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Biochemical and Genetic Analysis of a Pectate Lyase Gene from *Xanthomonas campestris* pv. *vesicatoria*

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Twenty-two percent of 522 strains of *Xanthomonas campestris* pv. *vesicatoria* were pectolytic on a sodium polypectate medium. Biochemical and genetic aspects of the pectolytic activity were further analyzed in strain XV56. The pectolytic activity was due to the secretion of a single pectate lyase with a pI of 8.8. From a genomic library of DNA of XV56, recombinant cosmids conferring pectolytic activity to nonpectolytic strains of *X. c.* pv. *vesicatoria* were identified. By subcloning, the pectate lyase gene was located within a *Pst*I restriction fragment of 1.4 kb. The pectate lyase gene of XV56 shared no homology to DNA of the soft-rotting bacterium, *Erwinia chrysanthemi*, but hybridized to the DNA of all xanthomonads tested, irrespective of their ability to degrade pectic compounds. Mutants deficient in synthesis of the pectate lyase were selected after treatment of cells with nitrosoguanidine. The mutants were not altered in their ability to evoke disease symptoms, their growth in plants, or the ability to induce the hypersensitive reaction in nonhost plants. Thus, the pectate lyase gene appeared to play no essential role in pathogenicity.

Additional keywords: bacterial spot of pepper and tomato.

The ability to degrade pectic polymers found in plant cell walls is a feature of several plant pathogenic bacteria. Biochemical and genetic aspects of pectolysis have been extensively studied in soft-rotting bacteria (Collmer and Keen 1986; Kotoujansky 1987), in which plant tissue maceration is mainly due to the secretion of the pectic enzymes: pectin lyases (PNL), pectate lyases (PL), and polygalacturonases (PG). The lyases cleave the alpha 1,4 bond between the galacturonic acid residues by transelimination, whereas the polygalacturonases cleave by hydrolysis. The preference for highly methoxylated substrates differentiates PNL from PL. Pectic enzymes are pathogenicity factors in soft-rotting bacteria; elimination of some of these enzymes reduces or abolishes the ability of the pathogen to induce disease (Payne *et al.* 1987; Liao *et al.* 1988; Boccara *et al.* 1989).

Diseases caused by xanthomonads do not involve extensive maceration of the host tissues, but pectolytic activity has been observed in these plant pathogens (Dye 1960; Starr and Nasuno 1967). The role of the pectic enzymes in the xanthomonads is unclear. Strains of some pathovars of *Xanthomonas campestris* (Pammel) Dowson, such as *X. c.* pv. *pruni*, *X. c.* pv. *begoniae*, and *X. c.* pv. *phaseoli* have no apparent pectolytic ability in culture (Starr and Nasuno 1967). Thus, pectinases produced in cultures, at least in some pathovars, are not essential to pathogenicity.

Pectic enzymes may be involved in pathogenicity of some xanthomonads, however. Infiltration of cotton leaves with culture filtrates of *X. c.* pv. *malvacearum* that retained pectolytic activity elicited a reaction thought to be similar to hypersensitivity (Hopper *et al.* 1975). In *X. c.* pv. *campestris*, a mutation of a gene involved in the export of pectate lyases and proteases yielded a nonpathogenic phenotype (Dow *et al.* 1987).

The presence of both pectolytic and nonpectolytic strains within *X. c.* pv. *vesicatoria* presented an opportunity to determine the importance of pectolytic enzymes in pathogenicity of that pathovar on tomato and pepper plants. A collection of strains from different geographical regions was screened for the ability to degrade pectic compounds. We report herein the initial characterization of a degradative enzyme and the cloning of the gene responsible for the pectolytic activity in strain XV56.

MATERIALS AND METHODS

A University of Florida collection of strains of *X. c.* pv. *vesicatoria* included 522 strains from various geographical origins (Table 1). The strains were pathogenic to either pepper or tomato and were stored in sterile tap water. The strains were recovered from storage by subculture at 30° C on nutrient agar, or on a medium consisting of lima bean agar (Difco Laboratories, Detroit, MI) and 1.5% water agar in 1:1 proportions. The mineral base of M9 (Maniatis *et al.* 1982) was used in the minimal media. Carbon sources were added at a concentration of 0.2%. Plates were solidified by adding Difco agar (15 g/L).

Screening for pectolytic activity was done on a modified PVC medium without crystal violet (Schaad 1988) or on Hildebrand's B medium (1971). When bacteria were tested for PNL activity, D and E media of Hildebrand (1971) were used. The skim milk-containing medium used in the detection of proteolytic activity was previously described (Schaad and Stall 1988). Pectic enzyme assays were made on filter-sterilized culture supernatants of bacteria grown to the stationary phase in a minimal medium containing polygalacturonic acid or glycerol as the sole carbon source.

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Escherichia coli and *Erwinia chrysanthemi* Burkholder *et al.* strains were cultured in Luria-Bertani (LB) medium (Maniatis *et al.* 1982) at 37° and 30° C, respectively. When necessary, antibiotics and/or an amino acid were added to the media at the following concentrations: tetracycline, 12.5 mg/L; rifampicin, 75 mg/L; ampicillin, 100 mg/L; chloramphenicol, 10 mg/L; kanamycin, 50 mg/L; nalidixic acid, 50 mg/L; spectinomycin, 10 mg/L; and methionine, 200 mg/L.

Plant material and inoculations. The tomato cultivar Bonny Best and pepper cultivar Early Calwonder were used in pathogenicity tests. Plants were grown in steamed peat-vermiculite mix in 10-cm pots in a greenhouse (20–35° C). A soluble 20-20-20 fertilizer (0.4 g/pot) was added to the plants on a biweekly schedule.

For inoculations, fresh cultures of *X. c.* pv. *vesicatoria* were centrifuged to form a pellet that was resuspended in sterile tap water and standardized spectrophotometrically to a concentration of 3×10^8 cfu/ml. Carborundum (600 grit) was then added to the bacterial suspension. Young, expanding leaves were rubbed with a cotton applicator soaked in the bacterial suspension. After inoculation, symptom development was observed periodically for 3 wk.

Plants of tomato cv. Bonny Best were also used to compare bacterial growth *in planta*. Bacteria from fresh cultures of *X. c.* pv. *vesicatoria* were resuspended in sterile tap water and standardized as before to 3×10^8 cfu/ml. The standardized suspension was diluted to a concentration of 3×10^4 cfu/ml for inoculum. The suspensions were infiltrated with a syringe and needle into the mesophyll of fully expanded leaflets. At 2-day intervals, 1 cm^2 of infiltrated plant tissue was collected and ground in 1 ml of sterile tap water to recover bacteria. Appropriate dilutions of this suspension were spread on nutrient agar and the number of bacterial colonies that developed were counted (Stall and Cook 1966). For each strain tested, six different leaflets were inoculated for each time interval sampled. After infiltration, plants were kept at 28° C in a growth chamber with a 12-hr illumination period.

The development of the hypersensitive reaction (HR) was tested on pepper cv. Early Calwonder, a nonhost species for strain XV56. Pepper leaves were infiltrated with a bacterial suspension (3×10^8 cfu/ml) as described above. Plants were kept at 28° C for 30 hr.

Pectic enzyme assays. 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 Dow *et al.* (1987). One unit of activity caused the liberation of 0.5 μM unsaturated product per minute. The procedure of Bertheau *et al.* (1984) was used to analyze PL enzyme production by electrofocusing culture supernatants in an ultrathin polyacrylamide gel (pH gradient from 3 to 10). Fifteen microliters of filtered supernatant was applied. Standard proteins (Bio-Rad Laboratories, Richmond, CA) were electrophoresed alongside the culture filtrates of *X. c.* pv. *vesicatoria* to estimate the pI of the PL enzymes. An agarose-pectate overlay was used to detect PL activities (Liao 1989). The overlay gel was prepared from a mixture of 0.8% agarose, 0.2% polygalacturonic acid in 100 mM of Tris (pH 8.5) containing 1 mM $CaCl_2$. The agarose-pectate gel was overlayed onto the electrofocusing gel that was neutralized in a Tris solution (100 mM, pH 8.5) for 30 min before the application. Incubation was carried out at 37° C for 3 hr. Activity of PL was visualized by soaking the electrofocusing gel in 1% mixed alkyltrimethyl ammonium bromide. Activities of PG (Lei *et al.* 1985) and PNL (Schlemmer *et al.* 1978) were assayed as previously described.

Chemical and insertion mutagenesis. Cells of XV56 were chemically mutagenized with nitrosoguanidine (NTG) by the procedure of Carlton and Brown (1981) and then plated on minimal medium containing glucose to eliminate auxotrophic mutants. Mutants deficient in pectolytic and proteolytic abilities were identified among 1,000 surviving bacteria using the media described above.

Insertion mutagenesis of pU49, which carries the pectate lyase gene of XV56, using pHoKmGus as the transposon was done by the method of Bonas *et al.* (1989). Briefly, *E. coli* HB101 (pHoKmGus, pSShe) was transformed with pU49 DNA. Transformants were mated with the Nal^r *polA* *E. coli* C2110 using pRK2073 as helper plasid. Transconjugants carrying pU49 with a transposon insertion were selected for their resistance to tetracycline, nalidixic acid, and kanamycin. The ability of these mutagenized plasmids to confer pectolytic activity was tested by mobilizing them into XV75-4 as described below.

Table 1. Geographical distribution of pectolytic strains from the collection of strains of *Xanthomonas campestris* pv. *vesicatoria*

Countries	Number of strains tested	Pectolytic strains (no.)
United States	331	1
Argentina	116	104
Taiwan	43	0
Brazil	10	4
Spain	9	1
Australia	5	2
New Zealand	1	1
El Salvador	1	0
Venezuela	1	0
Tonga	1	0

Table 2. Plasmids used in this study

Designation	Relevant characteristics[a]	Reference
pLAFR3	tet^r	Staskawicz *et al.* 1987
pLAFR6	pLAFR1 derivative containing *trp* terminators, tet^r	Bonas *et al.* 1989
pHoKmGus	KM^r, Amp^r, *tnpA*$^-$; promotorless B-glucuronidase gene	Bonas *et al.* 1989
pSShe	cm^r, *tnpA*	Stachel *et al.* 1985
pRK2073	Sp^r, *tra*$^+$, *mob*$^+$	Sawcyc *et al.* 1989
pXV6	pLAFR3 with insert from XV56 with PL activity	This study
pU49	pLAFR3 with 4.9-kb insert from pXV6 with PL activity	This study
pU14	pLAFR6 with 1.4-kb insert from pU49 with PL activity	This study

[a] tet, tetracycline; KM, kanamycin; Amp, ampicillin; cm, chloramphenicol; Sp, spectinomycin; r, resistant.

DNA manipulations. Plasmids used in this study are listed in Table 2. Plasmid DNA was obtained by the alkaline lysis method (Maniatis *et al.* 1982). Total DNA was isolated according to Boucher *et al.* (1987). A genomic library of DNA from strain XV56 was constructed as previously described (Minsavage *et al.* 1990). Briefly, DNA of XV56 was partially digested with *Sau*3AI and fragments of 15–25 kb were purified and ligated into the *Bam*HI site of pLAFR3 (Staskawicz *et al.* 1987). Ligated DNA was packaged with a packaging kit (Boehringer-Mannheim, Indianapolis, IN) and then transduced into *E. coli* DH5α. The recombinant clones were mobilized into strain XV75-4 by triparental mating (Ditta *et al.* 1980) using pRK2073 as a helper plasmid. Cosmids harboring a putative pectate lyase gene were identified by selecting transconjugants that caused a pit in media containing sodium polypectate (Schaad 1988). Further subcloning was done by the method described by Maniatis *et al.* (1982).

Procedures used to transfer DNA onto nitrocellulose membrane, to label probe, and to perform hybridization studies were described (Minsavage *et al.* 1990).

RESULTS

Pectolytic activity in a population of *X. c.* pv. *vesicatoria*. Pectolytic activity, as identified by the ability to degrade a polypectate gel, was found in 22% of the strains tested (Table 1). This trait was correlated with the geographical origin of isolation. Pectolytic strains represented 90% of those obtained from Argentina, but only one such strain was found among a total of 374 strains from the United States and Taiwan. Mixed populations were found in Oceania, Brazil, and Spain.

Both pectolytic and nonpectolytic strains caused a bacterial spot disease in tomato and/or pepper. However, the two groups induced different symptoms on the plants. Bacterial leaf spots initiated by the pectolytic strains were flat or sunken and surrounded by a water-soaked area. Nonpectolytic strains caused elevated spots with a whitish margin. The size and rate of expansion of the lesions were similar.

Identification of pectolytic enzymes. Culture supernatants of five the pectolytic strains of *X. c.* pv. *vesicatoria* were assayed for PL, PNL, and PG activities to identify the enzymes responsible for the pectolytic activity. No detectable PG or PNL activity was found in any supernatant. Absence of PNL activity in supernatants was correlated with the inability of strains to form pits on gels containing highly methoxylated pectic compounds. However, PL activity was detected after the addition of sterilized supernatants to a buffered solution containing polygalacturonic acid. This activity was present only after the strains were grown in the presence of the polygalacturonate. Supernatants of those strains grown in the absence a polygalacturonate lacked the ability to release unsaturated products efficiently from pectic polymers (Table 3). Electrofocusing revealed the presence of a single PL enzyme with a pI of 8.8 in the active supernatant of strain XV56. Electrofocusing also revealed three bands with pectolytic activity from supernatants of a strain of *X. c.* pv. *campestris*, two bands from supernatants of an an opportunistic xanthomonad, and four bands, as expected, from supernatants of a strain of *E. chrysanthemi*.

Cloning of the pectate lyase gene of XV56. Following mobilization of the library clones into XV75-4, a nonpectolytic strain of *X. c.* pv. *vesicatoria*, two out of 514 transconjugants acquired the ability to form pits in a pectate gel. One of these clones, pXV6, was further used in subcloning experiments. A 4.9-kb internal fragment of pXV6, obtained by digestion of the insert DNA with *Sau*3AI was subcloned into pLAFR3 and named pU49. This subclone still conferred pectolytic activity. To fix the boundaries of the pectate lyase gene, insertion mutagenesis of pU49 was performed. All of the seven independent insertions that inactivated pectolysis mapped within a 1.4-kb *Pst*I fragment. This *Pst*I fragment remained fully functional when cloned back into pLAFR6 (pU14), a cosmid containing *trp* terminators on either side of the multiple cloning site. This indicated that the entire pectate lyase gene, named *pelXV1* is within this 1.4-kb fragment.

Role of the pectate lyase gene in pathogenicity. Mutants of strain XV56 that were deficient in pectolysis were obtained after NTG treatment. Twenty-three out of 1,000 surviving colonies were deficient in pectolysis. However, most of these mutants (74%) were simultaneously altered in proteolysis. Six mutants were deficient in pectolysis, but retained the ability to degrade proteins. Such mutants, named Pel$^-$ mutants, were complemented by the introduction of pU14 (Table 3).

Cells of two of the Pel$^-$ mutants (strains XV4 and XVB) and cells of XV56 were inoculated into leaves of tomato. The mutants produced a bacterial spot disease characteristic of strain XV56. The mutants were not different from XV56 in the appearance of the spots or in the timing of symptom development. The growth rates of the bacteria in tomato leaves were not altered by the mutation as compared to

Table 3. Pectate lyase activity in the culture supernatants of strains of *Xanthomonas campestris* pv. *vesicatoria* grown in the presence of polygalacturonic acid or glycerol

Strain	Relevant characteristics	Pectate lyase activity (U/ml)	
		Polygalacturonate	glycerol
BV5 3a	Pectolytic strain from Corrientes, Argentina	0.20	0.01
BV21-1	Pectolytic strain from Corrientes, Argentina	0.24	0.01
BA27-1	Pectolytic strain from Buenos Aires, Argentina	0.18	0.00
0350	Pectolytic strain from Australia	1.11	0.05
XV56	Pectolytic strain from Brazil	0.31	0.00
XVB/pU49	Pel$^-$ mutant of XV56 carrying the PL gene from XV56	0.26	ND[a]
XV89-16/pU49	Nonpectolytic strain from United States carrying the PL gene from XV56	0.37	ND
XV89-16	Nonpectolytic strain from United States	0.00[b]	ND
XVB	Pel$^-$ mutant of XV56	0.00[b]	ND

[a] Not determined.
[b] No growth occurred with polygalacturonate as sole carbon source, glycerol was added to the medium.

the growth of XV56 (data not shown). Both mutants and the wild-type strain gave characteristic HR when infiltrated into a nonhost species (pepper cv. Early Calwonder). Thus, no alteration of pathogenicity was observed as the result of a loss of the pectolytic activity.

Hybridization of *pelXV1* to DNA of other bacteria. The 1.4-kb insert fragment of pU14 that contained *pelXV1* was used in hybridizations with DNA of pectolytic and nonpectolytic xanthomonads. The DNA of all xanthomonads tested, including nonpectolytic strains of *X. c.* pv. *vesicatoria*, hybridized to the probe (Fig. 1). However, the patterns of hybridizations differed among pectolytic strains of *X. c.* pv. *vesicatoria*, nonpectolytic strains of *X. c.* pv. *vesicatoria*, and strains of other pathovars of *X. campestris*. The probe did not detectably hybridize to DNA of the soft-rotting bacterium, *E. chrysanthemi* (Fig. 1).

The insert DNA of pU14 contained a region of about 0.5 kb (Fig. 2) in which transposon insertion did not inactivate pectolysis. Thus, the hybridization observed between the probe and the DNA of nonpectolytic strains of *X. c.* pv. *vesicatoria* could have resulted from homology with the DNA region outside the pectate lyase gene. To confirm that the pectate lyase gene was responsible for the detected homology, this 0.5-kb DNA region and part of the pectate lyase gene were deleted from the insert by an *Eco*RI digest. The resulting 0.8-kb *Eco*RI–*Pst*I fragment was used in hybridization. Once again, the probe hybridized to the nonpectolytic strains of *X. c.* pv. *vesicatoria*.

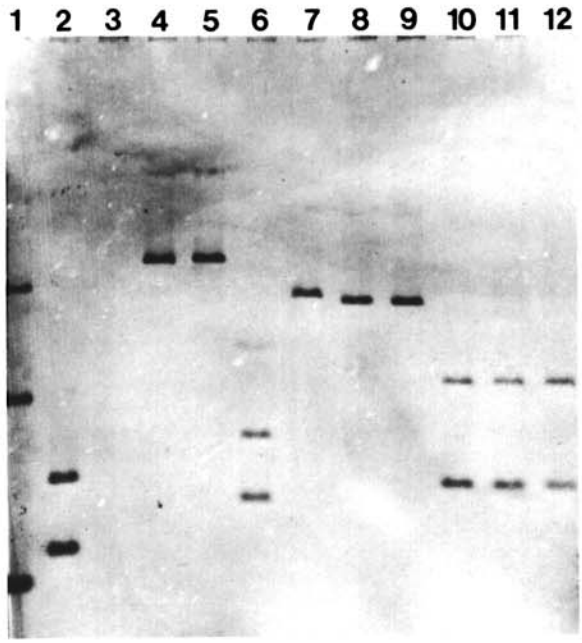

Fig. 1. Southern blot of *Eco*RI-restricted genomic DNA from strains of *Xanthomonas campestris* pv. *vesicatoria*, pathovars of *X. campestris*, opportunistic xanthomonads, and *Erwinia chrysanthemi* probed with the 1.4-kb insert of pU14. Lane: 1, opportunistic *X. campestris* strain 727 (pectolytic); 2, opportunistic *X. campestris* strain T55 (pectolytic); 3, *E. chrysanthemi*; 4, *X. c.* pv. *citrumelo* strain 80-1a (pectolytic); 5, *X. c.* pv. *dieffenbachiae* strain 2032 (pectolytic); 6, *X. c.* pv. *campestris* strain 33193 (pectolytic); 7, *X. c.* pv. *vesicatoria* strain XVP26 (nonpectolytic); 8, *X. c.* pv. *vesicatoria* strain XV75-3 (nonpectolytic); 9, *X. c.* pv. *vesicatoria* strain XV88-5 (nonpectolytic); 10, *X. c.* pv. *vesicatoria* strain BA28-1 (pectolytic); 11, *X. c.* pv. *vesicatoria* strain BA5-4a (pectolytic); 12, *X. c.* pv. *vesicatoria* strain XV56 (pectolytic).

DISCUSSION

X. c. pv. *vesicatoria* does not cause extensive maceration of host tissue, but the ability to degrade pectate was found in a portion of the strains tested. Among strains from some areas (Taiwan and United States), pectolytic activity was not found or was represented in a negligible portion of the population. However, pectolytic strains predominated in the collections from Argentina and were found with nonpectolytic strains in some other areas. The reasons for the nonrandom distribution of the pectolytic strains of *X. c.* pv. *vesicatoria* are not known.

The different symptoms of bacterial spot caused by the two groups of *X. c.* pv. *vesicatoria* cannot be attributed to the pectolytic activity. The Pel$^-$ mutants retained the ability to induce bacterial spot symptoms characteristic of the pectolytic strains. Moreover, Pel$^-$ mutants were not altered in disease development, growth in leaf tissue, or induction of HR on a nonhost plant. Thus, it appears that *pelXV1* of *X. c.* pv. *vesicatoria* is not essential for pathogenesis. It was previously shown that inactivation of the pectate lyase gene encoding production of the isozyme 1 in *X. c.* pv. *campestris* did not alter the pathogenicity of the mutants on seedlings or mature leaves of turnip (Dow *et al.* 1989). However, because *X. c.* pv. *campestris* produced three pectate lyase isozymes, inactivation of only one pectate lyase gene did not determine conclusively the function of pectolysis in pathogenicity. Nevertheless, a possible role for these genes in some accessory mechanism of pathogenicity cannot be excluded. Most likely, and as previously speculated for *Klebsiella* and *Yersinia* species, the PL of *X. c.* pv. *vesicatoria* may play a role in catabolic functions related to bacterial nutrition (Chatterjee *et al.* 1979; Manulis *et al.* 1988).

The pectolytic enzyme profile of XV56 is simple when compared with those of soft-rotting *Erwinia* species that synthesize and secrete multiple PLs and other pectic enzymes. This simple profile is not shared by all xanthomonads. Up to three PL isoenzymes are secreted by *X. c.* pv. *campestris* (Dow *et al.* 1987) and by some opportunistic xanthomonads. These xanthomonads are more efficient at pit formation on a polypectate gel. However, the weaker pectolytic activity in *X. c.* pv. *vesicatoria* cannot be entirely explained by the secretion of a unique PL enzyme. Some efficient soft-rotting bacteria secrete only one basic PL enzyme (Liao *et al.* 1988; Liao 1989). The pI of the PL enzyme of XV56 approaches that of the so-called "neutral

Fig 2. Restriction map of pU14, a 1.4-kb fragment of DNA that confers pectate lyase activity to strains that lack that activity in culture. Symbols for restriction enzymes are: E = *Eco*RI, H = *Hin*dIII, and P = *Pst*I. Spaces outside of insert are between restriction sites in vector. The closed triangles are positions of transposon insertions that inactivate activity. The open triangles are positions of insertions that do not inactivate activity. Bar = 0.1 kb.

PL" in strains of *Erwinia,* which might be responsible for weak pectolytic activity. In *E. chrysanthemi*, neutral PL isozymes are less efficient than the basic ones in plant tissue maceration (Roeder and Collmer 1985) and have little importance in pathogenicity (Boccara *et al.* 1988).

We also report the cloning of a pectate lyase gene (*pelXV1*) from *X. c.* pv. *vesicatoria* (*pelXV1*). A pectate lyase gene was previously cloned from a different pathovar of *X. campestris* (Dow *et al.* 1989). The active pectate lyase gene of *X. c.* pv. *campestris* contains 1.4 kb of DNA, which is more than that necessary for activity of the *pelXV1* gene of *X. c.* pv. *vesicatoria.* The degree of homology between the two pectate lyase genes still is to be determined. The *pelXV1* gene of the XV56 genome complements not only mutants deficient in pectolysis, but also confers pectolytic ability to nonpectolytic strains of *X. c.* pv. *vesicatoria.* In both cases, the recombinant bacteria produce and secrete the PL enzyme, indicating that nonpectolytic strains possess the set of genes essential for export of the PL enzyme. Possibly the export of the PL enzyme is achieved by the mechanism driving protease secretion. The recovery of mutants of *X. c.* pv. *vesicatoria* simultaneously deficient in pectolytic and proteolytic functions may mean that as in *X. c.* pv. *campestris* (Dow *et al.* 1987) and in *Pseudomonas viridiflava* (Burkholder) Dowson (Liao *et al.* 1988), proteases and pectinases share a common export mechanism.

The *pelXV1* gene of *X. c.* pv. *vesicatoria* hybridized to the DNA of all xanthomonads tested, regardless of their ability to use pectic compounds. It is possible that nonpectolytic strains of *X. c.* pv. *vesicatoria* possess a nonfunctional pectate lyase gene. The loss of this function would have no crucial effect since the pectate lyase gene plays no determinant role in pathogenicity. Another possibility is that the homologous DNA includes a functional pectate lyase gene, but the prerequisites for its expression were not fulfilled under the tested conditions. Usually, pectate lyase genes are either constitutively expressed (Liao *et al.* 1988) or inducible by pectic compounds (Reverchon and Robert-Baudouy 1987; Reverchon *et al.* 1986). However, numerous genes in plant pathogenic bacteria are specifically regulated by plant compounds (Stachel *et al.* 1986; Osbourn *et al.* 1987; Beaulieu and Van Gijsegem 1990). Regulation of some pectinases by plant products other than pectin derivatives can be considered. For examples, Collmer *et al.* (1991) reported that a strain of *E. chrysanthemi* deprived of all its pectin-inducible *pel* and *peh* genes still retained maceration ability. By cloning the DNA region homologous to the *pelXV1* from nonpectolytic strain of *X. c.* pv. *vesicatoria*, and by comparing these two regions, answers to those yet unresolved questions should be provided.

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