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Mu-*lac* Insertion-Directed Mutagenesis in a Pectate Lyase Gene of *Erwinia chrysanthemi*

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The *pelC* gene, which encodes one of the five major pectate lyase (PL) isoenzymes in *Erwinia chrysanthemi* 3937, designated PLc, was subcloned from a hybrid lambda phage into a pBR322 derivative and mutagenized with a mini-Mu-*lacZ* transposable element able to form fusions to the *lacZ* gene. One plasmid (pAD1) which had an inactivated *pelC* gene and a Lac$^+$ phenotype was selected in *Escherichia coli*. This plasmid was introduced into *Erwinia chrysanthemi*, and the *pelC*::mini-Mu insertion was substituted for the chromosomal allele by homologous recombination. This strain lacks the PLc isoenzyme. This *Erwinia chrysanthemi* strain has a Lac$^+$ phenotype that is inducible by polygalacturonate, as are the wild-type PL activities.

Erwinia chrysanthemi is an enterobacterium pathogenic for many plants, including monocots as well as dicots (22). The most usual infection symptom is a soft rot in which bacterial metabolic activities appear to be involved. Indeed, *E. chrysanthemi* produces various depolymerizing enzymes, such as pectinases (pectate lyases and pectin methylesterase) (2, 7–9), cellulases (endoglucanases) (2, 4, 9), and protease(s) (C. Vandersman, T. Andro, and Y. Bertheau, manuscript in preparation). The pectate lyase (PL) activity responsible for the degradation of the pectic components present in plant cell walls appears to be essential for plant tissue maceration (7, 9, 11, 13, 20) and has been shown to be due to several enzymes. In strain 3937, five major PL activities could be detected in culture supernatants by electrofocusing in ultrathin polyacrylamide gels (2).

The genes involved in the synthesis of the different PL isoenzymes were cloned in λ vector, and it has been shown that these PL isoenzymes are expressed from five different genes (*pelA*, *pelB*, *pelC*, *pelD*, *pelE*) which could be localized at two different regions in the chromosome map (15, 23). The understanding of the exact role played by each of these isoenzymes in the pectinolysis and the phytopathogenicity of *E. chrysanthemi* requires study of mutants affected in the synthesis of each of these PLs. All the mutants showing a Pel$^-$ phenotype so far obtained corresponded to mutants unable to secrete these enzymes (1). In this study we report insertion-directed mutagenesis in one of the five PL-encoding structural genes.

The *pelC* gene encoding PLc, one of the five major isoenzymes, was subcloned in a plasmid, and the hybrid plasmid was mutagenized in *Escherichia coli* with a mini-Mu transposable element, Mu dII1734 (6), which is able to form translational *lacZ* gene fusions through a transposition event. These gene fusions encode hybrid β-galactosidase (5). Mu dII1734 bacteriophage is deleted for the Mu *A* and *B* transposition genes, thus forming stable insertions that cannot undergo transposition unless complemented for these functions (6) and thus can be used for insertion-directed mutagenesis (A. Plessis, C. Robaglia, A. Diolez, A. Beyou, F. Leach, F. Casse-Delbart, and F. Richaud, Plasmid, in press).

A mutagenized plasmid with the *pelC* gene inactivated by the mini-Mu insertion and expressing the mini-Mu *lacZ* gene was selected. This plasmid was introduced into *Erwinia chrysanthemi*, and after homologous recombination events with the chromosomal *pelC* gene, a mutant lacking the PLc isoenzyme was identified. The study of *pelC* gene regulation can be independently studied by measuring the expression of the chromosomal *pelC-lacZ* hybrid gene encoding β-galactosidase activity. The synthesis of the PelC-LacZ hybrid protein is induced when polygalacturonate is present in the culture medium, as was shown for the synthesis of the set of PLs in *E. chrysanthemi* (10, 11).

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. Bacterial strains, phages, and plasmids used or constructed in this work are described in Table 1 (see also Fig. 3).

Media. The following media were used: L broth-rich medium (18) and M9 minimal medium (18) containing 2 g of glycerol per liter. When required, the media were solidified by using Difco agar (12 g per liter for plates and 6 g per liter for soft overlays). Levels of antibiotics used were 20 μg of kanamycin per ml and 50 μg of ampicillin per ml. When necessary amino acids were added to the media at a final concentration of 30 μg/ml.

For the PL plate assay, M9 agar medium was supplemented with 5 g of glycerol per liter, 1 g of yeast extract per liter, and 5 g of sodium polygalacturonate (Sigma Chemical Co.) per liter (PGT agar).

To score LacZ$^+$ clones, the β-galactosidase chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) was added to the L broth agar medium at a final concentration of 40 μg/ml.

Except when otherwise specified all incubations were carried out at 30°C for *Erwinia chrysanthemi* and at 37°C for *Escherichia coli*.

Chemicals and DNA electrophoresis. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories and were used as recommended by the suppliers. Horizontal gel electrophoresis was carried out in borate buffer as described by Maniatis et al. (17). Phage lambda DNA fragments from *Hin*dIII digests were used as size standard.

Preparation of DNA from λ 41. The λ 41 phage clone was plated on *Escherichia coli* C600 (between 10^6 and 10^7 phages per plate), on L agar medium supplemented with 10 mM $MgSO_4$. After 12 h, the plates were flooded with 6 ml of SM

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TABLE 1. Bacterial strains, bacteriophages, and plasmids

Strains, bacteriophages, and plasmids	Characteristics[a]	Source or reference
Escherichia coli		
C600	F⁻ *thi thr leuB lacY1 tonA21 supE44 hsdR* λ⁻ Str^r	P. Boistard
JM83	*ara* Δ(*lac pro*) *rpsL thi*, φ80 d*lacZ*ΔM15	24
POII1734	F⁻, *araD139 ara*::(Mucts)*3* Δ(*lac*)*X74 galU galK rpsL* with Mu dII1734	6
M8820 Mu	F⁻ *araD139* Δ(*ara leu*)*7697* Δ(*proAB-argF-lacIPOZYA*)XIII *rpsL* with Mu	6
G6	Hfr *his*	14
Erwinia chrysanthemi		
3937	Wild-type, isolated from Saintpaulia	Our collection
L10	A *lacZ* derivative of 3937	14a
AD1	A *pelC* derivative of L10	This study
Bacteriophages		
λ41	λ hybrid L47-1 phage producing PLb and PLc	15
Mu dII1734	Mu cts62 dII(Km^r *lacZYA*) with Δ(Mu *A B*)*Hin*dIII	6
Plasmids		
pUC9	Ap^r, *lacZ* (α region)	24
pUV50	5.9-kb *Hin*dIII fragment from λ 41 in pUC9	This study

[a] Abbreviations: Ap^r, ampicillin resistant; Str^r, streptomycin resistant; Km^r, kanamycin resistant; PLb and PLc, isoenzymes of pectate lyase.

buffer (17) and gently agitated at 4°C for 6 h. The buffer recovered from the plates contained about 10^{11} phages per ml. DNA from the purified phages was extracted by the method of Maniatis et al. (17).

Isolation of plasmid DNA. Mini-scale preparations of *Escherichia coli* plasmid DNA were made by the method of Birnboim and Doly (3). Large-scale preparations of *Escherichia coli* plasmid DNA were made from cleared lysates followed by density gradient centrifugation in a cesium chloride-ethidium bromide as described by Maniatis et al. (17).

Transformation. *Escherichia coli* and *Erwinia chrysanthemi* transformations were performed by the method of Lederberg and Cohen (16).

Plate assay for PL activity. The method used to rapidly screen the *Escherichia coli* clones for PL production was derived from the cup-plate technique, which is based on radial diffusion of the enzymes into a substrate (sodium polygalacturonate) bearing agar slab gels (12). Colonies were suspended in drops of 10 mM $MgSO_4$, and the drops were layered onto plates containing PGT agar. After 16 to 24 h at 37°C the plates were flooded with a saturated solution of copper acetate under gentle stirring. After 5 min translucent halos appeared around PL-producing clones.

Characterization of PL produced by the clones. The PL isoenzymes were measured in culture supernatants and whole cell extracts of stationary-phase cells. Whole cell extracts were prepared as follows: 1.5 ml of stationary-phase cultures was centrifuged and the pellets were suspended in 0.25 ml of double-distilled water. Lysozyme was added to a final concentration of 100 μg/ml. The mixture was incubated for 5 min at 4°C, and 25 μl of 250 mM EDTA was added. Samples of 10 to 20 μl from the culture supernatant or from the whole cell extracts were layered on an ultrathin polyacrylamide gel, and electrofocusing was performed in a gradient of pH 3 to 9.5. The PL activities were developed directly on the gel by a procedure described by Bertheau et al. (2).

β-Galactosidase activity. The β-galactosidase activity was measured, and units were calculated as described by Miller (18). For *Escherichia coli* G6, the β-galactosidase activity was measured in cultures induced with 1 mM isopropyl-β-D-thiogalactoside.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Samples of 35 to 50 μl from whole cell extracts of Lac⁺ mutants, prepared by the method of Silhavy et al. (21), were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 7% acrylamide–0.19% bisacrylamide gels. Gels were prepared and developed by the method of Pugsley and Schnaitman (19).

RESULTS

Subcloning of the *pelC* gene in plasmid pUC9. The DNA of the transducing phage λ 41 that produces the two major isoenzymes PLb and PLc (15) was purified and digested with *Hin*dIII. The resulting fragments were ligated with plasmid pUC9 linearized with *Hin*dIII and used to transform *Escherichia coli* JM83. Transformants harboring a recombinant plasmid were scored as white clones when plated on L agar medium supplemented with ampicillin and Xgal. About 2% of the transformants were recombinant clones and 17 of them produced a PL activity when patched on PGT medium (Fig. 1). All these clones were able to produce only the PLc

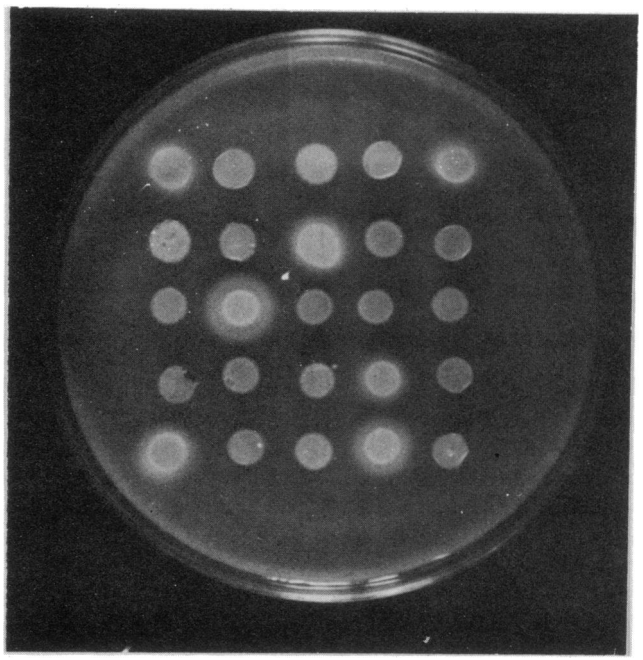

FIG. 1. Several randomly selected pUC9 recombinant clones plated on PGT agar containing 50 μg of ampicillin per ml. Seven PL-producing subclones were identified by the halos surrounding the colonies as described in the text.

isoenzyme, as was shown by electrofocusing of the whole cell extracts in polyacrylamide gels. The electrofocusing zymogram of the Pel$^+$ recombinant clone pUV50 is shown in Fig. 2.

Chimeric plasmids carrying the 5.9-kilobase (kb) *Hin*dIII fragment in the opposite orientation showed similar expression of *pelC* (data not shown). This result suggests that the *pelC* gene present in the cloned fragment is expressed from its own promoter.

An analysis of the deletion plasmids (Fig. 3) shows that *pelC* is encoded within the 2.4-kb *Hin*dIII-*Pst*I fragment and probably has a *Sma*I site within the structural gene. Further analysis of the 1.8-kb *Bam*HI-*Pst*I fragment showed that this fragment expressed *pelC* when cloned into the polylinker of pUC9 but only in one orientation. We conclude that in pAD50 the *pelC* promoter region is contained at least in part in the 590-base-pair *Hin*dIII-*Bam*HI restriction fragment and that the *pelC* gene is transcribed from the *Hin*dIII cloning site toward the *Pst*I site.

***pelC* insertion mutagenesis and *pelC-lacZ* gene fusion with Mu dII1734.** Insertion mutagenesis of the *pelC* gene and *pelC-lacZ* gene fusion were performed by using the Mu-derived bacteriophage transposon Mu dII1734 (Km^r *lacZYA*) by the procedure described by Castilho et al. (6). Phage Mu particles containing Mu dII1734 insertions on pAD50 were generated by inducing the helper phage Mu *c*ts62 in the Mu dII1734 lysogenic strain (POII1734) which was previously transformed with pAD50. Such insertions were selected by transducing the ΔLac *Escherichia coli* M8820 Mu with the resulting lysate. Infection of strain M8820 Mu resulted in the appearance of 2% strong blue transductant clones on Xgal L agar medium containing ampicillin and kanamycin. Of 30 Lac$^+$ transductants tested for PL activity, 12 were found to have acquired the Pel$^-$ phenotype. These 12 clones were expected to contain pAD50 with a Mu dII1734 inserted in the *pelC* locus. Restriction analysis with endonucleases *Hin*dIII, *Pst*I, and *Bam*HI of plasmid DNA isolated from these 12

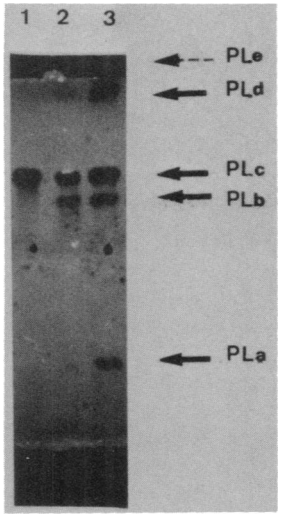

FIG. 2. Electrofocusing of the PLc-producing subclone in a thin polyacrylamide gel. The visible bands on the gel are due to the activity of the PL isoenzymes, revealed by the sandwich technique (2). Lane 1, 10 μl of whole cell extract of the PL-positive subclone harboring pUV50; lane 2, 20 μl of lysate of λ 41 phages; lane 3, 20 μl of 3937 culture supernatant (it should be noted that, in this gel, the PLe activity of pI higher than 9.5 is not visible). Phage lysate and whole cell extract were prepared as described in the text.

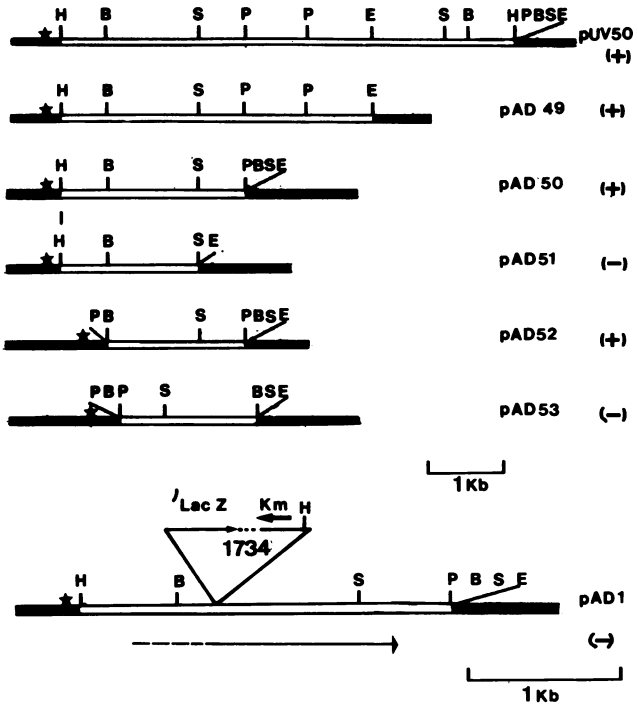

FIG. 3. Restriction maps of hybrid plasmids constructed in this study. *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sma*I restriction sites are indicated respectively by B, E, H, P, and S. Double lines are the inserted DNA segments, and heavy lines are vector pUC9. Stars represent the pUC9 *lac* promoter. pUV50 contains a 5.9-kb *Hin*dIII fragment from λ 41. pAD49, pAD50, and pAD51 are, respectively, *Eco*RI, *Pst*I, or *Sma*I deletions of pUV50. pAD52 and pAD53 are two *Bam*HI subclones (in opposite orientations) of pAD50 in pUC9. pAD1 is pAD50 with Mu dII1734 inserted in the 1.8-kb *Bam*HI-*Pst*I fragment. 1734 represents Mu dII1734 (not drawn to scale). The arrows indicate the orientation of transcription of the indicated genes. The approximate location of the *pelC* promoter is indicated by dotted lines. (+) or (−) indicate that the hybrid plasmid does or does not encode PLc.

clones showed that a Mu dII1734 insertion was present for 2 clones in nearly the same location in the 590-base-pair *Hin*dIII-*Bam*HI fragment, and for the other 10 clones the insertion was present in nearly the same location inside the 1.8-kb *Bam*HI-*Pst*I fragment. A more precise study of one of these last plasmids (pAD1) indicates that the Mu dII1734 insertion was located 240 base pairs after the *Bam*HI site (Fig. 3).

Expression of the hybrid gene in *Escherichia coli* was examined by analysis of β-galactosidase activity in strain M8820 Mu harboring pAD1. This strain makes 11,100 U of β-galactosidase, which is similar to the amount made in a wild-type *Escherichia coli* strain when fully induced. Inversion of the *Hin*dIII fragment of pAD1 containing the putative hybrid gene and the kanamycin determinant (plasmid pAD2) did not alter the Lac$^+$ character, indicating that expression of the *pelC-lacZ* hybrid gene was independent of the pUC9 *lac* promoter. Furthermore, an apparent hybrid β-galactosidase of 120,000 daltons is made by pAD1 or pAD2 (Fig. 4).

Transfer of the *pelC-lacZ* hybrid gene on the *Erwinia chrysanthemi* chromosome. Since some pBR322 derivatives have been shown to be unstable in *Erwinia chrysanthemi* 3937 (D. Expert, personal communication), it might be expected that after introduction of plasmid pAD1 in this

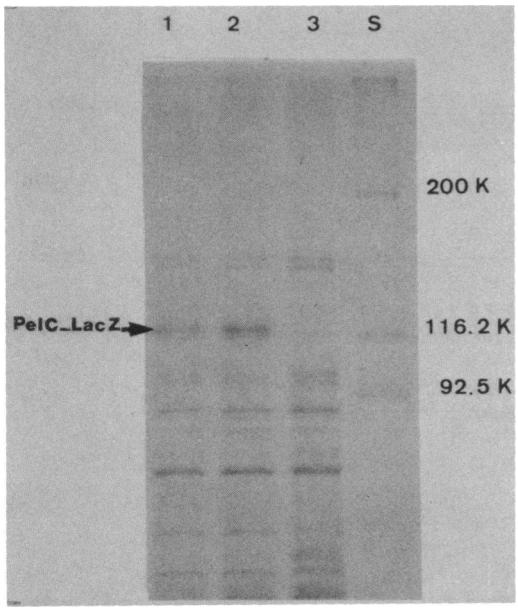

FIG. 4. Gel analysis of PelC-LacZ hybrid protein in *Escherichia coli*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7% acrylamide–0.19% bisacrylamide was carried out with 30-μl samples of cell extracts prepared by the method of Silhavy et al. (21). The strains were grown on minimal medium (M9 with glycerol as carbon source) supplemented with leucine and proline until the cultures had an optical density of 0.3 at 600 nm. Lanes 1 and 2, M8820 Mu strains transformed with, respectively, pAD2 (i.e., pAD1 with the *Hin*dIII fragment in opposite orientation) and pAD1; lane 3, Δ*lac* parental strain M8820 Mu. The apparent molecular sizes of protein standards (lane S) are indicated in kilodaltons (K).

strain, homologous recombinations would exchange the chromosomal wild-type *pelC* gene by its mutant allele in pAD1. Therefore, a Lac^- derivative of strain 3937, L10 (14a), was transformed with pAD1, and transformant clones were selected on Xgal L agar medium supplemented with kanamycin. All the Km^r transformants obtained were Lac^+ and Ap^r. One of these transformants clones was subcultured on kanamycin-containing medium, and an Lac^+ ampicillin-sensitive segregant was isolated (strain AD1). To verify that the Mu dII1734-induced mutation was indeed introduced in the chromosomal *pelC* locus, PL activities produced by strain AD1 were analyzed by electrofocusing as described above. A PL activity band corresponding to the PLc is clearly absent both in the whole cell extract and in the culture supernatant (Fig. 5). These results strongly suggest that mutant AD1 carries a Mu dII1734 insertion in the chromosomal *pelC* locus, resulting in a *pelC-lacZ* gene fusion.

Regulation of the hybrid *pelC-lacZ* gene expression in *Erwinia chrysanthemi* mutant strain AD1. In *Erwinia chrysanthemi*, PL synthesis is induced by the addition of polygalacturonate to the culture medium (7, 10, 11). Thus, the expression of the hybrid *pelC-lacZ* gene was studied by assaying β-galactosidase activity of the mutant strain AD1 upon induction with polygalacturonate.

For the AD1 ancestor strain (strain L10), another gene *lacB* is responsible for a low β-galactosidase level (10 U) which increases 4.7-fold when polygalacturonate is present in the culture medium. This result suggests that in *Erwinia chrysanthemi* the *lacB* gene is weakly induced by polygalacturonate or, most probably, by intermediates of pectate catabolism. A difference of β-galactosidase activity was detected between mutant strain AD1 and parental strain L10. This difference is probably due to the expression of the *pelC-lacZ* hybrid gene present in strain AD1. Expression of the hybrid gene corresponds to an approximate value of 4 U of β-galactosidase in minimal medium and to a value of 153 U of β-galactosidase when polygalacturonate is added to the minimal medium. From these results it can be infered that the *pelC-lacZ* gene expression is induced 37-fold by polygalacturonate, or by its degradation products, or both.

DISCUSSION

The *pelC* gene which encodes one of the two PL isoenzymes PLb and PLc of *Erwinia chrysanthemi* 3937, present in the transducing phage λ 41 (15), was subcloned in the multicopy plasmid pUC9. The cloned gene was normally expressed in *Escherichia coli* giving rise to active PL isoenzyme (with pI of 8.3 [2]) independent of its orientation in the cloning vector. This shows that the transcriptional and translational machineries of *Escherichia coli* recognize the DNA and RNA sequences that control gene expression in *Erwinia chrysanthemi*. Subclones producing the PLb isoenzyme were not isolated because of the presence of an *Hin*dIII restriction site inside the *pelB* locus (unpublished data).

Mini-Mu-*lac* insertion mutagenesis in the *pelC* gene was obtained in *Escherichia coli*. A resulting PelC-LacZ hybrid protein with an apparent molecular weight of 120,000 was detected in *Escherichia coli*. The *pelC* mutant allele was incorporated into the chromosome of *Erwinia chrysanthemi* 3937, and a mutant lacking the *pelC* protein was obtained.

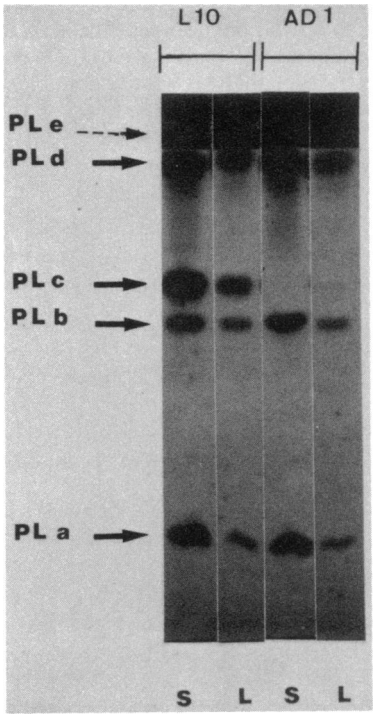

FIG. 5. Electrofocusing of PL produced by *Erwinia chrysanthemi* mutant strain AD1. Bacterial cultures were performed in glycerol minimal medium M9 supplemented with 0.5% (wt/vol) of sodium polygalacturonate. Abbreviations: S, culture supernatants; L, whole cell extracts. Cell extracts were prepared as described in the text. PLc is absent in strain AD1 both in the culture supernatant and in cell extracts.

This is the first report of insertion mutagenesis in a structural gene encoding a PL isoenzyme.

In *Erwinia chrysanthemi* induction by polygalacturonate requires the formation of intermediates of intracellular pectate catabolism and depends on oligogalacturonide lyase activity (10). In the *pelC*$^-$ mutant strain, *pelC-lacZ* hybrid gene expression appeared to be induced in the presence of sodium polygalacturonate. This provides a convenient system to study the regulation of the *pelC* gene, one of the five genes involved in pectinolysis. Results presented here suggest that *pelC* is induced 37-fold by the addition of polygalacturonate. Given the uncertainity in the determination of the uninduced basal activity and the presence of low levels of the interfering *lacB* activity, this factor is a first crude estimate for the induction factor of the *pelC* gene.

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