(pDR103) and *E. coli* JC2926(R64*drd*11, pLVC9) were performed by mixing 1 ml of a mid-log-phase culture of each strain. Tubes were centrifuged for 10 min in a clinical centrifuge, the supernatant was removed, and 2 ml of LB was carefully added without resuspending the pellet. The tube was incubated for 4 h at 30°C, and the pellet was resuspended and plated on LB agar containing tetracycline, ampicillin, chloramphenicol, and kanamycin.

Matings between *E. coli* HB101(pDR103, R64*drd*11, pLVC9) and *E. chrysanthemi* 1237 were done by the filter method of Chatterjee (4). Mid-log-phase cultures were mated on a membrane filter (0.45-μm pore size, 25-mm diameter; Millipore Corp., Bedford, Mass.). The filter was placed on pectate semisolid agar medium (36) and incubated at 30°C overnight. (Semisolid media lacking pectate may also be used for matings.) The cultures were washed twice in sterile diluting buffer (15 mM potassium phosphate [pH 7.0], 17 mM NaCl, 0.1 mM $CaCO_3$) and spread on M9 agar (25) containing 0.2% glucose, kanamycin, and ampicillin.

Selection for exchange recombination. *E. chrysanthemi* 1237 (pDR103) was grown in basal medium A-P (37)–0.2% glycerol–250 μM potassium phosphate (pH 7.0)–kanamycin at 30°C with shaking (200 rpm) for 32 h. The culture was then diluted and spread on LB-kanamycin plates. Isolated colonies were then replica plated on LB-ampicillin and LB-kanamycin plates to screen for Amp^s isolates.

Determination of pBR322 stability in phosphate-limited media. *E. chrysanthemi*(pBR322) was grown overnight in basal medium A-P supplemented with 70 mM potassium phosphate (pH 7.0), 0.2% glycerol, and ampicillin. The culture was then washed twice in the basal medium, and diluted to an optical density at 600 nm of 0.02 in LB or in a series of tubes of basal medium A-P supplemented with 0.2% glycerol and various levels (1 to 70 mM) of potassium phosphate, pH 7.0. Cultures were then incubated at 30°C with shaking (250 rpm) for 48 h and plated on LB and LB-ampicillin plates to determine the level of plasmid retention.

Determination of PL production. Cultures were grown to stationary phase in the minimal salts medium of Zucker and Hankin (42) supplemented with 0.2% glycerol and diluted to an optical density at 600 nm of 0.05 in the same medium with 0.5% polygalacturonic acid (Pfaltz and Bauer, Inc.) substituted for glycerol. Cultures were incubated at 30°C with shaking (200 rpm) for 36 h. Samples from three flasks of each strain were collected periodically and centrifuged, and the supernatants were stored at −20°C. Bacterial growth was monitored by optical density readings at 600 nm.

PL activity was determined by measuring the change in A_{230} of a reaction mixture containing 0.07% (wt/vol) polygalacturonic acid, 30 mM Tris hydrochloride (pH 8.5), 0.1 mM $CaCl_2$, and 6.7% enzyme sample. One unit of PL activity is defined as that amount of activity necessary to produce 1 μmol of product per min under optimal conditions at 25°C.

Detection of PL and protein in ultrathin-layer polyacrylamide IEF gels. Cultures were grown at 30°C to late-log phase in Zucker and Hankin medium supplemented with 0.5% polygalacturonic acid. The supernatant was concentrated by ultrafiltration through an immersible CX-10 membrane unit (molecular weight cutoff of 10,000; Millipore Corp.). The samples were desalted by exchanging several volumes of 10 mM Tris hydrochloride (pH 7.5) during the concentration procedure. Ultrathin-layer isoelectric focusing (IEF) was done as previously described (9). A 0.50-mm-thick 5% polyacrylamide gel containing 0.38 ml of pH 3 to 10 carrier ampholytes (Pharmacia, Uppsala, Sweden) per 5 ml of total volume was cast on a gel support film (100 by 125 mm) for acrylamide (Bio-Rad Laboratories, Richmond, Calif.). The gel was preelectrofocused for 30 min at a constant 2.0 W before 10-μl samples containing 0.005 U of PL activity were loaded onto the gel. The samples were focused for 1 h at a constant 4.5 W with a 2,000-V limit.

Ultrathin (0.35-mm) pectate-agarose overlay gels were prepared for detection of PL as previously described (9). The overlay gel was placed onto the IEF gel and incubated at room temperature for 75 s. The overlay gel was then stained in a ruthenium red solution (0.05%) for 20 min and air dried at room temperature.

Ampholytes were removed from IEF gels by soaking the gels for 30 min in a solution containing 11.5% trichloroacetic acid and 3.45% sulfosalicylic acid, followed by 30 min in 50% methanol and 30 min in water. The gels were then stained in 0.1% Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, Mo.)–50% methanol–10% acetic acid.

Virulence assays. Bacterial cells were prepared for inoculation as previously described (9). Store-bought russet potatoes were washed and surface sterilized by soaking 15 min in 0.8% (wt/vol) sodium hypochlorite–0.005% Triton X-100, rinsed in distilled water, and air dried. A modification of the procedure of Maher and Kelman (23) was used for the tuber inoculation. Disposable pipette tips (200 μl; VWR Scientific) containing 50 μl of bacterial suspension were inserted 15 mm into the potato tubers. The tubers were then incubated at

30°C in a sealed glass container with a constant flow of nitrogen. After 2 days the tubers were sliced through the axis of the injection site, and the macerated tissue was gently scraped out and weighed.

RESULTS

Insertional inactivation of the *pelC* gene. In vitro insertional mutagenesis of *pelC* was accomplished by using a partial *Sau*3A digest of the recombinant plasmid pCSR1. Of several recombinant plasmids containing *E. chrysanthemi* 1237 DNA that had been constructed (9), pCSR1 was chosen because it does not appear to encode any of the major PL isozymes other than PLc. The *pelC* gene had been localized to a 2.5-kb portion of pCSR1 (9). Because of the lack of useful restriction sites in this region (9), we decided to generate insertion sites with the tetranucleotide-recognizing restriction enzyme *Sau*3A. A 1.2-kb *kan*-containing fragment from pUC4K was ligated with near full-length pCSR1 molecules that had been linearized by partial digestion with *Sau*3A, thus inserting the *kan* determinant in various parts of the pectolytic plasmid. Of 140 Amp^r Kan^r transformants examined for pectolytic activity on pectate semisolid agar plates, 25 isolates failed to pit the medium, indicating that the *kan* gene had disrupted PLc production. Restriction analysis was performed on 10 of these isolates to determine the location of the insert and to ensure that no large deletions occurred as a result of the *Sau*3A digestion. One of the plasmids, pDR103, which appeared to have no deletion, was chosen for use in marker-exchange mutagenesis. The restriction analysis indicated that the *kan* gene was inserted into the 1-kb *Ava*I-*Eco*RI fragment of pCSR1 (Fig. 1). This fragment was invariably affected by the insertions (or attendant deletions) in all of the nonpectolytic plasmids analyzed.

Introduction of pDR103 into *E. chrysanthemi* and exchange recombination. Attempts to develop a transformation system for *E. chrysanthemi* 1237 were unsuccessful. Variations of the calcium chloride, rubidium chloride, and freeze-thaw procedures (12, 24, 25) did not yield transformants at useful frequencies (unpublished data). However, we were able to mobilize pDR103 into *E. chrysanthemi* with a conjugational helper system developed for pBR322 derivatives (40). An *E. coli* strain, JC2926, containing the helper plasmids R64*drd*11 and pLVC9 was mated with strain HB101(pDR103). The resulting *E. coli* strain containing pDR103, R64*drd*11, and pLVC9 was mated with *E. chrysanthemi*. pDR103 was mobilized to *E. chrysanthemi* at a frequency of 4×10^{-5} transconjugants per recipient.

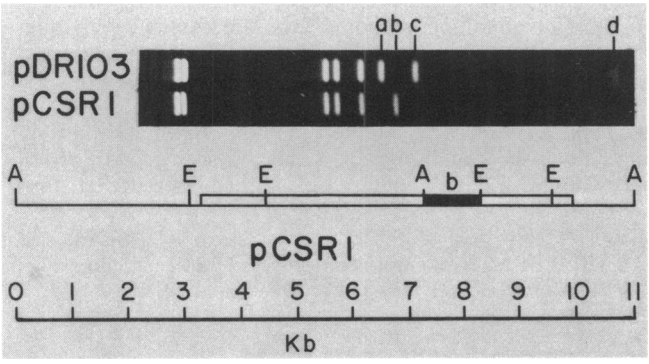

FIG. 1. Agarose gel (0.8%) showing fragments in an *Eco*RV-*Ava* 1 digest of pCSR1 and pDR103 (*pelC*::*kan*). The origin is at the left. *Eco*RV (E) and *Ava*I (A) sites in pCSR1 are shown in the restriction map. The region of vector DNA is depicted with a single line. Because there are two *Ava*I sites within the *kan* determinant, the pCSR1 fragment into which *kan* has been inserted (b) is cut into three fragments (a, c, d) by *Ava*I digestion of pDR103; fragment d is the internal *kan* fragment.

TABLE 2. Stability of pBR322 under phosphate-limiting growth conditions[a]

Growth medium	CFU/ml on:	
	LB	LB-ampicillin
LB	1.3×10^{10}	1.5×10^{10}
Basal medium A-P-0.2% glycerol-70 mM potassium phosphate	6.3×10^{10}	3.6×10^{10}
Basal medium A-P-0.2% glycerol-25 mM potassium phosphate	1.8×10^{9}	5.0×10^{7}
Basal medium A-P-0.2% glycerol-1.0 mM potassium phosphate	4.0×10^{8}	1.3×10^{4}

[a] After 48 h of growth in liquid medium, cultures were diluted and spread on LB and LB-ampicillin agar. Amp^r colonies represent cells that have retained the plasmid (pBR322).

Effective marker-exchange mutagenesis necessitates delivery of the mutated gene on an unstable replicon. Although pBR322 is stably maintained in *E. chrysanthemi* in media containing sufficient phosphate, the plasmid is lost when cultures are grown in phosphate-limited medium (Table 2). Transconjugants were grown in phosphate-limited medium containing kanamycin for 32 h, plated on LB-kanamycin plates, and replica plated on LB-ampicillin plates. Of 100 colonies tested, 99 were Amp^s, suggesting that exchange recombination had occurred and that the vector plasmid was no longer present. This was verified by hybridizing an *Eco*RV digest of *E. chrysanthemi* 1237-K3 chromosomal DNA with a biotin-labeled pUC4K probe (Fig. 2). Controls consisted of *Eco*RV digests of pCSR1, pDR103, and *E. chrysanthemi* 1237 chromosomal DNA. The *Eco*RV profiles of pCSR1 and pDR103 were identical except that the 4-kb band (Fig. 2, band c) containing *pelC* in pCSR1 was replaced by a 5.2-kb band (band a) in pDR103 due to the *kan* gene insert. The probe hybridized with both the pDR103 band containing the *kan* gene and a corresponding band in mutant 1237-K3 (the slight misalignment between these two bands in Fig. 2 was not observed in another Southern blot of these samples). Because pUC4K is a pBR322 derivative (41), it hybridized to vector DNA as well as to the *kan* gene insert. Hybridization was observed with the pBR322-containing band (b) in pCSR1 and pDR103. There was no corresponding hybridization with 1237 and 1237-K3 DNA, indicating the absence of the vector.

Analysis of the phenotype of mutant 1237-K3. *E. chrysanthemi* 1237-K3 was tested for the specific loss of PLc from the PL isozyme profile. Concentrated extracellular enzyme preparations from 1237 and 1237-K3 were subjected to IEF and then stained for PL activity with a pectate agarose overlay and for protein with Coomassie brilliant blue R. 1237-K3 is missing a band of activity and a major protein corresponding to the *pelC* isozyme (Fig. 3). No differences in the protein or PL isozyme profiles of the mutant and the wild-type were detected in the acidic portion of the gel, although two minor protein bands with apparent isoelectric points around 7.5 and 9.0 are also missing from the mutant profile.

To assess the effect of the *pelC* mutation on induction of PL synthesis and ability to utilize pectate, strains 1237 and 1237-K3 were grown in a medium containing polyga-

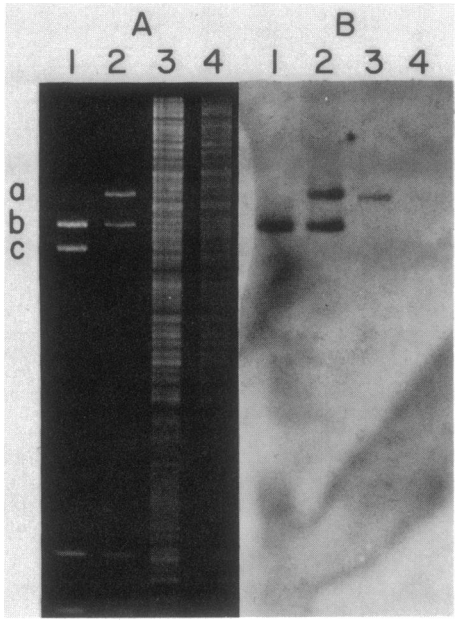

FIG. 2. Southern blot of pCSR1, pDR103, and *E. chrysanthemi* 1237-K3 and 1237 DNA. (A) *Eco*RV digests resolved by a 0.8% agarose gel. (B) Hybridization of a biotinylated pUC4K probe with a nitrocellulose transfer of the DNA shown in panel A. Lanes: 1, pCSR1; 2, pDR103; 3, 1237-K3 genomic DNA; and 4, 1237 genomic DNA. Band a contains the *kan* gene inserted into *pelC*, band b contains pBR322 DNA, and band c contains the functional *pelC* gene.

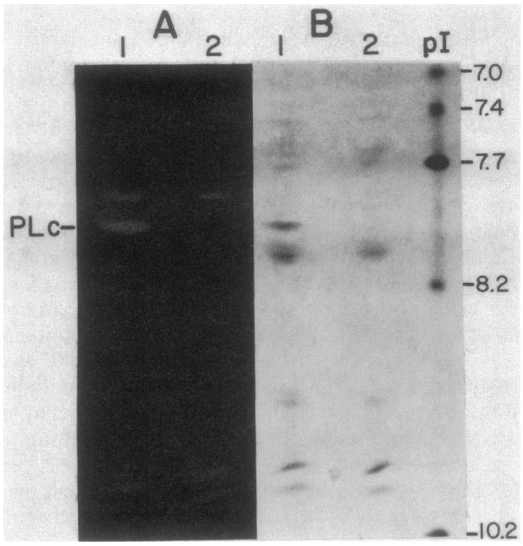

FIG. 3. IEF profile of major PL isozymes and extracellular proteins produced by *E. chrysanthemi* 1237 and 1237-K3. Concentrated extracellular proteins from *E. chrysanthemi* cultures grown in polygalacturonic acid minimal medium were resolved by IEF in ultrathin-layer polyacrylamide gels. (A) Zones of clearing in a ruthenium red-stained pectate-agarose overlay corresponding to bands of PL activity in the IEF gel. (B) IEF gel stained for protein after completion of the activity staining. Lanes: 1, *E. chrysanthemi* 1237; 2, *E. chrysanthemi* 1237-K3.

lacturonic acid as the sole carbon source. The rates of growth and PL induction in 1237-K3 were not significantly reduced in comparison with those of the wild-type parent. The maximum optical densities at 600 nm of replicate cultures of the two strains, attained at 32 h of incubation, were 2.52 ± 0.03 (mean and standard deviation) for 1237 and 2.50 ± 0.12 for 1237-K3. The extracellular PL activity at this time was 0.563 ± 0.075 U/ml for 1237 and 0.513 ± 0.054 U/ml for 1237-K3.

The effect of the *pelC* mutation on the virulence of *E. chrysanthemi* was assayed by injecting whole tubers with strains 1237 and 1237-K3 at various inoculum levels (Table 3). After 2 days of incubation at 30°C under anaerobic conditions, the macerated tissue from each injection site was weighed. A comparison of regression lines that were fit for the data from each strain showed no difference in their macerative ability.

DISCUSSION

Marker-exchange mutagenesis of one of the several genes encoding PL isozymes in *E. chrysanthemi* was accomplished by a simple procedure employing a *pel* gene cloned in pBR322. Marker-exchange mutagenesis (34) has proven to be a useful tool for manipulating genes that do not confer an easily screened phenotype but for which a cloned copy is available. Successful use of the technique requires a means for introducing the insertionally inactivated cloned gene into the target host and on the subsequent instability of the delivery replicon (11, 34, 40). We adapted available techniques to permit the use of pBR322 as a vehicle for marker-exchange mutagenesis in *E. chrysanthemi* (and presumably other erwinias). pBR322 is a convenient plasmid for manipulating cloned genes in *E. coli* and has previously been introduced into erwinias by transformation (15, 21). The plasmid can be mobilized easily and with high frequency into *E. chrysanthemi* by conjugation with an *E. coli* strain containing the helper plasmids R64*drd*11 and pLVC9, which provide *tra* and *mob* functions, respectively (40). Although pBR322 is stable in *E. chrysanthemi*, even in the absence of antibiotic selection pressure, we found that the plasmid is rapidly lost from small batch cultures grown in phosphate-limited medium (Table 2). By taking advantage of this effect, high frequencies of exchange recombination can be obtained without introduction of an incompatible plasmid (34) or prior subcloning into a plasmid unable to replicate in *E. chrysanthemi* (11, 40).

Jones et al. (17) had previously reported that pBR322 is not stably maintained in chemostat cultures of *E. coli* under phosphate-limited conditions. This instability has been postulated to be due to the lack of an efficient segregation mechanism in pBR322 (17). Under normal growth conditions the high plasmid copy number ensures continued distribution among the daughter cells. Under limited growth conditions, however, the plasmid copy number is reduced, thus

TABLE 3. Maceration of potato tubers by *E. chrysanthemi* 1237 and 1237-K3

CFU injected/ml	Wet wt (g) of macerated tissue from injection site[a]	
	1237	1237-K3
6×10^7	0.81 ± 0.36	0.87 ± 0.34
6×10^6	0.71 ± 0.25	0.76 ± 0.31
6×10^5	0.59 ± 0.23	0.53 ± 0.23

[a] Values represent mean ± standard deviation from at least 24 injections sites per bacterial concentration. Procedures for inoculation and incubation of potato tubers are given in the text.

diminishing the probability that the plasmid will be distributed to both daughter cells during cell division. A particular advantage of this phenomenon for genetic manipulation of erwinias is that it permits recombinant pBR322 derivatives containing erwinia genes to be stably maintained for complementation studies or, alternatively, lost for marker-exchange mutagenesis.

High-resolution IEF and activity stain techniques have revealed a striking complexity in the extracellular PL isozyme profiles of *E. chrysanthemi* strains (2, 9). At least 12 bands of activity (previously labeled PLa through PLl) were discerned in the strain 1237 profile (9). This complexity presents a problem in naming the corresponding *pel* genes. Four of the several bands that electrofocus above pH 7 are markedly stronger than the others (9; Fig. 3). Another, less-intense band is distinguished by its low pI of ca. 5.0 (9). On the basis of genetic evidence (9) or markedly different isoelectric points we estimate that at least four of the strain 1237 PL isozymes are encoded by different genes. Genetic evidence for five *pel* genes has recently been obtained with two other strains of *E. chrysanthemi* (19, 39). In accordance with the recommendations of a European Molecular Biology Organization Workshop on Soft-Rot Erwiniae (Marseille-Luminy, France, 23–27 July 1984), we are naming the *pel* genes alphabetically beginning with the most acidic isozyme. Consequently, the PL isozyme previously labeled PLg (9) is here described as PLc encoded by *pelC*.

Evaluation of the functional basis for the multiplicity of *pel* genes (or the selective advantage conferred to *E. chrysanthemi* by production of a specific isozyme) can be considered in the context of three distinguishable physiological activities of PL. (i) PL is a catabolic enzyme enabling *E. chrysanthemi* to rapidly utilize polygalacturonic acid as a sole carbon source. Since complex pectic polymers are a prevalent constituent of plant cell walls and consequently of plant litter, PL may play an important role in the saprophytic phase of the *E. chrysanthemi* life cycle. Indeed, the ability of soft-rot erwinias to compete with soil microorganisms for the utilization of pectic polymers permits enrichment of their population in soil samples amended with polygalacturonic acid (28). In polygalacturonic acid minimal media containing EDTA, which inhibits PL by chelating divalent cations essential for activity, *E. chrysanthemi* grows only slowly. Growth under these conditions is dependent on the products of another extracellular pectic enzyme, exo-poly-α-D-galacturonosidase (11). (ii) As an extracellular polymer-degrading enzyme, PL participates in its own induction through the generation of products that can be taken up by the cell (7, 8). It has been demonstrated in *Erwinia carotovora* that PL is poorly induced in polygalacturonic acid minimal media containing EDTA (38) and in *E. chrysanthemi* that PL is poorly induced when cultures are incubated with isolated plant cell walls in the presence of EDTA (10). (iii) Finally, PL has been implicated by substantial physiological and genetic evidence as being important to the plant pathogenic abilities of *E. chrysanthemi*.

PL isozymes produced by various soft-rot erwinias have been reported to differ with respect to their relative abilities to reduce the viscosity of pectate solutions and macerate and kill plant tissues (14) and to be differentially produced or inactivated in host tissues (31, 32). The PL isozymes produced by *E. chrysanthemi* 1237 were previously studied for the first three characters (14). The two major isozymes with isoelectric points above 9.0 more effectively reduced the viscosity of pectate solutions and macerated and killed host tissues than did the two isozymes with isoelectric points around 8.0. The individual isozymes within each pair were not resolved by the techniques used.

Insertional inactivation of *pelC* had slight effect on PL induction and culture growth by *E. chrysanthemi* in minimal medium containing polygalacturonic acid. There was no effect on virulence detectable by potato tuber injection assays (Table 3). Thus, although PLc is one of the major PL isozymes excreted by *E. chrysanthemi*, it makes no apparent contribution to the ability of the organism to utilize polygalacturonic acid or to cause plant disease. This finding affords two divergent interpretations. (i) There is redundancy in the PL isozymes, and *E. chrysanthemi* has a pectolytic capacity that exceeds the needs of the bacterium for pectate catabolism or pathogenicity (or similarly, the loss of one of the isozymes can be compensated for simply by overproduction of the others). (ii) Alternatively, the individual PL isozymes may have specialized activities that are essential to the organism in ecological niches that are not adequately represented by the standard tests we have used here. Further application of these genetic techniques will enable us to purify the PL isozymes for individual biochemical characterization and to specifically mutate additional *pel* genes. Ultimately, a deeper understanding of the biology of *E. chrysanthemi* will be required for an explanation of the functional basis for the proliferation of the PL isozymes.

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