Pathogenic Behavior of Pectinase-Defective *Erwinia chrysanthemi* Mutants on Different Plants

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The pathogenic behavior of *Erwinia chrysanthemi* strain 3937 and of several pectinase-defective mutants was analyzed on four different plants. The ΔPel mutant displayed a reduced virulence on all host plants tested. However, the individual *pel* and *pem* genes had, within host plants, a differential role in pathogenicity. On *Saintpaulia ionantha,* the genes of the *pelADE* cluster and the *pem* gene were essential for a full expression of *E. chrysanthemi* virulence, whereas the genes of the *pelBC* cluster did not seem to be involved in pathogenicity. On pea, only the PelA⁻ mutant exhibited a reduced virulence, and a mutant deleted of all the *pel* genes except *pelA* (PelBCDE⁻) was as virulent as the wild-type strain. On chicory leaf, the *pem* gene and all the *pel* genes except *pelE* were needed for virulence. On potato tuber, only the Pem⁻ mutant showed a reduced virulence. In addition to PLa, PLb, PLc, PLd, and PLe, we showed that another set of pectinases was produced *in planta.* Pectinases present in macerated tissues varied within host plants. This study brings strong evidence for a contribution of the different enzymes to the wide host range of strain 3937.

Erwinia chrysanthemi is a plant-pathogenic entorobacterium that causes soft rot on harvested crops and on growing plants. However, this species exhibits a certain degree of host specificity (Dickey 1979, 1981; Janse and Ruissen 1988); consequently, restriction fragment length polymorphism analysis allows the classification of *E. chrysanthemi* species based upon their pathogenic behavior on host plants (Boccara *et al.* 1992).

To efficiently macerate a host plant, *E. chrysanthemi* produces a set of depolymerizing enzymes such as pectinases, cellulases, and proteases (Kotoujansky 1987). Pectate lyases (PL), which cleave the α1,4-glycosidic bond in the pectin chain, play a central role in pathogenicity. In the presence of polygalacturonic acid, most *E. chrysanthemi* strains synthesize five PL isoenzymes (PLa, PLb, PLc, PLd, and PLe). The *pel* genes encoding these five isoenzymes are organized in two clusters on the bacterial chromosome. The *pelA, pelD,* and *pelE* genes are organized on one cluster, whereas the *pelB* and *pelC* genes are on the second one. The role of these individual *pel* genes in pathogenicity has been analyzed in *E. chrysanthemi* strain 3937, a strain isolated from *Saintpaulia ionantha.* Mutations in the *pelA, pelD,* and *pelE* genes result in a significant reduction of virulence on *S. ionantha,* although mutations in the *pel* genes of the second cluster do not alter bacterial virulence on the same plant (Boccara *et al.* 1988). Another depolymerizing enzyme, the pectin methylesterase (PME), removes the methoxyl groups from the pectin. It is also essential to bacterial infection on *S. ionantha* as mutants defective in the production of PME show a reduced virulence on *S. ionantha* (Boccara and Chatain 1989). Moreover, other pectinases could be involved in pathogenicity. Collmer *et al.* (1991) have recently identified a new set of pectinases that are produced during the growth of *E. chrysanthemi* strain EC16 on plant material.

The purpose of this study was to analyze the function of pectinases on different hosts. Pathogenicity tests involving several pectinase-defective mutants provide a first insight into the molecular basis of host specificity in *E. chrysanthemi.* The results strongly suggest that the different pectinases contribute to the wide host range of strain 3937.

RESULTS AND DISCUSSION

Pathogenic behavior of pectinase mutants on plants.

The virulence of mutants affected in pectinase production (Pel⁻ and Pem⁻ mutants) was tested on four different plant systems: two isolated plant organs—potato tuber and etiolated witloof chicory leaf—and two *in vitro*-grown plantlets—*Pisum sativum* and *S. ionantha.* The results of these pathogenicity tests are presented in Table 1. Only the ΔPel mutant displayed a reduced virulence on all host plants tested. However, mutations in the individual *pel* and *pem* genes differentially affected the expression of bacterial virulence within host plants. On potato tuber, only the Pem⁻ mutant showed a reduced virulence. On chicory leaf, the *pem* gene and all the *pel* genes except *pelE* were needed for virulence. On *S. ionantha,* as already described (Boccara *et al.* 1988; Boccara and Chatain 1989; Aymeric *et al.* 1989), the genes of the *pelADE* cluster and the *pem* gene were essential for a full expression of *E. chrysanthemi* virulence, whereas the genes of the *pelBC* cluster did not seem to be involved in pathogenicity. On

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pea, only the PelA$^-$ mutant exhibited a reduced virulence. A mutant deleted of all the *pel* genes but *pelA* (PelBCDE$^-$) was as virulent as the wild-type strain.

The potato tuber model.

All mutants deficient in the production of a single pectinase, except the PEM$^-$ mutant, efficiently macerated potato tuber. Other studies have shown that mutants of *E. chrysanthemi* impaired in virulence on whole plants cannot be discriminated by inoculation on potato tubers (Beaulieu and Van Gijsegem 1990, 1992). Moreover, inoculation on potato tuber was also unsuccessful in identifying mutants with reduced virulence in a related species, *E. carotovora* subsp. *atroseptica,* which causes black leg of potato (Hinton *et al.* 1989). Hinton *et al.* (1989) isolated Pep$^-$ mutants of *E. carotovora* subsp. *atroseptica* which were reduced both in the production and secretion of pectinases. These Pep$^-$ mutants showed a reduced virulence on potato plants but were not impaired in their ability to macerate potato tubers. This lack of discrimination is not due to the fact that the potato tuber is a plant organ, since other plant organs such as witloof chicory leaf proved to be good systems to quickly identify most mutants impaired in virulence functions (Beaulieu and Van Gijsegem 1992).

The chemical composition of a particular plant species probably contributes to the outcome of the interaction between the host and the bacteria. The reduced virulence on potato tuber of a mutant impaired in PME production might reflect this phenomenon. Indeed, tubers produced by hybrid plants derived from a protoplasmic fusion between cells of *Solanum tuberosum* and *S. brevidens* were analyzed for resistance to *E. carotovora.* The hybrids showing a resistant phenotype were found to have a higher degree of pectin methylation (Hedley and Pérombelon 1989).

Differential behavior of the pectinase mutants on other plants.

The pathogenicity tests carried out on chicory leaf as well as on *S. ionantha* and on pea plantlets allowed us to classify the mutants according to their pathogenic behavior. Only the mutant defective in the production of PLa exhibited a reduced virulence on the three host plants. Thus, the *pelA* gene appears to be the only *pel* gene to be involved in a general mechanism of virulence. Nevertheless, PLa has no macerating activity on plant tissues (Barras *et al.* 1987); PLa is produced in small amounts in minimal media supplemented with polygalacturonate, and it is responsible for only a small fraction of the total PL activity produced under this condition (Reverchon *et al.* 1986). Futhermore, the *pelA* gene expression is almost not induced by the addition of polygalacturonate (Reverchon *et al.* 1986). Recent study on depolymerization mechanisms of PL_s secreted by *E. chrysanthemi* showed that conversion of polygalacturonate to unsaturated oligomers by PLa is limited compared with conversion by the other PL isoenzymes (Preston *et al.* 1992). Preston *et al.* (1992) suggest that this limited conversion may reflect a preference of PLa for polymers over oligomers; this preference of PLa to degrade polymers could explain the importance of this enzyme in pathogenesis that would act at the early stages of infection.

The Pem$^-$ and the other Pel$^-$ mutants displayed a reduced virulence on either one or two of the host plants tested. Comparisons of the effects of PelD$^-$ and PelE$^-$ mutations are especially interesting since the sequences of the *pelD* and *pelE* structural genes show high homology (Van Gijsegem 1989). Despite this homology, insertion mutations into the *pelD* and *pelE* genes lead to different alterations in virulence. Whereas an insertion into the *pelD* gene had a negative effect on the bacterial ability to macerate chicory leaf and no effect on the ability to macerate potato tuber, an insertion into the *pelE* gene resulted in an increase of bacterial virulence on these plant organs. The upstream noncoding sequences of the *pelD* and *pelE* genes are divergent though three domains are conserved in the promoter region of the *pelD* and *pelE* genes (Van Gijsegem 1989; Reverchon *et al.* 1989). Moreover, the *pelE* promoter is complex and interacts with several regulatory proteins (Gold *et al.* 1992). Consequently the different pathogenic behaviors of the pectinase-defective mutants could result from sequential, differential, or interdependent expression of the *pel* genes within the plant species. To test this hypothesis, the PL content of fluids recovered from plant tissues in late phases of infection was analyzed by electrofocusing. The predominant PL isoenzymes in fluids from plant tissues infected by strain 3937 were actually found to be different within plant species. In potato and pea tissues, the five PL isoenzymes could be visualized

Table 1. Ratio of virulence between the mutants and the wild-type strain 3937

		Ratio of virulence efficiency on							
		Saintpaulia ionantha[a] (χ^2 Value)		*Pisum sativum*[a] (χ^2 Value)		Potato tuber[b] (*F* Value)		Chicory leaf[c] (*F* Value)	
Strain	Phenotype								
PMV4066	PelA$^-$	0.41	(16.70)*	0.47	(9.18)*	0.84	(1.78)	0.85	(7.50)*
AD(PMV4120)	PelBC$^-$	1.10	(0.38)	0.87	(0.53)	0.97	(0.01)	0.71	(22.85)*
PMV4070	PelD$^-$	0.47	(11.54)*	1.07	(0.12)	0.84	(2.50)	0.77	(19.15)*
PMV4071	PelE$^-$	0.19	(37.17)*	0.73	(2.18)	1.53	(20.83)*	1.22	(16.66)*
PMV4116	ΔPel	0	(201.80)*	0.41	(6.94)*	0.72	(11.00)*	0.69	(34.28)*
PMV4122	PelBCDE$^-$	ND[e]	ND	0.94	(0.28)	ND	ND	ND	ND
PMV4106	Pem$^-$	0.11	(70.62)*	0.77	(1.66)	0.74	(7.69)*	0.91	(2.45)

[a] Strain 3937 caused systemic disease on 18 out of 30 plants.
[b] The average weight of rotten tissues caused by strain 3937 was 16 g (18 repetitions).
[c] The average length of macerated tissues caused by strain 3937 was 2.6 cm (30 repetitions).
[d] Asterisks indicate values statistically different from the wild-type value ($P < 0.05$).
[e] Not determined.

without concentrating plant fluids. The amount of PLa appeared low when compared to the amounts of other PL isoenzymes. In fluids from infected *S. ionantha,* PLe could not be detected and the amount of PLa was small. In chicory leaf, only PLa and PLd were present in significant quantities (Fig. 1). Although predominant PLs in macerated tissue differed within plant species, little correlation between the preponderance of a particular enzyme in the late phase of infection and its significance in symptom development could be established. However, the abundance of an enzyme may not directly reflect its impact in pathogenesis. It is also possible that some enzymes are produced in early stages of the disease but are lost during late phase of infection. The kinetics of PL production *in planta* remains to be analyzed.

Residual pectinase activity in the ΔPel mutant.

It was shown by hybridizing the genome of the ΔPel mutant to plasmid DNA carrying the *pelADE* and *pelBC* clusters that PMV4116 was deleted of the *pelA, pelB, pelC, pelD,* and *pelE* genes. This mutant retained in part the ability to macerate plant organs. The maceration ability of the mutant on isolated organs was still unexpectedly strong (between 69 and 72% of the wild-type activity). A similar observation was reported by Ried and Collmer (1988) for a mutant of *E. chrysanthemi* strain EC16 that was deleted of all the structural *pel* genes. Pectinases such as exopolygalacturonase and exopolygalacturonate lyase have been characterized in *E. chrysanthemi* strain EC16 (Collmer and Keen 1986; Brooks *et al.* 1990; He and Collmer 1990) but no evidence was found for a role of these enzymes in maceration. However, Collmer *et al.* (1991) have recently identified a new set of PL_s which are produced by *E. chrysanthemi* strain EC16 only when the bacteria are grown on plant material or in the presence of plant extracts. Maceration caused by PMV4116, the ΔPel mutant of strain 3937, probably resulted from the action of the plant-inducible pectinases. To estimate PL activity *in planta,* fluids recovered from macerated plants was added to polygalacturonate, and the liberation of unsaturated uronides was measured spectrophotometrically. PL activity was relatively high in fluids from potato (1.5 U/ml) and chicory tissues (0.75 U/ml) macerated with the ΔPel mutant; the level of PL activity accounted for approximatively one third of the activity measured in tissues macerated with the wild-type strain. Compared with PL activity *in planta*, PL activity in the supernatant from cultures of the ΔPel mutant grown in the presence of succinate as the sole carbon source was very low (0.01 U/ml) and no pectate lyase could be detected by electrofocusing even when the supernatant was 10 times concentrated (data not shown). The amount of PL activity measured in supernatants of the wild-type strain grown in the same conditions was at least 25 times greater. PL activity in the wild-type strain increased about six times after addition of polygalacturonate. In contrast, there was no increased of PL activity in the supernatant of the ΔPel mutant grown in the presence of polygalacturonate (Table 2).

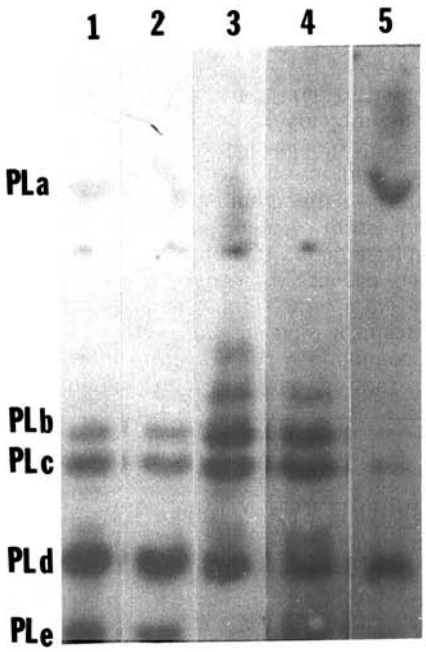

Fig. 1. Electrofocusing on a thin polyacrylamide gel of 3937 culture supernatant (lane 1) and of plant fluids from potato tuber (lane 2), *Saintpaulia ionantha* plantlet (lane 3), pea plantlet (lane 4), and witloof chicory leaf (lane 5) infected by the wild-type strain 3937.

Fluids from potato and chicory tissues infected by the ΔPel mutant were analyzed by electrofocusing. None of the five activity bands detected in the supernatant of the wild-type strain was present, but other bands were detected (Fig. 2). However, the position of the new bands differed depending on the plant fluids analyzed. This suggests a differential production of plant-inducible pectinases within host plants. The role in the pathogenicity process of these plant-inducible pectinases is not established yet. However, the failure of a ΔPel mutant to cause systemic disease on whole plants suggests that the sole production of these enzymes is not sufficient to allow the invasion of a whole plant by the bacteria. Interestingly, the pectinases encoded by these plant-inducible genes are likely to be exported from the bacterial cell by the same secretion mechanism that drives the export of other PL isoenzymes. Indeed, an Out^- mutant that was shown to be deficient in the secretion of cellulases and of PLa, PLb, PLc, PLd, and PLe induced no symptoms on plant organs (Beaulieu and Van Gijsegem 1992).

This study shows that the role of the different pectinases in pathogenesis is host dependent, and it suggests an in-

Table 2. Comparison of pectate lyase (PL) activity in culture supernatants and in plant fluids between *Erwinia chrysanthemi* strains 3937 and PMV4116

	PL activity (U/ml)			
Strains	**Succinate[a]**	**Poly-galacturonate[b]**	**Potato tissues**	**Chicory tissues**
3937	0.25[c]	1.60	4.30	2.44
PMV4116	0.01	0.01	1.50	0.75

[a] Bacteria were grown in minimal containing succinate as the sole carbon source.
[b] Bacteria were grown in minimal containing succinate and polygalacturonate as carbon source.
[c] Values are the mean of three repetitions.

volvement of pectinases in host specificity. The multiple pectinases undoubtedly provide an advantage to the bacterium by furnishing a considerable amount of nutrients derived from the degradation of plant cell wall constituents. However, this multiplicity of pectinases, whose production can be modulated by specific hosts, might also reflect the evolutionary response of the bacterium to the wide diversity of plant species.

MATERIALS AND METHODS

Bacterial strains.

Bacterial strains used in this study are listed in Table 3. Strain PMV4116, deleted of all five *pel* genes, was obtained using the method described by Ried and Collmer (1988). The *nptI-sacB-sacR* cartridge was first inserted into the *Bam*HI site of the *pelB* gene carried by pAD50 (Diolez and Coleno 1985). The resulting plasmid was introduced into *E. chrysanthemi* strain 3937, and the mutated allele was introduced into the *E. chrysanthemi* chromosome by marker exchange. Excision of the cartridge was then selected on the basis of sucrose tolerance. A kanamycin-sensitive and sucrose-tolerant clone (PMV4112) containing a 6-kb deletion that removed both the *pelB* and *pelC* genes was obtained. Plasmid pH553, which carries the *pelADE* gene cluster (Reverchon *et al.* 1986) was similarly mutated by inserting the *sac* cartridge into the *Bgl*II site of the *pelE* gene. The resulting plasmid was then introduced into strain PMV4112. Using the same procedure, a clone containing a deletion of 4 kb removing the *pelADE* gene cluster (PMV4116) was isolated (Fig. 3).

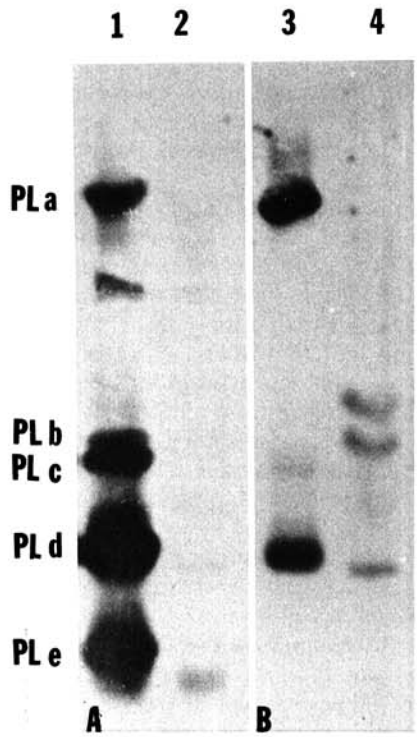

Fig. 2. Electrofocusing on a thin polyacrylamide gel of plant fluids from potato tuber (A) and from chicory leaf (B) infected by PMV4116 (lanes 2–4) or by the wild-type strain, 3937 (lanes 1–3).

Table 3. Bacterial strains used in this study

Strain	Relevant characteristics	Reference
PMV4066	*pelA*::Ω	Boccara *et al.* 1988
AD(PMV4120)	*pelB*::MudIIPR13 *pelC*::MudII1734	Diolez 1986
PMV4070	*pelD*::MudII1734	Boccara *et al.* 1988
PMV4122	Δ(*pelB pelC pelD pelE pem*)	Favey *et al.* 1992
PMV4116	Δ(*pelADE*) Δ(*pelBC*)	This work
PMV4106	*pem*::(MudIIPR13)	Boccara and Chatain 1989
PMV4071	*pelE*::Ω	Boccara *et al.* 1988
3937	Wild-type strain isolated from *Saintpaulia ionantha*	Lemattre and Narcy 1972

Media.

Bacterial strains were kept on L agar (Maniatis *et al.* 1982). Ceria broth (Glansdorff 1965) was the mineral base of all minimal media. Carbon sources were added to minimal media at a concentration of 0.2%. Antibiotics were used at a concentration of 20 μg/ml. Media were solidified by adding Difco agar (15 g/L).

Plant culture conditions and pathogencity tests.

Plantlets of *S. ionantha* 'Blue Rhapsody' were axenically cultured as described by Enard *et al.* (1988). Axenic pea plantlets were obtained by soaking seeds of *Pisum sativum* 'Proval' in a solution of sodium hypochlorite (0.5 M) and Triton X-100 (0.5%) for 30 min, rinsing three time with sterile tap water for 5 min, and germinating the sterilized seeds on 6% agarose plates. The germinated seeds were then transferred to tubes containing a Murashige and Skoog (MS) basal salt solution (Flow Laboratories, McLean, VA) solidified with 0.8% agar.

Plant tissue maceration tests were performed on potato tubers (*Solanum tuberosum*) and on etiolated witloof chicory leaves (*Cichorium intybus*) as previously described (Beaulieau and Van Gijsegem 1992). Holes were made in plant organs with a pipetman tip and the wounded tissues were inoculated with 50 μl (potato tuber) or 5 μl (witloof chicory leaf) of a bacterial suspension (1×10^8 cfu/ml).

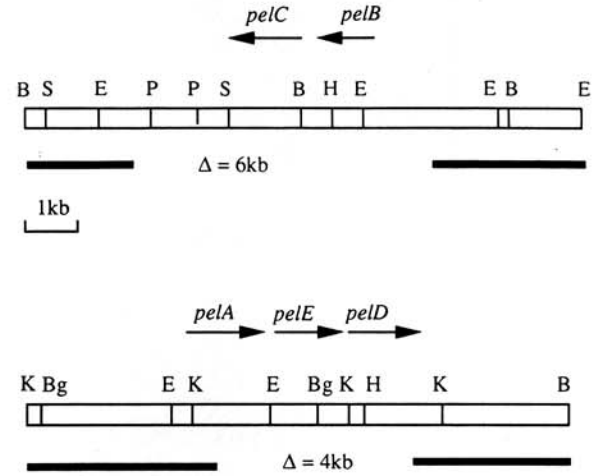

Fig. 3. Extent of the deletions in the *pel* genes in PMV4116 strain. The location of major restriction sites is indicated. Abbreviations: B, *Bam*HI; Bg, *Bgl*2; E, *Eco*R1; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I.

Potato tuber maceration was scored by measuring the weight of rotten tissues after 120 hr of incubation at 30° C; witloof chicory maceration was estimated by measuring the maximal length of macerated tissue after 24 hr of incubation at 30 C. The maceration abilities of the mutants and the wild-type strain were compared statistically by the ANOVA test (Little and Hill 1978).

Pathogenicity tests were performed on both axenic *S. ionantha* and pea plantlets. Two-month-old *S. ionantha* and 8-cm-high *in vitro*-grown pea plantlets were infected as described by Enard *et al.* (1988). At least 25 plantlets were inoculated with different strains. Fifteen or eight days after infection (saintpaulia and pea, respectively), the number of plantlets presenting systemic disease was scored. Statistical comparison between the virulence rate of the mutants and the wild-type strain was carried out with a χ^2 test (Little and Hill 1978).

PL activity assays.

Plant fluids from infected tissues were recovered by grinding these tissues and then removing plant debris by successive centrifugations at 10,000 rpm for 5 min. PL assays were made on filter-sterilized culture supernatants of bacteria grown in minimal media containing polygalacturonate or succinate as the carbon source or on plant fluids. The liberation of unsaturated products due to PL activity was monitored by the increase of absorbance of ultraviolet light (wavelength of 235 nm) according to the method of Van Gijsegem (1986). The procedure of Bertheau *et al.* (1984) was used to analyze PL enzyme production by electrofocusing in an ultrathin polyacrylamide gel (pH gradient from 3 to 10).

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