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Mu Insertion Directed Mutagenesis In Two Pectate Lyase Genes of *Erwinia chrysanthemi*

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Abstract

The *pelC* gene, which encodes one of the five major pectate lyase (PL) isoenzymes in *Erwinia chrysanthemi* 3937, designed PLc, was mutagenized with a mini-Mu-*lacZ* transposable element able to form fusions to the *lacZ* gene. This *E. chrysanthemi* strain lacks the PLc isoenzyme and has a Lac+ phenotype that is inducible by polygalacturonate, as is the set of PL activities in *E. chrysanthemi*. The *pelB* gene, which encodes the PLb major isoenzyme was mutagenized with a mini-Mu transposable element that can form gene fusions to the neomycin phosphotransferase-encoding region. Secondary mutants resistant to kanamycin in the absence of polygalacturonate were selected. Such mutants produced other pectate lyase isoenzymes in the absence of the inducer. Finally, a mutant strain both lacking the PLb and Plc isoenzymes was obtained.

Keywords: *Erwinia chrysanthemi*, pectate lyase gene, gene fusions, mutagenesis

Abbreviations: Pectate lyase: PL; kanamyan resistance: Km^R, Chloramphenicol resistance: Cm^R

1. Introduction

Erwinia chrysanthemi is an enterobacterium responsible for soft-rot of many plants. In infected plants as well as in culture supernatants, the bacterium produces pectinases, cellulases and proteases that depolymerize various plant cell wall components. These enzymes are secreted by *E. chrysanthemi* and recovered in the culture supernatant (Andro et al., 1984). The pectate lyase activity responsible for the degradation of the pectic components

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Table 1.

Mini-Mu	Characteristics and references
MudIII734 (Km^R, *'lacZYA*)	confers kanamycin resistance (Km^R) able to form translational *lacZ* gene fusions encoding functional hybrid galactosidase. (Castitilho et al., 1984)
MudIIPR13 (Cm^R, *'lacZYA*)	confers chloramphenicol resistance (Cm^R) able to form translational *lacZ* gene fusions. P. Ratet, unpublished results.
MudIIPR3 (Cm^R, *'nptI*)	confers chloramphenicol resistance (Cm^R) able to form translational *nptI* (neomycin phosphotransferase 1 encoding region) gene fusions, conferring a Km^R phenotype (Ratet and Richaud, 1986)

present in plant cell walls appears to be essential for plant tissue maceration. In strain 3937, pathogen to *Saint-paulia ionantha*, five major PL activities were detected by electrofocusing in ultrathin polyacrylamide gels (Bertheau et al., 1984). Five different genes (*pelA*, *pelB*, *pelC*, *pelD* and *pelE*) encoding the five PL isoenzymes (PLa, PLb, PLc, PLd and PLe) were cloned and were localized at two different regions of the chromosomal map (Kotoujansky et al., 1985). To understand the role played by these isoenzymes in the pectinolysis and in the pathogenicity of *Erwinia chrysanthemi*, insertion-directed mutagenesis of two PL-encoding structural genes was performed using different mini-Mu transposable elements.

2. Materials and Methods

Bacteriophages Bacteriophage Mu-derived transposons used in this work are described in Table 1.

Media

The following media were used: L broth rich medium and M9 minimal medium containing 2 g of glycerol per liter (M9Y medium). When required, the media were solidified by using Difco agar (15 g per liter). For the pectate lyase production, M9Y medium was supplemented with 5 g of sodium polygalacturonate per liter. To score LacZ+ clones, the β-galactosidase chromogenic substrate 5-bromo-4-chloro-3-indolyl- D-galactoside (Xgal) was added to the L broth agar medium at a final concentration of 40μg/ml.

For *E. chrysanthemi*, all incubations were carried out at 30°C.

Characterisation of PL produced by the clones

The PL Risoenzymes were measured in culture supernatants and whole cell extracts of stationary phase cultures. Whole cell extracts were prepared as follows: 1:5 ml of stationary-phase cultures were centrifuged and the pellets were resuspended in 0.25 ml of double distilled water. Lysozyme was added to the final concentration of 100μg/ml. The mixture was incubated for 5 min at 4°C and 25μl of 250 mM EDTA was added. Samples of 10 to 20μl from the culture supernatant or from the whole cell extracts were layered on a ultrathin polyacrylamide gel and electrofocusing was performed in a 3–9 pH gradient. The PL activities were developed directly on the gel according to the procedure of Bertheau et al. (1984).

3. Results

Mu insertion directed mutagenesis in pelB and pelC genes

Erwinia chrysanthemi pelB or *pelC* genes were cloned and mutagenized in *E. coli* with 3 different Mu bacteriophage derived transposons by the procedure described by Castilho. Mini-Mu used (a) lack the Mu transposase genes thus making inserts that cannot further transpose unless complemented with the Mu transposase genes, (b) are able to form translational gene fusion through a transposition event when inserted into a target gene in the proper orientation and phase (Castilho et al., 1984; Ratet and Richaud, 1986).

Hybrid plasmid bracketed by two mini-Mu transposons in direct orientation were generated and packaged by inducing the helper phage Mucts in a *E. coli* JM108-derived, hybrid plasmid-carrying strain lysogenic for mini-Mu and Mucts62 (Ratet and Richaud, 1986). Resulting lysates were used to transduce the *E. coli* M8820 strain (Castilho et al., 1984) and tansductants were selected on L-broth medium containing appropriate antibiotics. Four different plasmids were selected: pAD1 and pAD2 with the *pelC* gene inactivated by the mini-Mu insertion and expressing the mini-Mu *lacZ* gene; pAD3 with an inactivated *pelB* gene and a Lac$^+$ phenotype and pAD7 which has an inactivated *pelB* gene and a Km^R phenotype by expressing the mini-Mu *nptI* gene. Plasmids pAD1, pAD3 or pAD7 was then introduced into *E. chrysanthemi* and the *pelC*::mini-Mu or the *pelB*::mini-Mu insertion was substituted for the chromosomal allele by homologous recombinations. Four

different mutant strains were obtained (AD1, AD3, AD8, AD9, Fig. 1). The mutant strain ADI lacks the PLc isoenzyme. This strain carries a MudII1734 insertion in the *pelC* locus resulting in a *pelC::lacZ* gene fusion. The mutant strain AD3 lacks the PLb isoenzyme and carries a MudIIPR13 insertion in the *pelB* locus resulting in a *pelB::lacZ* gene fusion. The mutant strain AD8 lacks the PLb isoenzyme and carries a MudIIPR3 insertion in the *pelB* locus resulting in a *pelB::npt1* gene fusion.

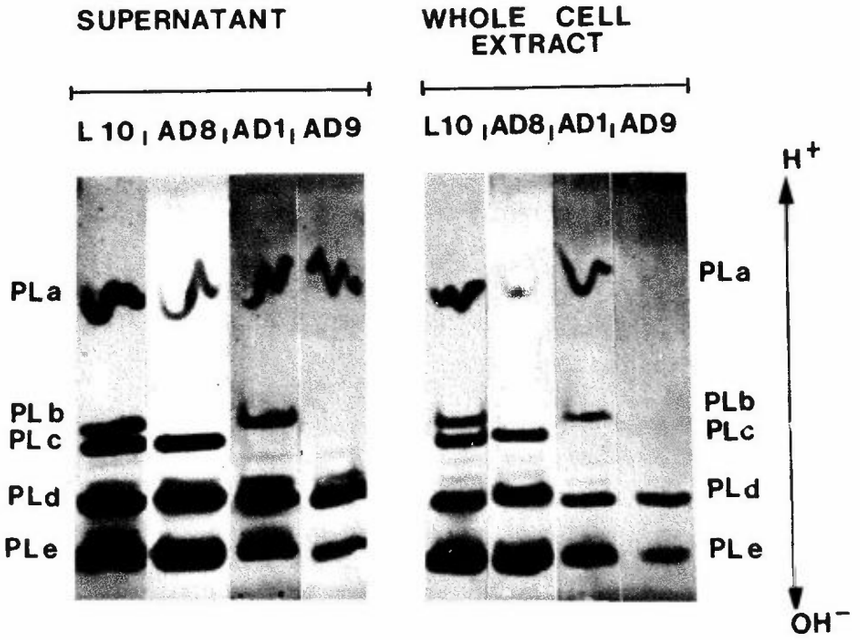

Figure 1. Electrofocusing of PL produced by *E. chrysanthemi* mutant strains. The visible bands on the gel are due to the activity of the PL isoenzymes revealed by the sandwich technique (Bertheau et al., 1984). L10 is a *lacZ* derivative of 3937. PL a, b, c, d, and e refer to the locations of the corresponding PL activity. PLc is absent from both culture supernatants and cell extracts in strain AD1 and AD9). PLb is absent from both compartments in strain AD8 and AD9.

The PelC$^-$ mutant strain was transformed with plasmid pAD3 containing the *pelB::lacZ* hybrid gene. After homologous recombination events with the

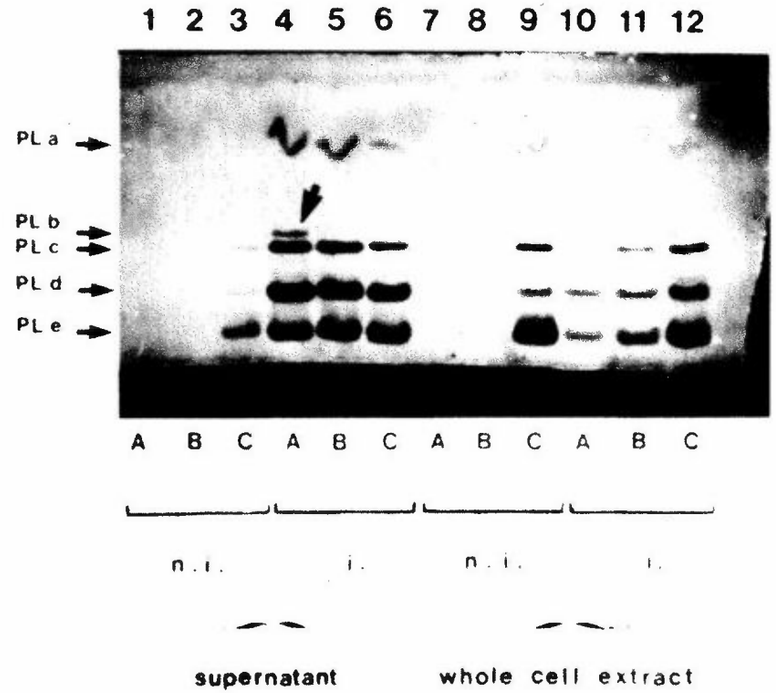

Figure 2. Electrofocusing of pectate lyase activities produced by *E. chrysanthemi* strains. Strains were growing M9Y (noninduced [n.i.] conditions) or M9YPG (induced [i] conditions). Lanes A, B, C, correspond to strains L10, AD8, and AD81. The arrow in lane 4 points to the PLb. Supernatants and whole cell extracts were analyzed by electrofocusing as described by Bertheau et al. (1984). The regulatory mutant strain (strain AD81) produce PLa, PLc, PLd, PLe in the supernatant and in cell extract in the absence of the inducer.

chromosomal *pelB* locus, a mutant both lacking the PLb and PLc isoenzymes was selected (strain AD9).

pelB regulatory mutants of E. chrysanthemi

In *E. chrysanthemi*, PL synthesis is induced by the addition of polygalacturonate in the culture medium. The mutant strain AD8 carrying a chromosomal *pelB::npt1* gene fusion has a kanamycin resistant phenotype that is inducible by polygalacturonate. This result suggests that the *pelB::npt1* hybrid gene is sensitive to regulation as is the wild type *pelB* gene.

Secondary mutants resistant to kanamycin (100μg/ml) in the absence of polygalacturonate were selected. One of these, AD81, was chosen for further studies. In AD81 (Fig. 2), intracellular and extracellular pectate lyase a, c, d, and e isoenzymes were detected without induction by polygalacturonate (lanes 3 and 9). Under the same conditions, these activities were barely detectable in the parent strain L10 and AD8 (lanes 1, 2, 7, and 8).

The existence of constitutive mutants producing four different isoenzymes suggests that a *trans*-acting regulatory gene has been altered in strain AD81 since the structural chromosomal genes for the PL a, c, d, and e isoenzymes are at different locations and all were constitutively expressed in the absence of the inducer. This is the first report of pleiotropic constitutive mutants that indicates at least one common regulatory gene for expression of *pel* genes in *E. chrysanthemi*.

4. Conclusion

Insertion-directed mutagenesis in two pectate lyase genes of *Erwinia chrysanthemi* was obtained using mini-Mu transposable elements able to form gene fusions. This provides a convenient system:

- to study the role of these 2 different isoenzymes in phytopathogenicity
- to independently study regulation of each of these 2 genes by measuring the expression of the chromosomal hybrid gene encoding β-galactosidase activity.

Furthermore, a pleiotropic constitutive mutant producing 4 different pectate lyases was obtained, indicating there is at least one common regulatory gene for expression of *pel* genes in *Erwinia chrysanthemi*.

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