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An Extracellular Pectate Lyase is the Pathogenicity Factor of the Soft-Rotting Bacterium *Pseudomonas viridiflava*

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***Pseudomonas viridiflava* is a soft-rotting pathogen of harvested vegetables. The genetic and biochemical evidence presented here demonstrates that an extracellular pectate lyase (PL) of *P. viridiflava* is responsible for the maceration of plant tissues. The PL from culture supernatant was purified to apparent homogeneity by ammonium-sulfate precipitation and ion-exchange chromatography. The purified PL was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and thin-layer polyacrylamide gel isoelectric focusing, and was shown to contain a single polypeptide with M_r 42 kD and pI 9.7. This PL preparation was devoid of pectin lyase, polygalacturonase and protease activities, and readily macerated plant tissues. *P. viridiflava* was mutagenized with Tn*5* by using pSUP1011 as a mobilizing vector. Two classes of mutants were altered in pectolytic ability. The Pel$^-$ mutants were apparently defective in the synthesis of PL, whereas the Out$^-$ mutants were pleiotropically altered in the export of PL and protease. Both Pel$^-$ and Out$^-$ mutants failed to induce soft rot in five unrelated plants. Southern blot analyses of the restriction enzyme-generated genomic DNA revealed that the Pel$^-$ (M1-4) and the Out$^-$ (M1-2) mutants carried single copies of Tn*5*. IS*50*, in addition to Tn*5*, was also detected in several mutants. Our observations indicate that PL, but not protease, is responsible for the pathogenicity of *P. viridiflava*. In addition, the data show that PL and protease share a common export system in this bacterium.**

Additional keywords: Tn*5* transposition, pathogenicity determinants.

Pseudomonas viridiflava is a phytopathogenic fluorescent pseudomonad characterized by its ability to macerate potato slices and by the absence of oxidase and arginine dihydrolase activities (Billing 1970). The organism has been associated with field outbreaks of several plant diseases, including internal stem rot of tomato, wet rot of pea, and soft rot of cauliflower, cabbage, and lettuce (Wilkie *et al.* 1973). However, this organism appears to be more important as a post-harvest pathogen than as a disease-causing agent in the field. For example, Liao and Wells (1987) recently reported that *P. viridiflava* accounted for more than 10% of bacterial spoilage of vegetables at produce markets.

Despite several studies of the extracellular enzyme (Cabezas de Herrera and Jurado 1975; Hildebrand 1971), the pathogenicity factors of *P. viridiflava* have not yet been identified. *P. viridiflava* has been shown to lose virulence in culture (Billing 1970; Burkholder 1930), although the genetic mechanism for this variability is not known. The ability of the organism to produce pectate lyase (PL) but not polygalacturonase (PG) has also been detected (C.-H. Liao, unpublished). These observations suggested that the pectic enzyme system, presumed to be involved in the soft-rot pathogenicity of *P. viridiflava*, probably was less complex than that of soft-rotting erwinias (Chatterjee and Vidaver 1986; Collmer and Keen 1986; Kotoujansky 1987). The soft-rot pathogenicity of *Erwinia* spp. has been attributed to an assortment of pectic enzymes, including PG (Lei *et al.* 1985; Willis *et al.* 1987), pectin lyase (Chatterjee and Vidaver 1986), and multiple species of PL (Pupillo *et al.* 1976; Van Gijsegem 1986).

Transposon mutagenesis is a useful tool for generation of selectable, single-site mutations in various bacteria (De Bruijn and Lupski 1984; Jorgensen *et al.* 1979). This technique has been sucessfully used to elucidate gene functions related to pathogenicity of several phytopathogenic bacteria (Mills 1985), including various pathovars of *P. syringae* (Anderson and Mills 1985; Cuppels 1986; Morgan and Chatterjee 1985; Panopoulos *et al.* 1985). *P. viridiflava* is closely related genetically and physiologically to *P. syringae* (Billing 1970). Therefore, it was reasonable to expect that the transposon-mobilization systems previously proven useful for *P. syringae* could be utilized in the mutagenesis of *P. viridiflava*.

The objectives of this study were: to define some of the conditions that affected PL production in *P. viridiflava*; to purify and characterize the extracellular PL; and to isolate PL-deficient mutants by transposon (Tn*5*) mutagenesis and then test their tissue-macerating activity. Our data show that *P. viridiflava* produces a single PL species that controls its pathogenicity. A preliminary account on the results was reported at the 1987 American Phytopathological Society annual meeting.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains of *P. viridiflava* and *E. coli* used are listed in Table 1. Two plasmids were used in the study: pSUP1011 (Simon *et al.* 1983) for Tn*5* mutagenesis and pRZ102 (Jorgensen *et al.* 1979) as the source of Tn*5* probe.

Media and culture conditions. *E. coli* strains were grown at 37° C in L broth (Maniatis *et al.* 1982) and *P. viridiflava* at 28° C in nutrient broth (Difco) or MY medium (pH 7.2)

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containing K_2HPO_4 (0.7%), KH_2PO_4 (0.2%), $MgSO_4.7H_2O$ (0.02%), $(NH_4)_2SO_4$ (0.1%), yeast extract (0.1%), $CaCl_2$ (1 mM), and various carbon sources as indicated. Antibiotics were used at the following concentrations: Kanamycin (Km) 50 μg/ml, rifampicin (Rif) 50 μg/ml, chlorampehicol (Cm) 25 μg/ml, streptomycin (Sm) 100 μg/ml, and spectinomycin (Sp) 300 μg/ml. Nutrient agar (NA) (Difco) was used for routine cultivation.

Purification of PL. *P. viridiflava* strain SF 312 was grown in MY medium containing 0.4% citrus pectin (Sigma Chemical Co., St. Louis; Grade 1) at 20° C for 48 hr. Cells were removed by centrifugation (10,400 × *g*, 20 min), and the supernatant was treated with ammonium sulfate. The precipitate formed at 50–85% saturation was collected by centrifugation, resuspended in 50 mM Tris-HCl (pH 7.5) buffer, and dialyzed against the same buffer at 4° C for 18 hr. The dialyzed enzyme preparation was applied onto a DEAE-cellulose column (1.5 × 20 cm), previously equilibrated with 50 mM Tris-HCl (pH 8.0) buffer. The step-wise elution was carried out with the buffer containing 0.05, 0.10, 0.20, or 0.30 M NaCl. Five-ml fractions were collected and each fraction assayed for PL activity. Fractions with PL activity were pooled and further concentrated with the Centricon 10 microconcentrator (Amicon, Danver, MA).

Assay of PL activity. The spectrophotometric method (Zucker and Hankin 1970), which measured the absorbance of the enzymatic end-product (unsaturated uronide) at 232 nm, was carried out at 30° C by using the Gilford Model 260 spectrophotometer. An increase in absorbance of 1.73 was considered to represent the formation of 1 μmole of unsaturated uronide (Keen *et al.* 1984). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmole of unsaturated uronide product at 30° C per min in the reaction mixture containing 100 mM Tris-HCl (pH 8.5), 0.25% sodium polygalacturonate (Sigma), and 1 mM $CaCl_2$. Specific activity was calculated by dividing the total enzyme units by the number of cells (10^{10} colony-forming units). Protein concentration was determined according to the Lowry's procedures included in the Protein Assay Kit from Sigma. Activities of PG (Lei *et al.* 1985) and pectin lyase (Schlemmer *et al.* 1987) were assayed according to the methods previously described. Proteolytic activity was tested on NA-skim milk medium to be described below.

Table 1. Bacterial strains and plasmids

Strains	Relevant characteristics[a]	Reference and source
P. viridiflava		
SF 312	Wild type, pathogenic	Isolated from rotten squash, Liao and Wells 1987
13223	Wild type, pathogenic	Isolated from dwarf bean, ATCC
SF 312 A	Spontaneous Rif^r derivative of SF 312	This paper
13223 A	Spontaneous Rif^r derivative of 13223	This paper
M1-2	Tn*5* insertion mutant of SF 312 A; nonpathogenic, Out$^-$	This paper
M1-4	Tn*5* insertion mutant of SF 312 A; nonpathogenic, Pel$^-$	This paper
M1-5	Similar to M1-4	This paper
M1-10	Similar to M1-2	This paper
Arg-1	Tn*5*-induced Arg$^-$ auxotroph, derived from SF 312 A	This paper
Eps-1	Tn*5*-induced Eps$^-$ mutant, derived from SF 312 A	This paper
E. coli		
SM 10	C 600 *thi thr leu recA*, Muc$^+$ chromosomally integrated, RP_4-2-Tc::Mu contains pSUP1011	Simon *et al.* 1983
HB101	*hsdR hsdM pro leu thi lacY recA* Sm^r Km^r, contains pRZ102	Jorgensen *et al.* 1979
Plasmid		
pSUP1011	Cm^r, Km^r, possibly Sm^r; 12.1 kb	Simon *et al.* 1983
pRZ102	Sm^r, Km^r 12.3 kb	Jorgensen *et al.* 1979

[a]Rif, rifampicin; Sm, streptomycin; Km, kanamycin; Cm, chloramphenicol. Out$^-$ and Pel$^-$, defective in export and synthesis of pectate lyase, respectively. Eps$^-$, defective in mucoid phenotype.

Effect of carbon sources on PL production. *P. viridiflava* strains were grown in MY medium containing one of the following carbon sources: glucose (0.2%), glycerol (0.2%), pectin (0.4%), and polygalacturonate (0.4%). After incubation at 28° C for a given period of time, cells were separated from the culture medium by centrifugation (10,000 × *g*, 10 min). The supernatant was assayed for PL activity. The cell pellet was washed twice and resuspended in 50 mM Tris-HCl (pH 7.2) buffer. Cells were disrupted with an ultrasonicator and cell debris removed by centrifugation (35,000 × *g*, 1 hr). The clear supernatant was removed and used to determine the cell-bound PL activity.

Pathogenicity assay. The ability of bacterial stains to macerate plant tissues was tested on detached parts of five different plants, including slices of potato tubers, celery petioles, and fruits of bell pepper, tomato, and squash. General procedures for preparation of testing plant materials and bacterial inocula have been previously described (Liao and Wells 1986). To assay the macerating ability of the purified PL, 3 μl of enzyme preparation containing 4.2 U was directly applied onto the surface of a potato slice. The maceration zone was measured one day after incubation at 20° C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and thin-layer polyacrylamide gel isoelectric focusing. SDS-PAGE was performed according to the methods described (Laemmli 1970). The polyacrylamide gel containing acrylamide (10%), bisacrylamide (0.33%), and SDS (0.1%) was used. The sample containing 3–7 μg of protein was added to the well. The molecular weight standard purchased from the Bio-Rad Laboratories (Richmond, CA) was included to estimate the molecular weight of the protein sample.

Isoelectric focusing (IEF) was performed at 10° C by using the LKB 2117 multiphor II electrophoresis unit (LKB/Pharmacia, Piscataway, NJ). Thin-layer (1 mm) polyacrylamide gel plates (pH 3.5–9.5) containing ampholine (2.4%) and gel concentration of 5% were obtained from the LKB Instrument. The polyacrylamide gel plate (11.0 × 12.2 cm) was prefocused at 9 W for 30 min. Samples (3–8 μl) were applied directly onto the gel and focused at 12 W for 40 min. NaOH (0.1 M) and phosphoric acid (0.1 M) were used as the catholyte and anolyte, respectively. The IEF marker, purchased from FMC Corp. (Rockland, ME), was included to estimate the isoelectric pH (pI) of the protein sample.

Transposon mutagenesis. *E. coli* SM10 (pSUP1011) and *P. viridiflava* (SF 312 A) were grown separately to a cell density of 5.0×10^8 cfu/ml. One ml each of the donor and the recipient culture were mixed in an Erlenmeyer flask (125 ml)

and subsequently incubated at 28° C for 3 hr with gentle shaking (10 rpm). At the end of incubation, 0.1-ml aliquots of a series of 10-fold dilutions of the conjugation mixture were plated onto the *Pseudomonas* agar F (Difco) supplemented with rifampicin and kanamycin. Rif^r Km^r transconjugants isolated from the selective medium were screened for phenotypic changes on four diagnostic agar media. CVP (Cuppels and Kelman 1974) and MM-9 (Liao and Wells 1986) media were used to detect pectolytic mutants, and NA supplemented with 5% skim milk (Difco) was used to detect proteolytic mutants. For detection of auxotrophs, MY medium containing 0.2% glycerol (as a carbon source) but without yeast extract was used. Nutritional requirements of the auxotrophs were determined according to Davis *et al.* (1980). Prototrophic reversion tests were performed according to the procedures described (Morgan and Chatterjee 1985). Rif^r Km^r transconjugants were also inoculated to the NA-chloramphenical medium to determine whether the acquired Km^r phenotype resulted from the replication of pSUP1011 or from the transposition of Tn*5* (Simon 1983).

Extraction of genomic DNA. Genomic DNAs were isolated and purified according to the methods described by Shepard and Polisky (1979). Before use, DNA preparations were usually purified and concentrated to the required concentration by the Elutip-d Mini-Column of Schleicher and Schuell (Keen, NH).

Restriction endonuclease digestion and agarose gel electrophoresis. Endonucleases (*Eco*RI, *Sst*I, and *Bgl*II) purchased from Bethesda Research Laboratories (Gaithersburg, MD) were used according to the manufacturer's suggestions. For complete digestion, the enzyme at the concentration of 5–10 U/μg of DNA was added, and the reaction mixture was incubated at 37° C for 16 hr. For separation of DNA, 0.5% agarose gel and Tris-borate buffer (Maniatis *et al.* 1982) were used throughout the study. Electrophoresis was conducted at 2–4 v/cm of gel for 4–16 hr, depending on the length of the gel. When desired, DNA fragments were electroeluted from the regular agarose or recovered from the low-melting agarose according to the methods described (Maniatis *et al.* 1982).

Sources of DNA used for preparation of Tn*5* probes. pRZ102, pSUP1011, and the 2.6-kb internal region of Tn*5* were used to detect Tn*5* sequences present in the genomes of the mutants. pRZ102 or pSUP1011 was amplified and isolated from the appropriate strain of *E. coli* according to the procedures described (Godson and Vapnek 1973). The 2.6-kb internal region of Tn*5* that encoded Km^r was obtained by digesting pRZ102 (or pSUP1011) with the endonuclease *Bgl*II (Jorgensen *et al.* 1979). DNA was labeled with two nonisotopic methods. The biotinated DNA probe developed by Leary *et al.* (1983) was commercially available as the "Blue Gene" kit from Bethesda Research Laboratories. The sulfonated DNA probe was developed based on the principle described by Sverdlov *et al.* (1974) and was recently available as the "Chemiprobe" from the FMC Corporation (Rockland, ME). Methods for labeling and detection were performed according to the procedures provided.

Southern blot analyses. Standard procedures previously described (Maniatis *et al.* 1982) were followed. Prehydrization and hybridization were conducted at 42° C in the Denhardt's solution containing 50% formamide. The denatured salmon DNA was added into the prehybridization mixture at the concentration of 100 μg/ml, and the denatured probe DNA was added into the hybridization mixture at 0.5 μg/ml.

RESULTS

Production of PL. PL activity was detected in supernatants obtained from cultures grown in various carbon sources and also in plant tissue extracts infected with *P. viridiflava* (SF 312 and 13223). At the mid stationary phase, the specific activities of PL were determined to be 7.8, 8.4, 10.5, and 13.7 U min^{-1} 10^{-10} colony-forming units in supernatants obtained from cultures grown in glucose, glycerol, polygalacturonate, and pectin, respectively. At the early log phase, 75% of the total PL activity (about 275 units) was detected in the sonicated cell extract. However, in the late stationary phase, the majority (94%) of the activity (about 820 units) was found in the culture supernatant.

Purification of PL. The extracellular PL was purified by ammonium sulfate precipitation and ion-exchange chromatography. About 65% of the total PL activity detected in the culture fluid was recovered in the precipitate formed at 50–85% saturation of ammonium sulfate. Nearly 75% of PL added onto the DEAE-cellulose (Cl^-) column was eluted and recovered in the buffer (50 mM Tris-HCl, pH 8.0) containing 0.05 M NaCl. The step-wise elution profile of the PL in the column is illustrated in Figure 1. The fractions containing PL were pooled, concentrated, and analyzed by SDS-PAGE and by thin-layer IEF in polyacrylamide gel. The occurrence of a single activity peak in DEAE cellulose fractions (Fig. 1) and a single band in SDS-PAGE and IEF gels (Fig. 2) indicate that the final preparation was homogenous and that *P. viridiflava*

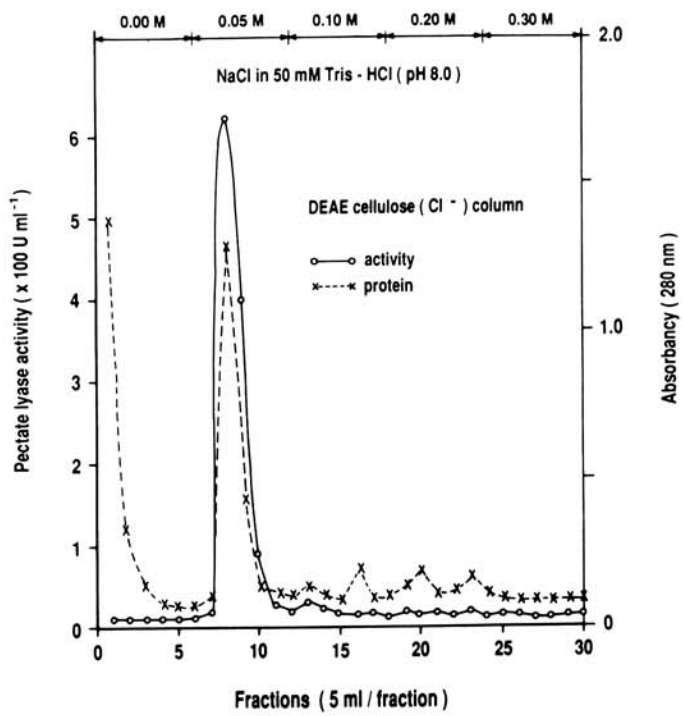

Fig. 1. Elution profile of pectate lyase from the DEAE-cellulose (Cl^-) column. The column was eluted with 50 mM Tris-HCl (pH 8.5) buffer followed by step-wise elution with buffer containing 0.05–0.30 M NaCl.

produced a single PL species. The molecular weight of the PL was estimated to be 42 kD (Fig. 2A), and the isoelectric pH of the PL was estimated to be 9.7 (Fig. 2B). No apparent activity of pectin lyase, protease, and PG was detected in the PL preparation. The purified PL readily macerated potato slices; a maceration zone of 14 mm in diameter was observed 1 day after the addition of 3 μl (1.4 U) of PL.

Isolation of Tn*5* insertion mutants. The Km^r marker encoded on Tn*5* was transferred from *E. coli* SM 10 (pSUP1011) to two strains of *P. viridiflava* (SF 312 A and 13223 A) at high frequencies (7.8×10^{-4}–1.3×10^{-5} per donor cell). A total of 3,350 Rif^r Km^r transconjugants of SF 312 A was isolated and subsequently screened for phenotypic changes. Only two transconjugants were resistant to chloramphenicol, indicating that the Km^r acquired by the rest did not result from the replication or integration of pSUP1011. About 1% (or 38 isolates) of the total transconjugants was auxotrophic. Auxotrophs requiring one of the following nutrients were identified: His, Trp, Ade, Arg, Leu, Cys, Ura, Pro, Ser, Tyr, Met, and Asn. Seven of 38 auxotrophs (or 18%) were identified as Met$^-$. The Arg$^-$, His$^-$, and Trp$^-$ auxotrophs were found to revert to prototrophs at the frequencies of 10^{-8}–10^{-9}. Five transconjugants, designated as Eps$^-$, had nonmucoid colony morphology on agar media. In addition, 10 transconjugants (later designated as Pel$^-$ or Out$^-$) defective in the degradation of pectate in the CVP medium were identified and further characterized.

Characterization of Pel$^-$ and Out$^-$ mutants. Based on their phenotypic properties on three diagnostic agar media, 10 transconjugants showing defects in pectolytic ability of CVP medium were divided into two groups. The first group (Pel$^-$) consisting of two isolates (M1-4 and M1-5) showed no pectolytic activity on CVP and MM-9 medium but exhibited proteolytic activity on NA-milk medium. The second group (Out$^-$) consisting of eight isolates, including M1-2 and M1-10, displayed neither the pectolytic nor the proteolytic activity. Figure 3 illustrates the typical phenotypic properties of the Pel$^-$ (M1-4) and the Out$^-$ (M1-2) mutants on two diagnostic media (MM-9 and NA-milk).

To determine the correlation between pectolytic activity and soft-rot pathogenicity, the 10 Pel$^-$ and Out$^-$ mutants were assayed individually for their macerating ability on detached parts of five different plants (potato, bell pepper, tomato, celery, and squash). None of the 10 mutants induced symptoms in any of the five plants tested. However, a restricted maceration (< 1 mm) was occasionally observed on the plant site inoculated with Out$^-$ mutants. A typical response of the bell pepper inoculated with the Pel$^-$ (M1-4) and the Out$^-$ (M1-2) mutant is illustrated in Figure 4. The loss of macerating ability thus correlated with the absence of pectolytic activity in each of the Pel$^-$ and Out$^-$ mutants.

The pleiotropic defect in pectolytic and proteolytic activities in Out$^-$ mutants (Fig. 3) suggested that the phenotype resulted from a mutation in a gene-specifying enzyme export. In contrast, we assumed that the Pel$^-$ Prt$^+$ phenotype resulted from a Tn*5* insertion in a gene for PL synthesis. To test these hypotheses, we measured the extracellular and intracellular PL activities of Pel$^-$ (M1-4) and Out$^-$ (M1-2) mutants grown in the presence of pectin. Typical results of one such experiment are summarized in Table 2. Because no PL activity was detected in sonicated cell extracts or in culture supernatants of the Pel$^-$ (M1-4) mutant throughout the growth cycle, it appears that Tn*5* may have inserted into a gene(s) directing the synthesis of PL. However, with the Out$^-$ mutant, high levels of PL activity were detected in sonicated cell extracts but none in supernatants of cultures at various growth stages.

Detection of Tn*5* sequences in the bacterial genome. To verify that Rif^r Km^r mutants were derived from the transposition of Tn*5*, biotinated and sulfonated pRZ102 DNA probes were used. As shown in Figure 5, hybridization bands were observed in the *Eco*RI-generated genomic fragments of mutants (M1-2, M1-4, Arg-1, and Eps-1) but not in the genomic fragments of the parent strain SF 312 A. The pattern of hybridization bands detected by the biotinated DNA probe (Fig. 5A) was identical to that detected by the sulfonated DNA probe (Fig. 5B). Although both DNA-labeling methods worked well, the sulfonated

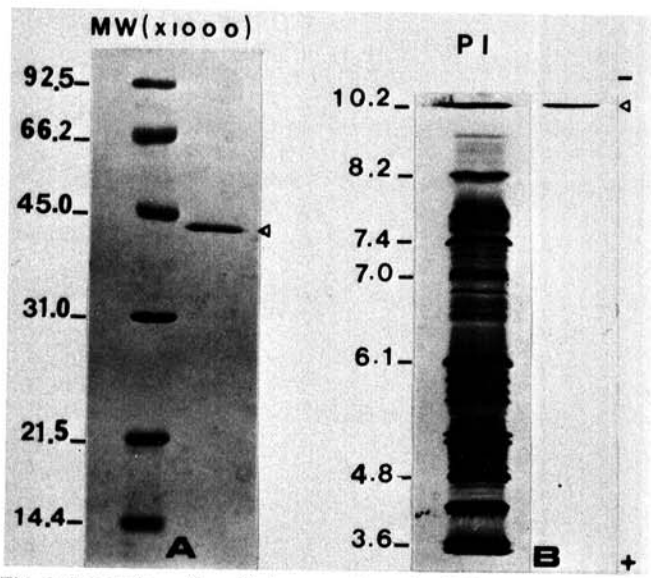

Fig. 2. Resolution of purified pectate lyase by SDS-PAGE (**A**) and by thin-layer polyacrylamide gel isoelectric focusing (**B**). The molecular weight markers (left lane, A) include: lysozyme (14.4 kD), soybean trypsin inhibitor (21.5 kD), carbonic anhydrase (31.0 kD), ovalbumin (45.0 kD), bovine serum albumin (66.2 kD), and phosphorylase B (92.5 kD). The pI markers (left lane, B) include: cytochrome C (10.2), whale myoglobin (8.2), horse myoglobin (7.4), horse myoglobin (7.0), carbonic anhydrase (6.1), ovalbumin (4.8), and aminoglucosidase (3.6).

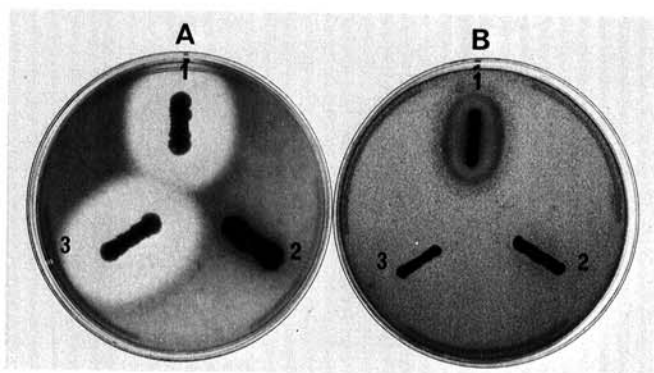

Fig. 3. Comparison of proteolytic and pectolytic activities of (1) the parent strain (SF 312 A), (2) the Out$^-$ (M1-2), and (3) the Pel$^-$ (M1-4) mutants of *Pseudomonas viridiflava* on two diagnostic agar media. A positive reaction in proteolytic activity is indicated by the formation of a clear zone on NA-milk medium (**A**) and a positive reaction in pectolytic activity indicated by the formation of a fuzzy zone on MM-9 (**B**) medium.

probe seemed to be somewhat more sensitive than the biotinated probe. The appearance of more than one hybridizing band in the *Eco*RI-generated genomic fragments of M1-2 (Fig. 5A and B, lane 1) and of Eps-1 (lane 4) mutant indicated the occurrence of secondary transposition of either Tn*5* or IS50.

Detection of 2.6-kb internal sequences of Tn*5* in the genome. The genomic DNA of the parent strain SF 312 A and of four mutants (M1-2, M1-4, Arg-1, and Eps-1) was digested with *Bgl*II and subsequently hybridized with the sulfonated pRZ102 probe. Results (Fig. 6) showed that although the pattern of hybridization bands varied with the strains, a hybridization band located at the 2.6-kb position was consistently detected in the genome of each mutant (lanes 1–4) but not in the genome of the parent strain (lane 5). This indicated that at least one copy of the Tn*5* sequences was present in the genome of each mutant. The variation in the number of hybridization bands further suggested that secondary transposition of either Tn*5* or IS50 did take place in some of the mutants.

Secondary transposition of IS50. To determine the presence of IS50 in addition to Tn*5*, the following experiment was conducted. Two blots were prepared using *Eco*RI-digested genomic DNAs of the parent strain SF 312 A and four mutants (M1-2, M1-4, Arg-1, and Eps-1). One blot was hybridized with the sulfonated pRZ102 probe and the other with the sulfonated, *Bgl*II-generated 2.6-kb probe. Because the 2.6-kb probe contained no IS50 sequences, it was expected that presence of IS50 as an independent segment in the genome would be recognized by the pRZ102 probe but not by the 2.6-kb probe. Results (Fig. 7) showed that some of hybridization bands in the *Eco*RI-generated genomic fragments of Eps-1 (Fig. 7A, lane 3) and M1-2 (Fig. 7A, lane 5) mutants were not recognized by the 2.6-kb probe (Fig. 7B, lanes 3 and 5). The "minor" bands not recognized by the 2.6-kb probe represented the IS50 sequences resulting from secondary transposition. The major bands (Fig. 7B) recognized by the 2.6-kb probe represented the Tn*5* sequences.

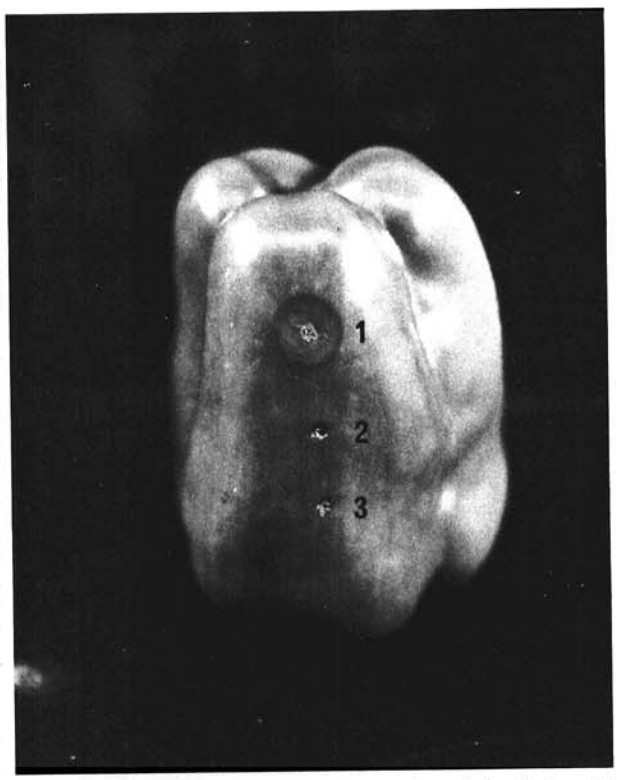

Fig. 4. Pathogenicity assays of the parent strain and the Out⁻ and Pel⁻ mutants of *Pseudomonas viridiflava* on bell pepper. Note that tissue maceration was caused by the parent strain SF 312 A (1) but not by the Out⁻ (M1-2) (2) and the Pel⁻ (M1-4) mutants (3).

DISCUSSION

Biochemical and genetic data presented here show that *P. viridiflava* produces a single PL species that determines its

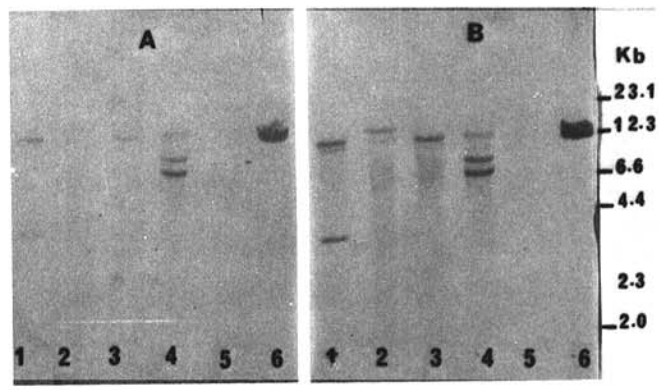

Fig. 5. Detection of Tn*5* sequences in *Eco*RI-generated genomic fragments of mutants. The pRZ102 labeled with (**A**) biotin-dCTP or with (**B**) sulfonated dCTP was used as the probe. Mutants examined were: Eps-1 (Eps⁻, lane 1), Arg-1 (Arg⁻, lane 2), M1-4 (Pel⁻, lane 3), M1-2 (Out⁻, lane 4). *Eco*RI-cleaved pSUP1011 was included as a reference (lane 6). Note that hybridization bands were detected in genomic fragments of mutants (lanes 1-4), but not of the parent strain SF 312 A (lane 5).

Table 2. Comparison of pectate lyase activities of the wild-type and Pel⁻ and Out⁻ mutants *Pseudomonas viridiflava* in culture supernatants (E) and in sonicated cell extracts (I)[a]

Incubation (hr)	Specific activity (U min^{-1} 10^{-10} cfu)[b]								
	Wild type (SF 312 A)			Pel⁻ (M1-4)			Out⁻ (M1-2)		
	E	I	% secreted	E	I	% secreted	E	I	% secreted
6	ND[c]	ND	ND	ND	ND	ND	ND	ND	ND
12	1.9	5.6	25.3	ND	ND	ND	ND	4.1	ND
18	8.7	4.3	66.9	ND	ND	ND	ND	3.9	ND
36	15.8	1.1	93.5	ND	ND	ND	0.02	5.7	0.35

[a] Grown in MY medium containing yeast extract (0.05%) and pectin (0.4%).
[b] See Materials and Methods for definition of the enzyme activity.
[c] ND, not detected.

pathogenicity. Thus, a relatively simple pectic enzyme system is involved in the elicitation of the soft-rotting disease by this bacterium. This contrasts with the production of an array of pectinases, including endo-pectin lyases, endo-polygalacturonases, and multiple species of endo-pectate lyases by most soft-rotting *Erwinia* (Chatterjee and Vidaver 1986; Collmer and Keen 1986). This multiplicity of pectinases has largely hindered the definition of the roles of the enzymes in pathogenicity. Recent evidence (Barras *et al.* 1987; Payne *et al.* 1987; Thurn *et al.* 1987), however, suggests that a single PL species (e.g., PLe of *E. chrysanthemi*) may play a dominant role, although the physiological basis for this response is not yet apparent. Based on the data available for various bacterial pathogens, including *P. viridiflava*, it appears that the basic PLs, but not the acidic or neutral (Roeder and Collmer 1985), are critical in tissue maceration and pathogenicity. Recently, a basic pectin lyase of pI 9.4, which cleaved pectin but not sodium polypectate, was also found to be the sole macerating factor in a soft-rotting strain of *P. fluorescens* (Schlemmer *et al.* 1987). Moreover, studies thus far indicate that the neutral and acidic PL species either are absent or constitute a very minor proportion of the total PL in spoilage bacteria that cause soft-rot diseases of harvested vegetables but not of field plants (this study; Schlemmer *et al.* 1987; C.-H. Liao, 1988 ASM annual meeting). It remains to be determined if the inability of the spoilage bacteria to infect living plants is due to the absence of neutral and acidic PLs.

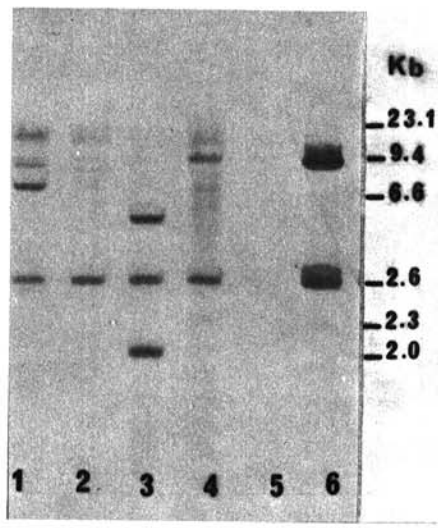

Fig. 6. Detection of the 2.6-kb internal fragment of Tn*5* in *Bgl*II-generated genomic fragments of mutants. Genomic DNAs of four mutants (Eps-1, lane 1; Arg-1, lane 2; M1-4, lane 3; M1-2, lane 4) and of the parent strain SF 312 A (lane 5) were digested with *Bgl*II and subsequently hybridized with the sulfonated pRZ102 probe. *Bgl*II-digested pSUP1011 was included as a reference (lane 6). Note that 2.6-kb internal sequences of Tn*5* was consistently detected in genomic fragments of mutants (lanes 1–4), but not of the parent strain (lane 5).

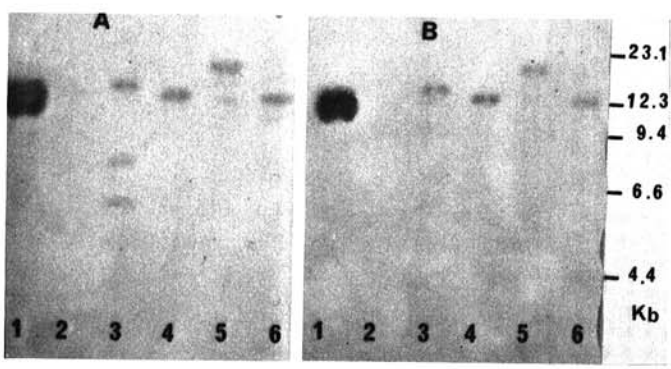

Fig. 7. Occurrence of secondary transposition of IS50 in the mutants Eps-1 and M1-2. Two sets of genomic DNA of the parent strain SF 312 A (lane 2) and of four mutants (Eps-1, lane 3; M1-4, lane 4; M1-2, lane 5; Arg-1, lane 6) were restricted similarly with the *Eco*RI. One set of genomic DNA was hybridized with the sulfonated pRZ102 probe (**A**) and another set with the sulfonated 2.6-kb fragment probe (**B**). Note that minor bands recognized by pRZ102 probe (lanes 3 and 5, A), but not by 2.6-kb fragment probe (lanes 3 and 5, B), represent IS50 sequences.

The production of a single PL species appears to be a trait common to *P. viridiflava* strains. In addition to the strain SF 312 examined in this study, seven other strains have been shown to produce in culture a single PL of nearly identical pI (9.7) (C.-H. Liao, 1988 ASM annual meeting). The ecological significance and the possible contribution to pathogen fitness of the production of a single PL by *P. viridiflava* and multiple PLs by *Erwinia* remain obscure. It is, however, unlikely that the PLs of *P. viridiflava* serve any catabolic function. None of *P. viridiflava* stains so far examined utilized polygalacturonate as a carbon source (C.-H. Liao, unpublished). The organism apparently does not possess the full complement of catabolic enzymes. In soft-rotting erwinias, pathogenicity also seems to be unrelated to polygalacturonate catabolism. No loss of pathogenicity was detected in mutants of *E. chrysanthemi* (Chatterjee *et al.* 1985) and of *E. carotovora* (C.-H. Liao, unpublished) that failed to utilize polygalacturonate as a carbon source. We therefore suggest that PL is produced by the pathogen for the sole purpose of degrading plant cell walls and thereby making nutrients within host cells accessible.

Several studies with *Erwinia* species have demonstrated the importance of pectinase export in pathogenicity (Andro *et al.* 1984; Chatterjee *et al.* 1979; Chatterjee *et al.* 1985; Chatterjee and Starr 1977; Thurn and Chatterjee 1985). Indeed, based on a comparative study of *Erwinia*, *Klebsiella*, and *Yersinia* PLs (Chatterjee *et al.* 1979), it was suggested that enzyme export was the primary factor contributing to the tissue-macerating ability of *Erwinia*. Likewise, protein export also appeared to affect pathogenicity of *Xanthomonas campestris* pv. *campestris* (Dow *et al.* 1987). The data with the Out$^-$ mutants now demonstrate the importance of enzyme export in pathogenicity of *P. viridiflava* in that the mutants invariably failed to macerate plant tissue. However, the export system of *P. viridiflava* appears distinct from those of *Erwinia* spp. In *Erwinia*, protease export is mediated by a pathway different from that for the export of PL, polygalacturonase, and cellulase (Andro *et al.* 1984; Barras *et al.* 1986; Thurn and Chatterjee 1985). In contrast, the Out$^-$ *P. viridiflava* strain was pleiotropically defective in the export of PL and protease (Fig. 3). This finding suggests that both of these enzymes share a common Out system. In addition, the total amount of PL produced by the mutant was reduced compared with the level in the Out$^+$ strain (Table 2). This may reflect a feedback control of the exported proteins, perhaps at the level of transcription, by the Out system of *P. viridiflava*. Clearly, much additional work is needed to

elucidate the molecular events in enzyme export. The relatively simple pectinase system and the occurrence of Tn*5*-insertion Out$^-$ mutants now make *P. viridiflava* an attractive system for the analysis of protein export.

The high frequency of the occurrence of secondary (multiple) transposition and some preference for the site of Tn*5* insertion in *P. viridiflava* merit comment. The predominance of the Met$^-$ mutants (i.e., 18% of the total auxotrophs) suggests that the *met* loci of *P. viridiflava* may represent hot spots for Tn*5* insertion. A similar site preference could also account for the high frequency of the occurrence of the putative Out$^-$ mutants. We should note that the existence of the preferential site (= hot spots) for the Tn*5* insertion has been detected in *X. c.* pv. *campestris* (Turner *et al.* 1984). In *P. viridiflava*, we obtained a number of mutants that carried either multiple copies of Tn*5* or a copy of IS50 in addition to a copy of Tn*5* (Figs. 5, 7). This probably resulted from secondary transposition of either the entire or a part of the Tn*5* element. The occurrence of secondary transposition of IS50 has also been detected in pathovars of *P. syringae* (Anderson and Mills 1985) and in *P. solanacearum* (Boucher *et al.* 1985). The basis for this seemingly aberrant behavior of Tn*5* in these *Pseudomonas* species is not known. Based on the knowledge of the temporal control of Tn*5* transposition (McCommas and Syvanen 1988; Rosetti *et al.* 1984), we suggest that a differential expression of the genes for transposase and the inhibitor of transposase, a differential stability of the corresponding mRNAs, or both, may account for the occurrence of secondary transpositions in these phytopathogenic bacteria.

In conclusion, the findings reported here and elsewhere (Chatterjee and Vidaver 1986; Kotoujansky 1987) reveal that the genes directing the production and secretion of PL are the two most critical pathogenicity determinants in *P. viridiflava* and in other soft-rotting bacteria as well. Our data, however, do not exclude the possibility that factors other than pectolytic activity are also required in the pathogenicity of *P. viridiflava*. Like other infectious diseases, the soft-rot pathogenesis may necessitate a series of concerted events, including infection, penetration, multiplication, and symptom expression. Theoretically, any changes in structural or catabolic functions that interrupt the above events would totally or partially abort the pathogenic response. Noteworthy in this context is the loss of pathogenicity in *E. chrysanthemi* resulting from the alterations in the outer membrane components (Expert and Toussaint 1985; Schoonejans *et al.* 1987) and in *E. carotovora* due to a deficiency in UDP glucose-pyrophosphorylase (Jayaswal *et al.* 1985). The use of generalized Tn*5* mutagenesis as demonstrated in this study should now facilitate the identification of other pathogenicity factors not related to PL activity of *P. viridiflava*.

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