Molecular Cloning, Characterization, and Mutagenesis of a *pel* Gene from *Pseudomonas syringae* pv. *lachrymans* Encoding a Member of the *Erwinia chrysanthemi* PelADE Family of Pectate Lyases

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The *pelS* gene from *Pseudomonas syringae* pv. *lachrymans* 859 was cloned by heterologous expression in nonpectolytic *P. syringae* pv. *syringae* BUVS1, using genomic DNA libraries constructed with two novel broad-host-range cosmid vectors, pCPP34 and pCPP47. Screening of *P. syringae* pv. *syringae* transconjugants for the ability to pit pectate media at pH 6.0 and 8.5 yielded several overlapping clones of the same DNA region. Ultrathin-layer isoelectric focusing gels, activity-stained with diagnostically buffered substrate overlays, revealed that this region encoded a single pectate lyase (PelS) with a pI of 9.4. *pelS* was subcloned from cosmid pCPP5020 and sequenced, revealing it to encode a member of the *Erwinia chrysanthemi* PelADE family, with highest similarity to *Pseudomonas viridiflava* PelV. A *pelS* probe hybridized at high stringency in DNA gel blots with total DNA from *P. syringae* pv. *lachrymans* strains 859 and Pla5, *P. syringae* pv. *tabaci*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *glycinea*, *P. fluorescens* (*marginalis*), *P. viridiflava*, and *Xanthomonas campestris* pv. *campestris*, but not with *P. syringae* pv. *pisi*, *P. syringae* pv. *syringae*, *P. syringae* pv. *tomato*, *P. syringae* pv. *papulans*, *E. chrysanthemi*, or *Ralstonia* (*Pseudomonas* or *Burkholderia*) *solanacearum*. The PelS sequence revealed an N-terminal signal peptide, whose processing in *Escherichia coli* was confirmed by protein sequence analysis. PelS was similar to *E. chrysanthemi* PelE in its substrate preference and ability to reduce the viscosity of pectate and to macerate potato tuber tissue. A *pelS*:: Ω Km^r mutation was marker-exchanged into *P. syringae* pv. *lachrymans* Pla5. *pelS* was also subcloned into the broad-host-range expression vector pML122 under control of the vector *nptII* promoter, and then transformed into *P. syringae* pv. *lachrymans* Pla5 to produce a strain overproducing PelS. Necrotic lesions developed in cotyledons following inoculation with all of the *P. syringae* pv. *lachrymans* Pla5 derivatives, regardless of their Pel phenotype. However, only cotyledons infected with *pelS*⁺ strains showed evidence of maceration and yielded Pel activity upon extraction. In

contrast, *pelS*⁺ *P. syringae* pv. *syringae* BUVS1(pCPP5020) produced no symptoms in cucumber cotyledons. Thus, PelS in *P. syringae* pv. *lachrymans* appears to alter the final symptoms in infected cucumber cotyledons without contributing to pathogenicity or altering host range.

Additional keyword: δ^{54} -dependent promoters.

Plant pathogenic bacteria differ widely in their capacities to produce pectic enzymes and to cause host tissue maceration. *Erwinia chrysanthemi* and *Erwinia carotovora* secrete many pectic enzymes and cause macerated soft rots in a wide range of plants (Barras et al. 1994). In contrast, the host-specific pathovars of *Pseudomonas syringae* have a weaker, variable pectolytic capacity; they typically cause foliar necroses rather than rots, and their inability to rot potato tuber tissue (which is highly sensitive to maceration by pectic enzymes) is a diagnostic character of the species (Gross and Cody 1985; Hirano and Upper 1990; Collmer and Bauer 1994). Molecular cloning and mutagenesis of *E. chrysanthemi* pectate lyase-encoding *pel* genes has revealed that the bacterium produces multiple isozymes varying in pI and inducing conditions (Hugouvieux-Cotte-Pattat et al. 1992; Kelemu and Collmer 1993; Barras et al. 1994; Alfano et al. 1995; Lojkowska et al. 1995), that isozyme multiplicity may contribute to the wide host-range of the pathogen (Beaulieu et al. 1993), and that no single isozyme is essential for maceration—although isozymes with very alkaline pIs, like PelE of strain EC16, are particularly important (Payne et al. 1987; Ried and Collmer 1988; Beaulieu et al. 1993). Relatively little is known about the *P. syringae* pectic enzymes, and their contribution to pathogenesis has awaited genetic testing.

The pectic enzymes of two other plant-pathogenic, fluorescent pseudomonads, *P. fluorescens* and *P. viridiflava*, have been partially characterized. Both bacteria are opportunistic pathogens that cause postharvest soft rots. Plant pathogenic *P. fluorescens* strains (often referred to as *P. marginalis*) produce pectin methylesterase, polygalacturonase, pectin lyase, and two pectate lyase (Pel) isozymes (Nasuno and Starr 1966; Schlemmer et al. 1987; Liao 1989). *P. viridiflava* produces a single Pel, which has a very alkaline pI, like the major Pel isozyme of *P. fluorescens* (Liao et al. 1988; Liao 1989). *pel* genes have been cloned from *P. fluorescens* and *P. viridiflava*

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(Liao 1991; Liao et al. 1992; Nikaidou et al. 1993), and DNA sequence analysis has revealed the *P. fluorescens* and *P. viridiflava* PelS to be members of the *E. chrysanthemi* PelADE family (Liao et al. 1996). The *P. viridiflava pel* gene has been mutated, revealing it to be essential for soft-rot pathogenesis (Liao et al. 1988).

At least some of the *P. syringae* pathovars also produce pectic enzymes. This is indicated by their ability to pit pectate gels that are differentially buffered to detect the activity of polygalacturonase (low pH) or Pel (high pH). Some pathovars, like *P. syringae* pv. *lachrymans*, cause pitting at both pHs, others, like *P. syringae* pv. *tomato* and *P. syringae* pv. *glycinea*, cause pitting only at low pH, and still others, like *P. syringae* pv. *syringae*, cause no pitting at either pH (Hildebrand 1971). Although the pectate gel pitting test is used in the identification of *P. syringae* pathovars (Palleroni 1984), it may underestimate the genetic potential of these bacteria to produce pectic enzymes. For example, *P. syringae* pv. *glycinea* has been reported to produce two Pel isozymes with alkaline pIs, even though its plate-pitting phenotype predicts that it produces only polygalacturonase (Magro et al. 1994). Reports that polygalacturonase can be extracted from plant tissues infected with *P. syringae* pv. *tomato* and *P. syringae* pv. *phaseolicola* raise the possibility that pectic enzymes may have some function in pathogenesis (Bashan et al. 1985; Longland et al. 1992).

To initiate investigation of the role of pectic enzymes in the plant pathogenicity of *P. syringae*, we have cloned and characterized *P. syringae* pv. *lachrymans pelS*, which encodes a Pel with a highly alkaline pI. The cloned *pelS* was used to probe other *P. syringae* pathovars for the presence of a homolog, to marker-exchange a mutation into *P. syringae* pv. *lachrymans*, and to determine the effects on pathogenicity of

heterologous expression of the gene in *P. syringae* pv. *syringae*. A preliminary account of portions of this work has been published (Collmer et al. 1991).

RESULTS

Cloning of a *P. syringae* pv. *lachrymans pel* gene by heterologous expression in *P. syringae* pv. *syringae*, using improved cosmid vectors.

A library of *P. syringae* pv. *lachrymans* strain 859 DNA fragments, partially digested with *Sau3AI*, was constructed in cosmid pCPP34 and transduced into *Escherichia coli* ED8767. The construction of pCPP34 is described in the Materials and Methods, and its useful features are presented in Figure 1. One thousand *E. coli* transductants were screened for pectolytic activity at pH 8.5 and pH 6.0 on pectate gel medium (Hildebrand 1971). No pectolytic clones were found, suggesting that any *pel* genes in the library were poorly expressed in *E. coli*. Consequently, the DNA library was conjugated en masse by triparental mating with *E. coli* DH5 α (pRK2013) into *P. syringae* pv. *syringae* BUVS1, a *recA* derivative of the bean pathogen B728a, which has no detectable pectic enzyme activity (Willis et al. 1988). Eight transconjugants with the ability to pit pectate gels at both pH values were identified among 700 tested. Activity-stained, isoelectric focusing gels revealed that all produced a single band of activity with a pI of approximately 9.4 when assayed at pH 8.5, but no band was visible when assayed at pH 5.5 in the presence of EDTA, which are conditions intended to reveal polygalacturonase activity but inhibit Pel activity (data not shown). This differential activity with diagnostically buffered, activity-staining overlays suggested that a single *pel* gene had been cloned (Ried and Collmer 1985).

Fig. 1. Two broad-host-range cosmid vectors used to clone a pectic enzyme gene from *Pseudomonas syringae* pv. *lachrymans* 859 by heterologous expression in nonpectolytic *P. syringae* pv. *syringae* BUVS1. Abbreviations: *cos*, cohesive site from λ ; *par*, partition locus from RK2; *oriV*, origin of replication from RK2; *oriT*, origin of transfer from RK2; *Tc^r*, tetracycline resistance gene from RK2; *Ap^r*, ampicillin resistance (*bla*) gene from pBR322; T7, T7 RNA polymerase promoter; T3, T3 RNA polymerase promoter. Restriction sites: H, *Hind*III; Nr, *Nru*I; S, *Sac*I; Xh, *Xho*I; Bg, *Bgl*II; X, *Xba*I; EV, *EcoRV*; C, *Cla*I; E, *Eco*RI; N, *Not*I; B, *Bam*HI; K, *Kpn*I; Sc, *Sca*I; Sp, *Sph*I; Sm, *Sma*I; Sa, *Sal*I; Bs, *Bsa*I; St, *Stu*I.

Restriction digests revealed that five of the eight pectolytic cosmids were different, but all contained overlapping *P. syringae* pv. *lachrymans* DNA inserts (not shown). One of the cosmids directing Pel production was chosen for further characterization and designated pCPP5020. Cosmid pCPP47, a more stable derivative of pCPP34 (Fig. 1), was used to construct a second library of *P. syringae* pv. *lachrymans* strain 859 DNA. Among 250 recombinant pCPP47 *P. syringae* pv. *syringae* BUVS1 transconjugants analyzed, two were pectolytic, produced the same Pel activity, and harbored inserts that overlapped with the previously cloned pectolytic cosmids. Thus, although we used a highly sensitive screening assay that would have detected any pectate-inducible Pel or polygalacturonase activity expressed in *P. syringae*, we found only a single *pel* gene. Various Pel⁺ subclones from pCPP5020 were subsequently constructed for sequencing (pCPP5022), marker-exchange mutagenesis (pCPP5021), expression from the *npIII* promoter in trans in *P. syringae* pv. *lachrymans* (pCPP2275), and T7 promoter/polymerase-dependent expression in *E. coli* (pCPP2191).

Nucleotide sequence of *P. syringae* pv. *lachrymans pelS* reveals a product with similarity to the Pels of highly pectolytic pseudomonads and PelE of *E. chrysanthemi*.

DNA sequencing of the *pelS* locus in pCPP5022 revealed an open reading frame (ORF) encoding a predicted protein of 40.3 kDa (Fig. 2). The deduced prePelS has a 29-amino-acid, N-terminal signal peptide (von Heijne 1985), whose processing would leave a 37.4-kDa mature protein. The predicted N-terminal 12 amino acids of mature PelS produced by an *E. coli* transformant were confirmed by protein sequencing (Fig. 2). A typical ribosome-binding site was located 10 bp upstream of the start of the *pelS* ORF. No σ^{70} promoter could be detected in the 124 nucleotides sequenced upstream of the *pelS* initiation codon. However, a potential σ^{54} promoter was discerned between 43 and 60 bp upstream of the start site. This sequence featured -24 GG and -12 GC motifs, with the latter preceded by typical TT residues (Shingler 1996). An inverted repeat was found downstream of *pelS*, but it was not followed by the T-rich region typical of rho-independent terminators. These observations suggest that the *pelS* operon may contain no other genes.

PelS showed strong similarity to members of the *E. chrysanthemi* PelE family (Fig. 3). Similarity was weakest in the signal peptide and C-terminal regions. As determined by the DNASTar program of Hein (1990), PelS was 79% similar to *P.*

viridiflava PelIV, 78% similar to *P. fluorescens* PelF, 76% similar to *X. campestris* pv. *malvacearum* PelX, and 42% similar to *E. chrysanthemi* PelE. The highly conserved regions included the four previously noted by Liao et al. (1996) for three other members of the PelE family from bacteria in the Pseudomonadaceae. Also conserved were 2 amino acids that are likely to play a key role in oligosaccharide cleavage, Asp¹⁶⁹ and Arg²⁶⁴ (Lietzke et al. 1996).

Sequences hybridizing with *pelS* are present in several other, but not all, *P. syringae* pathovars.

To determine the distribution of *pelS* among representative *P. syringae* pathovars and other bacteria, we probed a DNA gel blot of *EcoRI*-digested total DNA with a 1.2-kb *BsrGI* fragment from pCPP5022. This fragment contains the entire *pelS* ORF plus 29 bp of 5' DNA and 17 bp of 3' DNA. The *pelS* probe hybridized with DNA from *P. syringae* pv. *lachrymans* 859 and Pla5, *P. syringae* pv. *tabaci*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *glycinea*, *P. fluorescens* (*marginalis*) 1152 and 1772, *P. viridiflava*, and *X. campestris* pv. *campestris*, but not with *P. syringae* pv. *pisi*, *P. syringae* pv. *syringae*, *P. syringae* pv. *tomato*, *P. syringae* pv. *papulans*, *E. chrysanthemi*, or *Ralstonia* (*Pseudomonas* or *Burkholderia*) *solanacearum* CR10 and GMIT (Fig. 4). Hybridization was noticeably stronger with the *P. syringae* strains, and the same-sized *EcoRI* fragment carried *pelS* in both *P. syringae* pv. *lachrymans* strains.

P. syringae pv. *lachrymans* PelS and *E. chrysanthemi* PelE have similar enzymological properties.

PelS was purified from *E. coli* BL21(DE3) expressing *pelS* from the T7 promoter in pCPP2191. Bacteria were grown overnight in the presence of isopropyl- β -D-thiogalactopyranoside to induce production of the T7 polymerase, after which most of the Pel activity was in the culture supernatant (presumably leaked from the periplasm). PelS was then purified from culture supernatants by ammonium sulfate precipitation and affinity chromatography on a Sepharose 6B column (Davis et al. 1984). PelE was similarly obtained from culture fluids of *E. coli* JA221(pPEL748) (Keen and Tamaki 1986).

The pH optimum of PelS was between 8.0 and 8.5. The enzyme had approximately the same level of activity on polygalacturonic acid and pectin with 31 and 68% methylesterification (3.01, 3.23, and 2.81 units/ml, respectively), but sixfold lower activity on 93% methylesterified pectin. PelS and PelE had the same relative ability to reduce the viscosity of polygalacturonic acid (data not shown). To assess the relative maceration capacity of the two enzymes, potato tuber disks (0.7 $\times$ 10 mm) were incubated in Pel assay buffer containing identical dilution series of PelS and PelE, beginning with a highest level of 0.25 units/ml. After incubation for 4 h, the cohesiveness of the tissue was tested by teasing with toothpicks. The potato tissue was macerated at the same end-point dilutions in three independent tests, indicating that PelS and PelE were approximately equal in their maceration efficiency.

P. syringae strains altered in their production of *pelS* reveal no apparent role for PelS in virulence or determination of host range.

To mutate the *pelS* gene in *P. syringae* pv. *lachrymans*, we disrupted *pelS* in pCPP5021 by in vitro insertion of the Ω Km^r

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1  GACTATATTCAATCAACAGCAACATTCTGTTGCGCTACTGCCTTGGCA
51  ACAGGATGTTTGCTGTGCG ATACGCAATT AATAACTGCACGTTGTACA
101  CACATTAATCAAGGAATAGATTATATGTTGAAGCCTCATGGTTTAACGCC
      M L K P H G L T P
151  GCTCGCATTGACGGGCGGCATTCTTGATCGCTATTGTCTGTCAGCCTGT
      L A I T G G I L V S L L S V S L S
201  CTGCCCACGCCGAATCGCCACCGATGTTGCCACTACCGGTTGGGCTACT
      A H A E I A T D V A T T G W A T
251  CAAAACGGGGGCACCAAGGGTGGTTCGAGGGCAGCAGCAATAACATTTA
      Q N G G T K G G S R A A A N N I Y

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Fig. 2. DNA sequence of beginning of *pelS* and its predicted product. Putative σ^{54} promoter and ribosome-binding sequences are marked by double underlining and single underlining, respectively. Predicted PelS signal peptide is underlined. Amino acids at N terminus of mature PelS, confirmed by N-terminal sequencing, are double underlined. The *pelS* sequence has been assigned GenBank accession number U75414.

interposon (Fellay et al. 1987) and then marker-exchanged the mutation into *P. syringae* pv. *lachrymans* Pla5. This strain was used because we could not obtain transformants or transconjugants with strain 859. The *pelS* mutation in *P. syringae* pv. *lachrymans* CUCPB1773 was confirmed by both polymerase chain reaction and DNA gel blot analysis (data not shown). To heterologously express *pelS* in *P. syringae* pv. *lachrymans*, we recloned the *pelS* ORF from pCPP5022 into pML122 such that *pelS* was expressed from the vector *nptII* (*aphII*) promoter. The resultant plasmid pCPP2275 was electrotransformed into *P. syringae* pv. *lachrymans* Pla5 to produce a strain that produced more PelS than the wild type (Table 1).

P. syringae strains with altered abilities to produce PelS were tested for their pathogenicity and ability to cause maceration in cucumber cotyledons and potato tuber tissue (Table 2). *P. syringae* pv. *syringae* BUVS1 (a pathogen of bean) did not produce any symptoms in cucumber cotyledons, even when carrying *pelS*⁺ cosmid pCPP5020. *P. viridiflava* was similarly nonpathogenic in cucumber cotyledons, although it was alone among the strains tested in causing maceration in potato tuber tissue. In contrast, all *P. syringae* pv. *lachrymans* strains were pathogenic regardless of their PelS phenotype.

In an attempt to detect any minor contribution of PelS to the virulence of *P. syringae* pv. *lachrymans*, we inoculated cucumber cotyledons with Pla5 and CUCPB1773 at concentrations of 10^4 and 10^5 CFU/ml, which were estimated from pilot tests to be near the ED₅₀ for *P. syringae* pv. *lachrymans* Pla5.

The ability of several plant-pathogenic members of the Pseudomonadaceae to macerate potato tubers and other fleshy plant tissues is associated with their production of large amounts of a highly alkaline member of the *E. chrysanthemi*

Fig. 3. Comparison of the predicted amino acid sequences of *Pseudomonas syringae* pv. *lachrymans* PelS, *P. viridiflava* PelV (Liao et al. 1996), *P. fluorescens* PelF (Liao et al. 1996), *Xanthomonas campestris* pv. *malvacearum* PelX (Liao et al. 1996), and *Erwinia chrysanthemi* PelE (Keen and Tamaki 1986). The sequences were aligned by the DNASTar method of Hein (1990).

PelADE family. The newly characterized PelS of *P. syringae* pv. *lachrymans* is a member of this group. However, the bacterium does not macerate potato tuber tissue, and although PelS appears to be the only pectic enzyme produced (and causes discernible maceration in infected cucumber cotyledons), the enzyme does not appear to affect the pathogenicity or host specificity of *P. syringae* pv. *lachrymans*.

Direct comparison of PelE and PelS revealed no fundamental differences in enzymological properties that would indicate different roles in pathogenesis. Most importantly, PelS and PelE were able to macerate potato tuber tissue with equal efficiency. Thus, the inability of *P. syringae* pv. *lachrymans* to rot potato tuber tissue must result from differences in Pel deployment and/or deficiencies in other bacterial adaptations to host-promiscuous pathogenesis. In fact, PelS is produced at a very low level by *P. syringae* pv. *lachrymans*, and this prevented us from determining whether the protein was specifi-

cally secreted by the pathogen. However, all other members of the PelADE family that have been analyzed in this regard appear to be secreted by the type II pathway (Barras et al. 1994), and PelS is like them in possessing an N-terminal signal peptide that is cleaved upon Sec-mediated translocation to the periplasm of *E. coli* transformants.

The distribution of *pelS* homologs among different pathovars of *P. syringae* was generally consistent with the observations of Hildebrand (1971). As predicted from the differential pitting of pectate gels at alkaline pH, *P. syringae* pv. *tabaci* possessed a *pelS* homolog, whereas *P. syringae* pv. *syringae*, *P. syringae* pv. *pisi*, and *P. syringae* pv. *tomato* did not. However, *P. syringae* pv. *glycinea* and *P. syringae* pv. *phaseolicola*

Table 1. Pel activity of *Pseudomonas syringae* pv. *lachrymans* wild-type and *pelS* mutant strains in culture and in planta

<i>P. syringae</i> pv. <i>lachrymans</i> strain	Pel activity	
	In culture ^a	In planta ^b
Pla5 (wild-type)	0.045	0.131
CUCPB1773 (<i>pelS</i> ::ΩKm ^r Pla5)	0.000	0.006 ^c
Pla5(pCPP2275 <i>pelS</i> ⁺)	0.220	0.498

^a Pel activity (units/ml) of overnight (early stationary phase) cultures grown in King's B medium.

^b Pel activity (units/cm²) in lesions 2 days post inoculation.

^c Not significantly above the background variation caused by plant extracts.

Fig. 4. DNA gel blot of total DNA from representative *Pseudomonas syringae* pathovars and other bacteria hybridized at high stringency with a *pelS* probe. *Eco*RI-digested DNA fragments were resolved in a 0.7% agarose gel, transferred to Immobilon N membrane (Millipore, Bedford, MA), and then probed with the ³²P-labeled, 1.2-kb *Bsr*GI *pelS* open reading frame fragment. Strains (abbreviations): *P. syringae* pv. *lachrymans* (Psl), *P. syringae* pv. *tabaci* (Psta), *P. syringae* pv. *syringae* (Pss), *P. syringae* pv. *phaseolicola* (Psph), *P. syringae* pv. *glycinea* (Psg), *P. syringae* pv. *pisi* (Pspi), *P. syringae* pv. *tomato* (Psto), *P. syringae* pv. *papulans* (Pspa), *P. fluorescens* (marginalis) (Pf), *P. viridiflava* (Pv), *Erwinia chrysanthemi* (Ech), *Xanthomonas campestris* pv. *campestris* (Xcc), *Ralstonia solanacearum* (Rsol).

Fig. 5. Symptoms in cucumber cotyledons produced by *Pseudomonas syringae* pv. *lachrymans* Pla5 and *pelS* mutant CUCPB1773. Cotyledons were inoculated 4 days previously with 1×10^5 CFU/ml of the indicated strains or mock inoculated with 10 mM MgCl₂ (control), then photographed through a Wratten 29 filter to enhance black and white visualization of the chlorotic lesions. Partially macerated cotyledon inoculated with *P. syringae* pv. *lachrymans* Pla5 has a wrinkled appearance, the result of handling the seedling in preparation for photography.

Table 2. Plant reactions to bacteria differing in Pel production

Strain	Pel phenotype	Cucumber cotyledon		Potato tuber maceration ^c
		Necrotic lesion ^a	Maceration ^b	
<i>Pseudomonas syringae</i> subsp. <i>syringae</i> BUVS1	–	–	–	ND ^d
<i>P. syringae</i> subsp. <i>syringae</i> BUVS1 (pCPP5020 <i>pelS</i> ⁺)	+	–	–	ND
<i>P. viridiflava</i>	+	–	–	+
<i>P. syringae</i> pv. <i>lachrymans</i> Pla5	+	+	+	–
<i>P. syringae</i> pv. <i>lachrymans</i> CUCPB1773 (<i>pelS</i> ::ΩKm ^r)	–	+	–	–
<i>P. syringae</i> pv. <i>lachrymans</i> Pla5 (pCPP2275 <i>pelS</i> ⁺)	++	+	+	–

^a + denotes the appearance of any necrosis within 5 days after inoculation.

^b + indicates that the cotyledon tissue disintegrated when teased apart with toothpicks; if the tissue was merely flaccid, it was scored as –.

^c + denotes maceration detectable by probing with toothpicks in the tissue surrounding the small well in the tuber slice where the inoculum was applied 2 days previously.

^d Not determined.

were not predicted by this assay to produce Pel activity (Hildebrand 1971), but they hybridized strongly with the *pelS* probe. Actually, *P. syringae* pv. *glycinea* NCPPB 2411 had already been shown with activity-stained, isoelectric focusing gels to produce two Pel isoforms with pIs of 9.0 and 9.5 (Magro et al. 1994). Because we analyzed different strains, we cannot determine if the multiple bands of Pel activity from NCPPB 2411 reflect two *pel* genes or are an artifact of isoelectric focusing, as we previously noted with *E. chrysanthemi* Pels (Collmer et al. 1985). Similarly, bean leaf lesions containing compatible *P. syringae* pv. *phaseolicola* NCPPB1341 reveal polygalacturonase activity but not Pel activity after 2 days (Longland et al. 1992). It is possible that *pelS* is cryptic in *P. syringae* pv. *phaseolicola* strains or simply missing from strain NCPPB1341. Because we sometimes had trouble detecting the low level of Pel activity in *P. syringae* pv. *lachrymans*, we feel that it is likely that pectic enzyme genes may be distributed more widely than predicted by gel-pitting assays. As expected from the levels of sequence similarity, *pelS* hybridization was observed with other species: *P. fluorescens*, *P. viridiflava*, and *X. campestris* (but not *R. solanacearum*). Apparent homologs of the *E. chrysanthemi pelE* gene have also been reported in all *X. campestris* pathovars tested (Beaulieu et al. 1991; Liao et al. 1996). The wide, but scattered, distribution of this gene raises the possibility that it has been transferred horizontally among these plant-pathogenic bacteria. Further evidence for this hypothesis would come if discrepancies were found in phylogenetic trees based on the sequences of additional *pelE* homologs and on 16S rDNA (Smith et al. 1992).

pelS joins a growing list of genes controlling potential *P. syringae* disease factors (e.g., toxins, phytohormones, and ice nucleation activity) that are variable in their distribution and effect on pathogenesis among the pathovars (Gross 1991; White and Zeigler 1991; Edwards et al. 1994). Hirano and Upper (1990) have noted the remarkable pathogenic diversity of *P. syringae* strains. The possibility that some of this genetic diversity has arisen through horizontal gene transfers with other bacteria sharing the plant niche is intriguing.

Our observations suggest that *pelS* is the only gene encoding an endolytic pectic enzyme carried by *P. syringae* pv. *lachrymans* and that the gene is expressed in planta: all of the recombinant cosmids conferring pectolytic activity to *P. syringae* pv. *syringae* BUVS1 contained *pelS*; only subclones carrying *pelS* had pectolytic activity; and wild-type *P. syringae* pv. *lachrymans* produced Pel activity and maceration in cucumber leaves, whereas *pelS* mutants did not. Because exolytic pectic enzymes produce a weaker phenotype in pectate pitting assays and do not cause maceration, we cannot dismiss the possibility that *P. syringae* pv. *lachrymans* produces one or more of these enzymes.

The regulation of *pelS* has not been characterized. Based on the relatively weak pitting of pectate semisolid agar and the low levels of Pel activity in broth cultures, Pel production by *P. syringae* pv. *lachrymans* is minor compared with that of soft-rot pathogens like *P. viridiflava* and *E. chrysanthemi*. In an attempt to increase PelS production, we cultured *P. syringae* pv. *lachrymans* 859 in *hrp*-derepressing minimal medium (Huynh et al. 1989) and in M9 minimal medium supplemented (in place of glucose) with 10% (vol/vol) extract of autoclaved cucumber fruit, 1% polygalacturonic acid, or 1% of highly

methylesterified pectin. None of these resulted in a substantial increase in Pel production over that observed with King's B or nutrient broth medium (data not shown), and the possibility remains that *pelS* is strongly expressed only in planta.

The DNA sequence suggests that *pelS* is expressed in a σ^{54} (RpoN)-dependent manner. The latter point is intriguing because putative σ^{54} -dependent promoters have been found upstream of several genes associated with pathogenicity in *P. syringae*, including some *hrp* (hypersensitive response and pathogenicity), *avr* (avirulence), and *tbl* (tabtoxin biosynthesis) genes (Willis et al. 1994). *tblA* is one of several genes regulated by LemA, a positive regulator required by *P. syringae* for lesion formation but not parasitic multiplication or elicitation of the hypersensitive response (Barta et al. 1993). Interestingly, Pel production in *P. viridiflava* is dependent on a LemA homolog (Liao et al. 1994). This suggests the testable hypothesis that *pelS* expression in *P. syringae* pv. *lachrymans* is similarly regulated by LemA and is thus a component of a battery of factors, including toxins and proteases, that are associated with the formation of disease lesions.

Pectic enzymes elicit defense responses in plants through the generation of oligouronide products with elicitor activity (Collmer and Keen 1986). Indeed, pretreatment of tobacco leaf tissue with low levels of purified *E. chrysanthemi* PelC can induce resistance against challenge with the compatible pathogen *P. syringae* pv. *tabaci* (Baker et al. 1986). Furthermore, the polygalacturonase isolated from bean leaves infected with *P. syringae* pv. *phaseolicola* is a potent elicitor of bean phytoalexins (Longland et al. 1992). Given these observations, we were puzzled to observe that the watersoaked lesions resulting from the compatible interaction of *P. syringae* pv. *lachrymans* with cucumber cotyledons are at least partially macerated. As noted by Longland et al. (1992), defense responses are elicited in the later stages of compatible interactions, and the contribution of pectic enzymes to defense elicitation at a late stage may not make much difference. Alternatively, *P. syringae* appears to actively suppress defense responses in compatible interactions (Jakobek et al. 1993), and it may thereby block defense expression that would result from low levels of pectic enzyme activity early in the interaction.

Finally, it is interesting to contrast the contributions to virulence of the homologous *E. chrysanthemi* PelE, *P. viridiflava* PelV, and *P. syringae* pv. *lachrymans* PelS proteins. *E. chrysanthemi* EC16 has a spontaneous deletion in *pelD*, which encodes an alkaline pI homolog of PelE that is present in most *E. chrysanthemi* strains (Tamaki et al. 1988). Mutation of *pelE* in strain EC16 reduces maceration virulence by 50% (Payne et al. 1987). The potential importance of PelE in maceration is further supported by the observation that *E. coli* strains highly expressing cloned *pelE* are able to macerate potato tuber tissue (Keen and Tamaki 1986; Payne et al. 1987). Similarly, mutation of *P. viridiflava pel* abolishes the ability of the bacterium to produce any symptoms in its market vegetable hosts (Liao et al. 1988). Thus, PelE-like proteins appear to be key determinants of the host-promiscuous, soft-rot pathogenicity of these two taxonomically distant bacteria. Given the scattered distribution of PelE homologs among *P. syringae* pathovars, the challenge now is to learn how this plant-destructive protein contributes to the fitness of these bacteria and why it does not disrupt a pathogenic life style based on host-specific compatibility rather than rapid host killing and maceration.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids are listed in Table 3. *E. coli* strains were grown in LM broth (Hanahan 1985) or on LM agar plates at 37°C. For PelS production, *E. coli* was grown at 28 to 30°C. *P. syringae* strains were grown in King's B (King et al. 1954) broth or agar medium at 28 to 30°C. Initial screening of DNA libraries in *P. syringae* pv. *syringae* BUVS1

and *E. coli* transductants was done on the pectate medium of Hildebrand (1971) at pH 6.0 and 8.5. The pectate semisolid agar medium of Starr et al. (1977) was used to assay PelS production by *E. coli* transformants during subcloning. Antibiotics were used where appropriate at the following concentrations (μg/ml): ampicillin (100, but 200 for pET21 and derivatives), gentamycin (10), kanamycin (50), rifampicin (40), spectinomycin (50 for *E. coli*, 100 for *P. syringae* pv. *lachrymans*), and tetracycline (10).

Table 3. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics	Reference or source
<i>Escherichia coli</i>		
ED8767	<i>supE44 supF58 hsdS3(r_B⁻m_B⁻) recA56 galk2 galT22 metB1</i>	Sambrook et al. 1989
DH5α	<i>supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> NaI ^r	Hanahan 1985; Life Technologies, Inc. (Gaithersburg, MD)
BL21(DE3)	F ⁻ <i>ompT hsdS_B(r_B⁻m_B⁻) gal dcm</i> (DE3)	Studier et al. 1990; Novagen (Madison, WI)
JA221(pPEL748)	Plasmid carries <i>Erwinia chrysanthemi pelE</i> highly expressed from triple <i>lac</i> UV5 promoters	Keen and Tamaki 1986
<i>Pseudomonas syringae</i> strains		
pv. <i>lachrymans</i> 859	Wild type	Leben 1988
pv. <i>lachrymans</i> Pla5	Wild type	S. V. Beer
pv. <i>lachrymans</i> CUCPB1773	<i>pelS</i> ::ΩKm ^r derivative of Pla5	This work
pv. <i>syringae</i> B728a	Wild type; Rif ^r	Willis et al. 1988
pv. <i>syringae</i> BUVS1	<i>recA4</i> ::ΩSp ^r Sm ^r derivative of B728a	D. K. Willis
pv. <i>syringae</i> 61	Wild type	Huang et al. 1988
pv. <i>tabaci</i> 11528	Wild type	American Type Culture Collection
pv. <i>phaseolicola</i> 343	Wild type	M. Sasser
pv. <i>glycinea</i> race 4	Wild type	N. T. Keen
pv. <i>pisii</i> 11	Wild type	M. M. Atkinson
pv. <i>tomato</i> DC3000	Wild type	D. A. Cuppels
pv. <i>papulans</i> 19	Wild type	T. J. Burr
<i>P. fluorescens</i> (<i>marginalis</i>) 1152	Wild type	R. S. Dickey
<i>P. fluorescens</i> (<i>marginalis</i>) 1772	Wild type	R. S. Dickey
<i>P. viridiflava</i> PV-5	Wild type	N. T. Keen
<i>Erwinia chrysanthemi</i> AC4150	NaI ^r derivative of EC16	Chatterjee et al. 1983
<i>Xanthomonas campestris</i> pv. <i>campestris</i> B-24	Wild type	R. S. Dickey
<i>Ralstonia solanacearum</i> CR10	Wild type	C. Stockwell
<i>R. solanacearum</i> GMIT	Wild type	C. Stockwell
Plasmids		
pMP92	Broad-host-range IncP vector, Tc ^r	Spaink et al. 1987
pSUP106	pACYC184-derived cosmid, Cm ^r Tc ^r	Priefer et al. 1985
pMTL24	Plasmid vector, Ap ^r	Chambers et al. 1988
pWE15	Cosmid vector, Ap ^r	Wahl et al. 1987
pCPP34	Broad-host-range cosmid derived from pMP92, tandem <i>cos</i> ⁺ , Tc ^r	This work
pBR322	Plasmid vector, Ap ^r Tc ^r	Bolivar et al. 1977
RK2	IncP replicon, Ap ^r Km ^r Tc ^r Tra ⁺	D. Coplin
pCPP47	Broad-host-range cosmid derived from pCPP34, tandem <i>cos</i> ⁺ , par ⁺ , Tc ^r	This work
pRK2013	ColE1 replicon, Km ^r , Tra ⁺	Figurski and Helinski 1979
pBluescript II KS(-)	Plasmid vector, Ap ^r	Stratagene
pML122	RSF1010-derived broad-host-range expression vector, Gm ^r	Labes et al. 1990
pET21	T7 promoter/polymerase expression vector, Ap ^r	Novagen
pSF6	Broad-host-range IncW cosmid vector, Sp ^r Sm ^r	Selvaraj et al. 1984
pHP45ΩKm	pBR322 derivative carrying Km ^r interposon	Fellay et al. 1987
pCPP5020	pCPP34 carrying <i>pelS</i> ⁺ 35.9-kb fragment from <i>P. syringae</i> pv. <i>lachrymans</i> 859	This work
pCPP5021	<i>pelS</i> ⁺ 4.5-kb <i>Sau3AI</i> fragment from pCPP5020 in pBluescript II KS-	This work
pCPP5022	<i>pelS</i> ⁺ 2.5-kb <i>Sau3AI</i> fragment from pCPP5020 in pBluescript II KS-	This work
pCPP2326	pSF6 carrying <i>pelS</i> ::ΩKm ^r <i>XbaI-EcoRI</i> fragment from pCPP5021	This work
pCPP2275	<i>pelS</i> ⁺ 2.5-kb <i>XbaI-HindIII</i> fragment from pCPP5022 in pML122	This work
pCPP2191	pET21 carrying 2.5-kb <i>EcoRI-NotI</i> fragment from pCPP5022 with <i>pelS</i> downstream of T7 promoter	This work

General DNA manipulations.

General DNA manipulations were performed by standard techniques (Sambrook et al. 1989). Plasmid DNA was isolated by a modified alkaline lysis procedure (Feliciello and Chinali 1993). *E. coli* DH5 α was transformed according to Hanahan (1985). *E. coli* BL21(DE3) and *P. syringae* pv. *lachrymans* Pla5 were transformed by electroporation according to the following procedure. Bacteria were grown overnight on LM agar (to reduce exopolysaccharide production). A loopful of cells was suspended in 1.5 ml of ice-cold 10% glycerol, 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 6.8. The bacteria were washed 3 times and finally resuspended in the glycerol solution. Two microliters of miniprep DNA was added, and the bacteria were electroporated at 12.5 kV/cm, 200 Ω , 25 μ F with a Bio-Rad (Richmond, CA) Gene-Pulser electroporator. DNA gel blot hybridization was done at 65°C with standard techniques (Sambrook et al. 1989), except that prehybridization and hybridization were done in Hyb-9 solution (Gentra Systems, Inc., Research Triangle Park, NC).

Construction of cosmids pCPP37 and pCPP47.

To construct cosmid vectors that were easier to manipulate and able to clone DNA inserts larger than those achievable with the pLAFR series of vectors (Friedman et al. 1982), we first investigated several small, broad-host-range plasmids for stability in *P. syringae* pv. *syringae*. Of those tested, pMP92 (Spaink et al. 1987) was the most stable in the absence of antibiotic selection and was chosen for cosmid construction. Tandem *cos* sites separated by a unique blunt-end restriction site, and thereby suitable for the rapid cloning procedure of Bates and Swift (1983), were incorporated into the vector. Two *cos* sites originally isolated as 400-bp *Pst*I fragments from pSUP106 (Priefer et al. 1985) were cloned in tandem, with an *Stu*I site separating them, in pMTL24 (Chambers et al. 1988), resulting in pMTL24cos2. pMP92 was previously modified by removing the unique *Stu*I site by brief digestion with *Bal*31 and religation. An *Eco*RI fragment from pWE15 (Wahl et al. 1987), containing a *Bam*HI site flanked by T3 and T7 promoters and *Not*I sites designed for rapid mapping was put into the *Eco*RI site of the modified pMP92. The tandem *cos* sites from pMTL24cos2 were then incorporated into the vector, producing pCPP34.

We encountered three problems with pCPP34 and recombinant derivatives: (i) recombination between the *cos* sites frequently resulted in deletion of one of them; (ii) many derivatives with inserts were unstable without antibiotic selection; and (iii) deletion of insert DNA was sometimes observed. These problems were diminished with the following modifications. First the *bla* (ampicillin resistance) gene was excised from a partial *Bsp*HI digest of pBR322 (Bolivar et al. 1977) and ligated into the *Stu*I site between the tandem *cos* sites to produce pCPP37. *bla* contains unique *Sca*I and *Bsa*I sites that can be used in the rapid cosmid-cloning procedure. Second, a 3.2-kb *Sac*II-*Cla*I fragment with the partition region from plasmid RK2 was ligated between the *Xho*I and *Cla*I sites of pCPP37 to generate pCPP47. This plasmid had better stability in the absence of antibiotic selection. Stability tests involving repeated transfers in King's B broth without antibiotics revealed that losses per generation were 0.13% for pLAFR5, 0.25% for pMP92, 0.49% for pCPP34, 0.55% for pCPP37, and 0.06% for pCPP47. Furthermore, deletions were encoun-

tered much less frequently, with only one in 20 cosmids with random inserts showing evidence of deletion in 70 generations.

pCPP47 has the advantages of having (i) the broad-host-range IncP origin of replication from RK2, (ii) *oriT* from RK2, permitting conjugation in the presence of RK2 transfer functions, (iii) tetracycline resistance, which affords selection in most gram-negative bacteria, (iv) tandem *cos* sites for rapid library construction, (v) a unique *Bam*HI site flanked by T3 and T7 promoters and *Not*I sites for rapid expression and mapping studies, (vi) the RK2 partition region for increased stability in the absence of antibiotic selection, (vii) a copy number approximately twice that of pLAFR vectors, facilitating plasmid isolation, and (viii) finally, a small size, 12.3 kb (10.8 kb when packaged following deletion of one *cos* site and the *bla* region), which permits the cloning of DNA inserts up to 41 kb. pCPP47 has now been used successfully to clone *hrp* genes from *P. syringae* pv. *syringae* B728a, *P. syringae* pv. *tomato* DC3000, and *P. syringae* pv. *glycinea* race 4 (A. O. Loniello, D. W. Bauer, and A. Collmer, unpublished results).

Construction of cosmid libraries

of *P. syringae* pv. *lachrymans* 859 DNA.

Total DNA of *P. syringae* pv. *lachrymans* 859 was prepared according to Silhavy et al. (1984). The DNA was partially digested with *Sau*3AI and separated on a 0.3% agarose gel. Fragments of 35 to 45 kb were excised in agarose plugs and electroeluted with an Elutrap device (Schleicher and Schuell, Keene, NH). pCPP34 was digested with *Bam*HI and *Stu*I, whereas pCPP47 was digested with *Bam*HI and *Sca*I. The size-fractionated *P. syringae* pv. *lachrymans* DNA was ligated to the vector arms of the respective plasmids in the presence of 5 mM ATP to inhibit blunt-end ligation. The DNA was packaged into λ with the Stratagene Gigapack Gold kit (Stratagene, La Jolla, CA). The cosmids were transduced into *E. coli* ED8767 and selected by growing in LM medium with tetracycline. Cosmids were conjugated from *E. coli* ED8767 into *P. syringae* pv. *syringae* BUVS1 by triparental matings with *E. coli* DH5 α (pRK2013) as the helper.

Subcloning and sequencing *pelS*.

pCPP5020 was partially digested with *Sau*3AI, and the fragments were ligated into the *Bam*HI site of pBluescript II KS- (Stratagene). The DNA was transformed into *E. coli* DH5 α , and 200 white colonies were tested for Pel activity on pectate semisolid agar plates (Starr et al. 1977). Three positive clones were found. One clone, pCPP5021, contained a 4.5-kb insert and was used to generate a *pelS* mutation (described below). Another, pCPP5022, contained a 2.5-kb insert and was used for sequencing.

Both strands of a 1,397-bp region of the *P. syringae* pv. *lachrymans* 859 DNA in pCPP5022 were sequenced by a combination of manual sequencing with the Sequenase Version 2 kit (U.S. Biochemical, Cleveland, OH) and automated sequencing on an ABI 373A sequencer (Applied Biosystems, Foster City, CA) at the Cornell Biotechnology Center. Deletions for manual sequencing were made in pCPP5022 with the ExoIII Erase-a-Base kit (Promega, Madison, WI). Oligonucleotide primers for automated sequencing were obtained from Integrated DNA Technologies (Coralville, IA). DNA sequences were analyzed with the DNASTar (Madison, WI) software package.

Construction of *P. syringae* pv. *lachrymans* CUCPB1773, pCPP2275, and pCPP2191.

pCPP5021 was digested with *Bcl*I, which cuts once in the *pelS* gene. The Ω Km^r interposon was excised as a *Bam*HI fragment from pHP45 Ω Km (Fellay et al. 1987), isolated from an agarose gel with the Prep-a-Gene kit (Bio-Rad) and then ligated into the *Bcl*I site in pCPP5021. The resultant plasmid was propagated in *E. coli* DH5 α , and then the *pelS*:: Ω Km^r *Xba*I-*Eco*RI fragment was excised and cloned into pSF6 (Selvaraj et al. 1984), which had been digested with the same enzymes. The correct construction of the resultant plasmid, pCPP2326, was confirmed in *E. coli* DH5 α transformants, and then the plasmid was transformed into *P. syringae* pv. *lachrymans* Pla5 by electroporation. Repeated attempts to introduce pCPP2326 into *P. syringae* pv. *lachrymans* 859 by electroporation and conjugation failed. The *pelS*:: Ω Km^r mutation was marker-exchanged into the *P. syringae* pv. *lachrymans* Pla5 genome by growth, with several transfers, in King's B medium supplemented with kanamycin but not spectinomycin (the vector marker). Replicate plating revealed that 90% of the cells were Km^r but Sp^s. One mutant, CUCPB1773, was confirmed by polymerase chain reaction and DNA gel blot analysis to be *pelS*:: Ω Km^r.

The *pelS*⁺ ORF fragment was transferred as a 2.5-kb *Xba*I-*Hind*III fragment from pCPP5022 to pML122, such that *pelS* was expressed from the *npII* (*aphII*) vector promoter. The resultant plasmid, pCPP2275 was then introduced into *P. syringae* pv. *lachrymans* Pla5 by electroporation. The *pelS*⁺ 2.5-kb *Eco*RI-*Not*I fragment from pCPP5022 was recloned into pET21(+) to produce pCPP2191, which was electroporated into *E. coli* BL21(DE3).

Protein manipulations.

PelS for isoelectric focusing analysis was prepared by growing pectolytic *P. syringae* pv. *syringae* BUVS1 transconjugants overnight in 20 ml of LM medium (which supports much less exopolysaccharide production than King's B). Proteins in the culture supernatant were desalted and concentrated 30-fold with Centrprep (Amicon, Beverly, MA) and then submitted to activity-stained, ultrathin-layer isoelectric focusing analysis as previously described (Ried and Collmer 1985). The ampholytes were provided in pH 3 to 10 IEF Gel Servalyte Precotes (Serva Chemical Co., Paramus, NJ) with the pH 4.45 to 9.6 isoelectric focusing standards of BioRad used as pH gradient markers.

PelS was purified from *E. coli* BL21(DE3)(pCPP2191). Cells were grown at 37°C to an OD₆₀₀ of 0.8 in 1 liter of M9 medium containing 0.4% (wt/vol) casamino acids and 40 mg of thiamine, then isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 1 mM, and growth was continued with shaking at room temperature overnight. Bacterial cells were then removed by centrifugation at 5,800 $\times$ g for 20 min, and proteins in the supernatant were precipitated with 570 g of ammonium sulfate, harvested by centrifugation at 16,000 $\times$ g for 30 min, resuspended in 5 ml of 10 mM Tricine, pH 8.0, and then dialyzed overnight against the same buffer. The PelS preparation was then applied to a 5-ml column of Sepharose 6B that had been equilibrated with 10 mM Tricine, pH 8.0, and 0.1 mM CaCl₂. The column was eluted successively with a bed volume of 10 mM Tricine, pH 8.0, and 0.1 mM CaCl₂, then 10 mM Tricine, pH 8.0, and finally, 10 mM

Tricine, pH 8.0, 0.2 M NaCl. Fractions were collected after the last eluant was applied and assayed spectrophotometrically for Pel activity. Proteins in 100- μ l samples of peak fractions were concentrated with Strataclean resin (Stratagene) and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through a 12.5% homogeneous PhastGel (Pharmacia Biotech, Uppsala, Sweden), followed by staining with Coomassie brilliant blue. This preparation yielded a single major band corresponding to a protein with a molecular weight of approximately 38 and six faint bands.

PelS for determination of the N-terminal sequence was obtained by electrophoresing the peak fraction from the Sepharose 6B column through the wide-comb lane of a 10% polyacrylamide, 0.75-mm thick Hoefer Mighty Small gel (Hoefer Scientific, San Francisco, CA). Proteins were electrophoretically transferred to an Immobilon P^{8Q} (Millipore, Bedford, MA) membrane and stained with Coomassie brilliant blue R. The PelS band was excised and subjected to N-terminal sequence analysis at the Cornell Biotechnology Program Analytical Chemistry and Peptide/DNA Synthesis Facility.

Pel assays.

Pel activity was assayed at 25°C by the increase in absorbance at 235 nm of a reaction mixture containing 0.1% (wt/vol) polygalacturonic acid, 100 mM Tris-HCl, pH 8.5, and 1.5 mM CaCl₂ (Collmer et al. 1988). A unit of activity is defined as releasing 1 μ mol of unsaturated uronide per min. Additionally, viscometric assays were performed in size 300 Ostwald-Fenske viscometers with the substrate concentration increased to 1.5% (wt/vol). Polygalacturonic acid and variously methyl-esterified pectins were obtained from Sigma (St. Louis, MO). To assay Pel activity in planta, cucumber cotyledons were infiltrated with bacteria at a concentration of 1 $\times$ 10⁶ CFU/ml, as described below. After incubation for 2 days, 1-cm² pieces of tissue were removed with a cork borer and ground in 1 ml of water in a microfuge tube with a plastic pestle. Particulate material was sedimented by centrifugation, and the supernatant was assayed for Pel activity spectrophotometrically.

Plant assays.

Cucumber cv. Marketmore 76 seeds were planted in small flats in Metro mix (Northeast Horticultural Products, Scotts, Inc., Allentown, PA) and grown 8 to 10 days in a growth chamber at 24°C with 16 h of light until the first true leaves began to emerge. *Pseudomonas* strains were grown overnight on King's B agar medium. Bacterial cells were then resuspended in King's B broth, grown to an OD₆₀₀ of 0.8 (1 $\times$ 10⁹ CFU/ml), harvested by centrifugation at 5,800 $\times$ g, resuspended in 10 mM MgCl₂, centrifuged again, and finally resuspended and diluted to 10⁴ or 10⁵ CFU/ml in 10 mM MgCl₂. Cucumber cotyledons were infiltrated through stomates in the lower surface with approximately 100 μ l of each bacterial suspension, using a syringe without a needle. One cotyledon was infiltrated per plant and four plants were inoculated with each strain per experiment. The flats were placed in an aquarium with moist paper towels on the bottom, covered by a single layer of plastic wrap with several perforations, incubated in the growth chamber, and observed for 1 week.

Potato tuber maceration tests were performed by inoculating small wells in 1-cm-thick slices of potato cv. Russet with an inoculation loopful of cells from a fresh King's B agar

plate. Inoculated tuber slices were maintained on wet filter paper in petri dishes at 25°C for 2 days before being assayed for maceration.

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